Servier: Looking to the future

Innovation-driven partnerships

Issue coordinated by Emmanuel Canet and Pascal Touchon

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Drug therapies have been a major driving force behind medical progress in recent decades. Yet unmet needs remain in many areas of medicine. One of the outstanding challenges of the 21st century is to master diseases lacking effective treatment. Servier has taken up the gauntlet. Scientific and technological advances in the life sciences may not have revolutionized the drug discovery process, but they have had, and will continue to have, a substantial impact on our capacity to explore new therapeutic approaches to aging-related illnesses of great complexity, such as cancer, neurodegenerative disorders, and cardiovascular diseases, and to manage associated risk factors.

Now more than ever, meeting this challenge means calling upon the skills and wherewithal of academe and of biotechnology companies, plus high-level research in the pharmaceutical industry.

At Servier this pressing need goes hand in hand with the conviction, expressed at the outset by company founder Dr Jacques Servier, that unmet medical needs are most effectively addressed through the creation of sound research partnerships, imbued with mutual respect and confidence and driven by a steadfast commitment to the long term. The very structure of Servier, which is controlled by a foundation, empowers such lasting human, material, and financial investment.

Servier’s Research and Development partnerships are active both in France and internationally and cover all areas of expertise (see Figure page 268). By working toward the creation of an environment and of tools that foster inventiveness, these partnerships meet four objectives: (i) innovate by investigating new lines of research through greater understanding of the pathophysiological mechanisms underlying serious diseases; (ii) expedite the drug discovery process through the sharing of complementary proficiency and know-how; (iii) increase the creativity and competitiveness of our research through knowledge sharing and the ensuing cross-fertilization; (iv) identify promising drug candidates at different stages of the discovery process and seize the opportunity to create medical value by taking them from bench to bedside.

**New modes of operation**
Dynamic partnerships are one of the cornerstones of innovation and drug discovery and are for us essential, given growth in knowledge and the biological complexity of the diseases studied. No individual, group, or organization alone can hope to encompass all the stages leading from assimilation of the latest findings and of
the resulting hypotheses to the discovery of a drug candidate, its clinical testing, industrial production, and provision to doctors and patients. Medical breakthroughs, the main beneficiary of which will be the patient, are best achieved through research networks between academic organizations, university hospitals, public or private institutions, biotech companies, and pharmaceutical firms. Such partnerships involve technology transfer, data sharing, cross-fertilization, and pooling of knowledge and resources. In precompetitive or competitive projects, participants from academic institutions and the pharmaceutical industry pool their understanding, ideas, knowledge, skills, know-how, technologies, and resources, be they human, material, or financial.

Servier, biotechs, and partnerships
Servier has for many years embraced the collaborative spirit of working in networks and partnerships while respecting three principles that guarantee their success: (1) the human factor, notably trust and mutual scientific respect; (2) the quality of the science and the therapeutic potential of the project; and (3) the commitment of one and all to clearly defined goals.

What sets us apart is the manner in which we approach partnerships. As a company we naturally have economic imperatives. Yet our ambition and founding values are to bring added scientific and medical value to the patient, through the discovery and development of drug therapies that fulfill unmet needs. From the outset we have viewed ourselves as “a research institute as much as an industry” and bring to our partnerships learning, creativity, scientific proficiency, and hands-on experience.

What also distinguishes our partnerships, a corollary of the above, is that they are based on genuine exchange, on complementarity. For reasons of principle and ethics, and by conviction, we insist on developing our own expertise, thereby benefiting from vigorous in-house research. This enables us to identify the most original projects and the most creative researchers and so to choose the best partnerships. Once the partnership is in place, we seek synergy and complementarity through constant exchanges and mutual respect of our respective skill sets. This pooling of resources and skills is intended not only to develop drug candidates pinpointed by biotechs, but also to boost our capacity to develop drug candidates from our own research.

Thus, we are able to bring to an R&D project targeting therapeutic innovation the medical, scientific, and technological expertise and creativity of clinical and basic researchers, from academe and industry, thereby contributing to the success of this approach, the ultimate aim of which is the discovery of innovative drug therapies. The future and our collective success
depend on our capacity to generate new ideas and to ensure the continuum between basic and clinical research, so that translational research—from bench to bedside and back—contributes to the creation of innovative drug therapies and to the implementation of new medical and therapeutic practices.

Through our strategy of openness and partnerships, we aim to expedite the bench-to-bedside transfer of the latest findings and technologies, to test the relevance of a biological hypothesis, and to determine the biological basis of an observation recorded in a clinical setting or in a population of patients, so as to open new or back up existing lines of research. Drug discovery is a long and complex endeavor the success of which is contingent upon the commitment and collective intelligence of those who bring their skills and proficiency to the task in hand and who are resolved to meet this inspiring challenge. As exemplified in this special issue of Medicographia on “Servier: Looking to the Future. Innovation-Driven Partnerships,” the partnerships illustrated in the following pages bear witness to the vitality of our company and to its determination to promote medical progress and to bring innovative treatments to the patient’s bedside.

**Keywords:** biotechnology; drug discovery; oncology; partnership; pharmaceutical industry; pharmacology; pipeline

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ÉDITORIAL

Trois principes-clés caractérisent les partenariats Servier: (1) confiance et respect scientifique mutuels ; (2) qualité de la science et potentiel thérapeutique du projet ; et (3) objectifs communs clairement définis. De façon importante, les partenariats Servier s’appuient sur une recherche interne puissante en mesure d’identifier les projets les plus innovants, de travailler avec les chercheurs les plus créatifs et de fédérer les meilleures équipes au service du progrès thérapeutique. »

par E. Canet et P. Touchon, France

Servier : promesse d’avenir
Partenariats pour l’innovation

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S i le médicament a été au cours de ces dernières décennies l’un des vecteurs majeurs du progrès médical, il est de très nombreux domaines thérapeutiques où les besoins restent majeurs. L’un des principaux enjeux du XXIe siècle sera de contribuer au progrès thérapeutique dans des maladies aujourd’hui encore mal maîtrisées. Notre ambition au sein de Servier est de participer à ce défi. Les progrès scientifiques et technologiques dans le domaine des sciences de la vie, s’ils n’ont pas révolutionné le processus fondamental de découverte de médicaments ont, et auront, un impact majeur sur notre capacité à ouvrir de nouvelles voies thérapeutiques, et ce particulièrement dans des pathologies liées au vieillissement et d’une grande complexité telles que le cancer, les maladies neurodégénératives ou les maladies cardio-vasculaires et facteurs de risques associés.

Aujourd’hui encore plus qu’hier, relever ce défi nécessite de pouvoir bénéficier de l’ensemble des compétences et des moyens du monde académique, des sociétés de biotechnologie et d’une recherche interne de très haut niveau au sein des laboratoires pharmaceutiques.

Cette nécessité de plus en plus aiguë se double chez Servier de la conviction, affirmée par le Docteur Jacques Servier dès la création de l’entreprise qui porte son nom, que la manière la plus efficace de répondre aux besoins thérapeutiques non satisfaits passe par l’établissement de partenariats de recherche solides, marqués par le respect et la confiance mutuels, avec un engagement résolument axé sur le long terme. La structure même de Servier, qui est contrôlé par une fondation, permet un tel investissement humain, matériel et financier sur le long terme avec nos partenaires.

Les partenariats de recherche et développement Servier sont actifs tant au niveau national qu’international, et ne négligent aucun domaine d’expertise (Figure). Ils concourent à mettre en place l’écosystème et les outils nécessaires à l’innovation. Ils répondent à quatre objectifs : (1) innover en ouvrant de nouvelles voies de recherche par une meilleure compréhension des mécanismes physiopathologiques responsables des maladies sévères ; (2) accélérer le processus de découverte par la mise en commun d’expertises et de savoir-faire complémentaires ; (3) accroître la créativité et la compétitivité de notre recherche par le partage des connaissances et la fertilisation croisée qui en résulte ; et (4) identifier et saisir les opportunités de candidats médicaments innovants à différents stades de maturité avec pour objectif de créer de la valeur médicale en les développant jusqu’à leur mise à disposition des médecins et de leurs patients.
De nouveaux modes de fonctionnement

La dynamique des partenariats constitue une des clés de voûte de l’innovation et de la découverte de nouveaux médicaments. Elle s’impose à nous au vu de l’augmentation des connaissances et de la complexité biologique des pathologies à explorer. Aucun individu, groupe ou structure, ne peut prétendre embrasser seul le champ de toutes les étapes menant de l’intégration des connaissances et des hypothèses ainsi nouvellement élaborées à la découverte d’un candidat médicamenteux, à sa validation clinique, à sa production industrielle et à sa mise à disposition auprès des médecins et des malades. Le travail en réseau établi entre la recherche académique, le monde hospitalo-universitaire, les institutions publiques ou privées, les sociétés de biotechnologie, et les entreprises pharmaceutiques est le plus à même d’apporter de nombreuses innovations médicales dont le patient sera le bénéficiaire principal. Il s’agit dans le cadre des partenariats de transfert de technologies, de partage de connaissances, de fécondation croisée, de mutualisation du savoir et des moyens.

Ainsi les acteurs du monde académique et du monde pharmaceutique dans le cadre de projets précompetitifs ou compétitifs sont engagés à mettre en commun les intelligences, les idées, les connaissances, les compétences, les savoir-faire, les technologies, les ressources, qu’elles soient humaines, matérielles, ou financières.

Servier, biotechs et partenariats

Chez Servier, nous sommes entrés depuis de nombreuses années dans cet esprit collaboratif de partenariats en respectant trois principes garants de leur succès : (1) le facteur humain et en particulier la confiance et le respect scientifique mutuels ; (2) la qualité de la science et le potentiel thérapeutique du projet ; et (3) l’engagement réciproque de chacun des acteurs autour d’objectifs communs clairement définis.

◆ Notre spécificité, c’est l’esprit dans lequel nous abordons nos partenariats. Si nous sommes une entreprise, avec ses impératifs économiques, notre ambition et nos valeurs fondateuses sont de créer une véritable valeur ajoutée scientifique et médicale pour le malade par la découverte et le développement de médicaments qui répondent à des besoins majeurs. Dès l’origine nous nous sommes définis comme « une recherche au moins autant qu’une industrie » et nous mettons dans nos partenariats l’ensemble de notre savoir, notre créativité, nos compétences scientifiques, notre expérience pratique...

◆ Une autre caractéristique de nos partenariats, corollaire de la précédente, c’est que ceux-ci s’appuient sur un véritable échange, une complémentarité. Nous tenons par principe, par éthique et par conviction, à développer nos propres expertises et à bénéficier ainsi en interne d’une recherche puis-
santé. Ceci nous permet à la fois d’identifier les projets les plus originaux, les chercheurs les plus créatifs et ainsi de faire les meilleurs choix de partenariats. Une fois le partenariat en place, synergies et complémentarité sont recherchées par des échanges constants entre les deux partenaires, et ce, dans le respect mutuel des compétences de chacun. La mise en commun des moyens et des compétences de chacun vise non seulement à développer des candidats médicaments issus de sociétés de biotechnologies, mais également à renforcer notre capacité à développer les candidats médicaments issus de notre propre recherche.

Ainsi, avant tout, il s’agit de fédérer, autour d’un projet de Recherche et Développement dont l’objectif est l’innovation thérapeutique, les expertises médicales, scientifiques et technologiques et la créativité des chercheurs cliniciens et/ou fondamentalistes, académiques ou industriels et de contribuer au succès de cette démarche dont le but ultime reste la découverte de médicaments innovants. L’avenir et notre réussite collective dépendent en effet de notre capacité à générer de nouvelles idées et à assurer le continuum entre la recherche fondamentale et la recherche clinique afin que cette recherche dite « translationnelle » soit contributive à la création de médicaments innovants et à la mise en place de nouvelles pratiques médicales et thérapeutiques. Par une stratégie d’ouverture et de partenariats, nos objectifs sont de transférer et d’interpréter le plus vite possible les connaissances nouvelles et les nouvelles technologies vers des applications thérapeutiques, au bénéfice des patients ; tester la pertinence d’une hypothèse biologique et/ou déterminer les bases biologiques d’une observation faite en clinique ou dans une population de patients afin d’ouvrir ou de conforter de nouvelles pistes de recherches à visée thérapeutique.


Mots-clés : biotechnologie ; découverte du médicament ; industrie pharmaceutique ; oncologie ; partenariat ; pharmacologie ; pipeline


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Oncology
Innovation-driven partnerships

IMMUNOMODULATION AND CANCER

State of the Art – Cancer immunotherapy through checkpoint blockade: the future of cancer treatment
\textit{D. Pardoll, USA} \hspace{1cm} \text{Page 274}

Targeting B7-H3 in cancer
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EPIGENETICS AND CANCER

State of the Art – Epigenetic defects in cancer
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Clinical development of histone deacetylase inhibitors
\textit{M. Salcedo Magguilli, France} \hspace{1cm} \text{Page 300}

APOPTOSIS AND CANCER

State of the Art – The roles of programmed cell death in tumor development and cancer therapy
\textit{A. Strasser, Australia} \hspace{1cm} \text{Page 311}

Acting on the BCL2 dependence of cancer cells
\textit{O. Geneste, France} \hspace{1cm} \text{Page 319}
After decades of research and clinical trials aimed at harnessing a cancer patient’s immune system to attack their cancer, clinical efficacy with both vaccines and inhibitors of immune checkpoints has been demonstrated. The past few years have, therefore, become a turning point establishing active immunotherapy as a viable approach to cancer therapy. These advances have been fueled by basic molecular and cellular discoveries related to immune system activation, as well as study of the tumor microenvironment to identify resistance mechanisms that can be directly targeted. Future work will concentrate on targeting multiple pathways of immune regulation and developing rationally designed combinatorial approaches.

Without question, the major molecules to be successfully targeted in clinical cancer immunotherapy are the growing class of ligand-receptor pairs, commonly referred to as immune checkpoints. The notion of immune checkpoint blockade stems from many years of analysis of the immune microenvironment of cancer. These studies demonstrated upregulation within the tumor microenvironment of many inhibitory cytokines (eg, interleukin 10 [IL-10], transforming growth factor β [TGF-β]), ligands for inhibitory receptors on T cells (eg, programmed cell death protein 1 ligand 1 [PD-L1] and PD-L2) and metabolic enzymes (eg, indoleamine-pyrrole 2,3-dioxygenase [IDO] and inducible nitric oxide synthase [iNOS]) that consume amino acids essential for immune function or produce immune-inhibitory metabolites (Figure 1). A number of these inhibitory signals derive from inhibitory cell populations that accumulate in the tumor microenvironment, such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). In considering the mechanism(s) of action of inhibitors of various checkpoints, it is critical to appreciate the diversity of immune functions that they regulate. For example, the two immune checkpoint receptors that have been most actively studied in the context of clinical cancer immunotherapy, cytotoxic T lymphocyte–associated antigen 4 (CTLA-4, also known as cluster of differentiation 152 [CD152]) and programmed cell death protein 1 (PD-1, also known as CD279), regulate immune responses at very different levels and by very different mechanisms (Figure 2). The clinical activity of blocking antibodies for each of these receptors implies that antitumor immunity can be enhanced at multiple levels, and that combinatorial strategies can be intelligently designed, guided by mechanistic considerations and preclinical models. This review will focus particular attention upon the CTLA-4 and PD-1 pathways, since they were the two checkpoints whose inhibition has revolutionized clinical cancer im-

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Cancer immunotherapy through checkpoint blockade: the future of cancer treatment – Pardoll

The CTLA-4 checkpoint—a global regulator of T-cell activation

CTLA-4, the first immune checkpoint receptor to be clinically targeted, is expressed exclusively on T cells, where it primarily regulates the amplitude of the early stages of T-cell activation. CTLA-4 knockout (KO) mice die within three weeks from immune destruction of multiple organs, which attests to its critical role as an inhibitory regulator of T-cell–dependent immune responses. Primarily, CTLA-4 counteracts the activity of the T-cell costimulatory receptor CD28. CD28 does not affect T-cell activation unless the T-cell receptor (TCR) is first engaged by a cognate antigen. Once antigen recognition occurs, CD28 signaling strongly amplifies the TCR signal to activate T cells. CD28 and CTLA-4 share identical ligands, CD80 (B7.1) and CD86 (B7.2). Because CTLA-4 has a much higher overall affinity for both ligands, its expression on the surface of T cells dampens the activation of T cells by both outcomes delivering inhibitory signals to the T cell. The specific signaling pathways by which CTLA-4 blocks T-cell activation are still under investigation, although a number of studies suggest that activation of the phosphatases, Src homology region 2 (SHP2) and protein phosphatase 2A (PP2A) are important in counteracting kinase signals induced by TCR and CD28.

However, CTLA-4 also confers “signaling-independent” T-cell inhibition through sequestration of CD80 and CD86 from CD28 engagement, as well as active removal from the antigen presenting cell (APC) surface. The central role of CTLA-4 in maintaining T-cell activation in check is dramatically demonstrated by the systemic immune hyperactivation phenotype of CTLA-4 KO mice.
Even though CTLA-4 is expressed by activated CD8 killer T cells, the major physiologic role of CTLA-4 appears to be through distinct effects on the two major subsets of CD4 T cells; downmodulation of helper T cell (TH) activity and enhancement of Treg suppressive activity. CTLA-4 blockade results in a broad enhancement of immune responses dependent on TH and conversely, CTLA-4 engagement on Tregs enhances their suppressive function. CTLA-4 is a target gene of the transcription factor Foxp3, the expression of which determines the Treg lineage and Tregs therefore express CTLA-4 constitutively. While the mechanism by which CTLA-4 enhances the inhibitory function of Tregs is not known, Treg-specific CTLA-4 KO or blockade significantly inhibits their ability to regulate both autoimmunity and antitumor immunity. Thus, in considering the mechanism of action for CTLA-4 blockade, both enhancement of effector CD4 T-cell activity and inhibition of Treg-dependent immune suppression are likely important factors.

Clinical application of CTLA-4 blocking

Blockade of CTLA-4 as a general strategy was initially questioned because there is no tumor specificity to expression of the CTLA-4 ligands (other than certain myeloid and lymphoid tumors) and also because the dramatic lethal autoimmune/hyperimmune phenotype of CTLA-4 KO mice predicted a high degree of immune toxicity associated with blockade of this receptor. However, Allison and colleagues used preclinical models to demonstrate that a therapeutic window was indeed achieved when CTLA-4 was partially blocked with antibodies. The initial studies demonstrated significant antitumor responses without overt immune toxicities when mice bearing partially immunogenic tumors, particularly melanomas, were treated with anti-CTLA-4 antibodies as single agents. Poorly immunogenic tumors did not respond to anti-CTLA-4 as a single agent, but did respond when anti-CTLA-4 was combined with a granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced cellular vaccine. These findings suggested that, if there was an endogenous antitumor response present in the animals after tumor implantation, CTLA-4 blockade could enhance that endogenous response, which ultimately induced tumor regression. In the case of poorly immunogenic tumors, which do not induce significant endogenous responses, the combination of a vaccine and an anti-CTLA-4 antibody could induce a strong enough immune response to slow tumor growth, and in some cases, eliminate established tumors. These preclinical findings encouraged the production and testing of two fully human anti-CTLA-4 antibodies, ipilimumab and tremilimumab, which began clinical testing in 2000. As with virtually all anticancer agents, initial testing was as a single agent in patients with advanced disease, not responding to conventional therapy. Both antibodies produced objective clinical responses in roughly 10% of melanoma patients, but also immune-related toxicities involving various tissue sites in 25% to 30% of patients, with colitis being a particularly common event. The first randomized phase 3 clinical trial to be completed was for tremilimumab in patients with advanced melanoma. In the trial, 15mg/kg tremilimumab was given every three months as a single agent and compared with dacarbazine (DTIC), a standard melanoma chemotherapy treatment. The trial showed no survival benefit with this dose and schedule relative to DTIC. However, ipilimumab fared better. Even though the two antibodies appear to have similar intrinsic activity, response rates in phase 2 trials, and immune toxicity profiles, ipilimumab was more carefully evaluated at different doses and schedules. Additionally, more careful definition of algorithms for improved clinical management of the immune toxicities (using steroids and tumour necrosis factor (TNF)–blockers) mitigated the overall morbidity and mortality associated with immunologic toxicities.

The toxicity rate for ipilimumab is quite significant (14% to 30% grades 3-5 in various studies) and is generally immunologic in nature, implying that it is “on target”. This was predicted from the dramatic lethal hyperimmune/autoimmune phenotype of the CTLA-4 KO mice. The most common toxicities with both ipilimumab and tremilimumab are cutaneous (rash) and colitis. However, hepatitis, pneumonitis, hypophysitis, and thyroiditis are also observed. Interestingly, while there is evidence that clinical responses might be associated with immune-related adverse events, this correlation is modest.

Finally, in a randomized three-arm clinical trial of patients with advanced melanoma that received either a melanoma-specific glycoprotein 100 (gp100) peptide vaccine alone, the gp100 vaccine plus ipilimumab, or ipilimumab alone, there was a 3.5 month survival benefit for patients in both groups receiving ipilimumab (ie, with or without the gp100 peptide vaccine),
compared with the group receiving peptide vaccine alone.32
As the first therapy ever to demonstrate a survival benefit for
patients with metastatic melanoma (DTIC was approved based
on response rate and has never been shown to provide a
survival benefit in melanoma) ipilimumab was Food and Drug
Administration (FDA) approved for treatment of advanced
melanoma in 2010.

More impressive than the mean survival benefit was the effect
on long-term survival: 20% of the ipilimumab treated patients
survived beyond two years (compared with 5% of patients
receiving the peptide vaccine alone).32 In this and other studies,
the proportion of long-term survivors is higher than the
proportion of objective responders. The finding of ongoing re-
sponses and survival long after completion of a relatively short
course of therapy (4 doses of 10mg/kg over 3 months) support the
concept that immune-based therapies might reeducate the
immune system to maintain tumors in check after completion of the therapeutic intervention.

As with all oncology agents that benefit a limited proportion of
treated patients, there has been much effort in defining biomarkers predictive of clinical response to anti–CTLA-4 treat-
ment. To date, no such pretreatment biomarker has been
validated to the point where it could be applied as part of
standard-of-care therapeutic decision-making, though insights have emerged from identification of certain posttreat-
ment immune responses that seem to correlate with clinical
outcome.33-35

An important feature of the anti–CTLA-4 clinical responses that distinguishes them from conventional chemotherapeutic
agents and oncogene-targeted small molecule drugs is their kinetics. While chemotherapy and tyrosine kinase inhibitor
(TKI) responses commonly occur within weeks of initial ad-
ministration, the response to immune checkpoint blockers is slower and, in a number of patients, delayed (up to 6 months
after treatment initiation). In some cases, metastatic lesions actually increase on computed tomography (CT) or magnetic
resonance imaging (MRI) scans prior to regressing. These findings demand a reevaluation of response criteria for immu
notherapeutics that does not use conventional time-to-
progression or objective response evaluation criteria in solid
tumors (RECIST), which were developed based on the expe-
rience with chemotherapy agents and as the primary measure of drug efficacy.36

Biology of the PD-1 checkpoint—a pathway that functions within the tumor microenvironment

Another immune checkpoint receptor, PD-1, is emerging as a
promising target, emphasizing the diversity of potential mol-
ecularly defined immune manipulations capable of inducing antitumor responses by the patient’s own immune system.
In contrast to CTLA-4, the major role of PD-1 is to limit the
activity of T cells in the peripheral tissues at the time of an
inflammatory response to infection, and to limit autoimmuni-
ty.37-43 This translates to a major immune resistance mecha-
nism within the tumor microenvironment.44-46 PD-1 expression is induced when T cells become activated.38 When engaged by
one of its ligands, PD-1 inhibits kinases involved in T-cell activation via the phosphatase SHP2,47 although additional
signaling pathways are also likely induced, and because PD-1 engagement inhibits the TCR stop signal, this pathway could
modify the duration of T cell/APC or T cell/target cell contact.57
Similar to CTLA-4, PD-1 is highly expressed on Tregs, where
it may enhance their proliferation in the presence of ligand.55
Because many tumors are highly infiltrated with Tregs that likely further suppress effector responses, PD-1 pathway block-
ade may also enhance antitumor responses by diminishing the number and/or suppressive activity of intratumoral Tregs.

The two ligands for PD-1 are PD-L1 (B7-H1, CD274) and PD-
L2 (B7-DC, CD273).37,49-51 These B7 family members share
37% sequence homology and arose via gene duplication, po-
sitioning them within 100kB of each other in the genome.51
Recently, an unexpected molecular interaction between PD-L1 and CD80 was discovered,52 whereby CD80 expressed on
T cells (and possibly APCs) can potentially behave as a recep-
tor rather than a ligand, delivering inhibitory signals when en-

gen by B7-H1,53,54 the relevance of this interaction in tumor
immune resistance has not yet been determined. Finally, geo-
etic evidence from PD-1–deficient T cells suggests that both
PD-L1 and CD80 may bind to a costimulatory receptor ex-
pressed on T cells.52 These complex binding interactions are reminiscent of the CD80/CD86 ligand pair, which binds the
costimulatory CD28 expressed on resting T cells and the in-
hibitory CTLA-4 expressed on activated T cells, though, as
stated above, PD-1 predominantly regulates effector T-cell ac-
tivity within tissue and tumors while CTLA-4 predominantly
regulates T-cell activation. Understanding the role of these various interactions in given cancer settings is highly relevant
for selection of both antibodies and recombinant ligands for
use in the clinic.

PD-1 is more broadly expressed than CTLA-4; it is induced on
other activated non–T cell subsets, including B cells and NK
cells,55,56 limiting their lytic activity. Thus, while PD-1 block-
ade is typically viewed as enhancing the activity of effector
T cells in tissues and in the tumor microenvironment, it likely
also enhances NK activity in tumors and tissues and may also enhance antibody production, either indirectly or through di-
rect effects on PD-1 positive B cells.57

In addition, chronic antigen exposure, such as occurs with
chronic viral infection and cancer, can lead to high levels of per-
sistent PD-1 expression, which induces a state of exhaustion
or anergy among cognate antigen-specific T cells. This state,
which has been demonstrated in multiple murine and human
chronic viral infections, appears to be partially reversible by
PD-1 pathway blockade.58 Finally, while the PD-1 pathway
PD-1 is expressed on a large proportion of tumor-infiltrating lymphocytes (TILs) from many different tumor types. Some of the enhanced PD-1 expression among CD4 TILs reflects a generally high level of PD-1 on Tregs, which, as noted above, can represent a large fraction of intratumoral CD4 T cells. Increased PD-1 expression on CD8 TILs may either reflect an anergic/exhausted state, as has been suggested by decreased cytokine production by PD-1 positive vs PD-1 negative TILs from melanomas.

Just as PD-1 is highly expressed on TILs from many cancers, the PD-1 ligands are commonly upregulated on many different human tumors. On solid tumors, the major PD-1 ligand to be expressed is PD-L1. Forced expression of PD-L1 on murine tumors inhibits local antitumor T-cell responses. Indeed, this combination of findings provides the basis for PD-1 pathway blockade to enhance antitumor effector function in the tumor microenvironment. As immunohistochemistry techniques and flow cytometry analysis of surface expression has been employed, it has become clear that the selective upregulation of PD-1 ligands in various human tumor types is heterogeneous at a number of levels. Expression patterns of PD-1 ligands may very well be critical in choosing suitability for therapeutic blockade of this pathway, since its primary role in cancer is thought to be immune inhibition within the tumor microenvironment, and PD-1 only inhibits lymphocyte function when it is engaged by cognate ligand.

Initially, the majority of melanoma, ovarian, and lung cancer samples were reported to have high expression of PD-L1 and subsequently, many other human cancers were reported to upregulate PD-L1. In addition to tumor cells, PD-L1 is commonly expressed on myeloid cells in the tumor microenvironment. An initial report in renal cancer demonstrated that expression of PD-L1 on either tumor cells or infiltrating leukocytes in primary tumors predicted a worse prognosis, ie, decreased overall survival relative to PD-L1 negative tumors. Since that report, analyses of various tumors have suggested that PD-L1 status can either correlate with poor prognosis, better prognosis, or show no correlation with prognosis. Variability in immunohistochemistry technique, cancer type, stage of cancer analyzed (most analyses are of primary, not metastatic lesions), and treatment history in the analyzed cohort all likely contribute to the wide range of reported outcomes. While most of the analyses of PD-1 ligand expression has focused on PD-L1, PD-1 has also been reported to be upregulated on a number of tumors. It is highly upregulated on certain B-cell lymphomas such as primary mediastinal, follicular cell B-cell lymphoma, and Hodgkin's disease. Upregulation in these lymphomas is commonly associated with gene amplification, or rearrangement to the class II transactivator (CIITA) locus, which is highly transcriptionally active in B-cell lymphomas.

Given the heterogeneity of expression and potential relevance as a biomarker for blockade of the PD-1 pathway, it is important to understand the signals that induce expression of PD-1 ligands on tumor cells, and also hematopoietic cells, within the tumor microenvironment. Two general mechanisms for regulation of PD-L1 have emerged: innate and adaptive. For some tumors, such as glioblastoma, it has been demonstrated that PD-L1 is driven by constitutive oncogenic signaling pathways in the tumor cell. Expression on glioblastomas is enhanced upon deletion or silencing of phosphatase and tensin homolog (PTEN), implicating the phosphatidylinositol-3-kinase (PI3K)-AKT pathway. Similarly, constitutive anaplastic lymphoma kinase (ALK) signaling, observed in certain lymphomas and occasionally in lung cancer, has been reported to drive PD-L1 expression via signal transducer and activator of transcription 3 (STAT3) signaling.

The alternative mechanism for PD-L1 upregulation on tumors that has emerged from both clinical and preclinical studies reflects their adaptation to endogenous tumor-specific immune responses, a process termed adaptive resistance. In adaptive resistance, the tumor utilizes the natural physiology of the PD-1 ligand induction for tissue protection in the face of an immune response to infection in order to protect itself from an antitumor response. Expression of PD-L1 as an adaptive response to endogenous antitumor immunity can occur because it is induced on most cancers in response to interferons, predominantly γ-interferon, similar to what is observed in epithelial and stromal cells in normal tissues. This mechanism represents an alternative to the conventional drug resistance mechanisms that involve mutation of drug targets. It also contrasts with mechanisms of viral immune escape that involve mutation of immunodominant epitopes. The mechanism of adaptive resistance intrinsically implies that immune surveillance does exist even in some advanced cancers, but the tumor ultimately resists immune elimination by upregulating ligands for inhibitory receptors on tumor-specific lymphocytes that turn off antitumor responses within the tumor microenvironment.

A number of preclinical and clinical studies support the adaptive resistance hypothesis. Gajewski and colleagues have demonstrated that melanomas can be roughly divided into “inflammatory” and “noninflammatory” categories defined by expression of multiple inflammatory genes, including those involved in the interferon pathway. A recent study in melanoma demonstrated a very high correlation between cell surface PD-L1 expression on tumor cells and both lymphocytic infiltration and intratumoral γ-interferon expression. This correla-
Evidence of clinical activity for PD-1 blockade

Taken together, the general findings of increased PD-1 expression by TIL and increased PD-1 ligand expression by tumor cells created an important rationale for the capacity of antibody blockade of this pathway to enhance intratumoral immune responses. This was validated through many murine tumor studies demonstrating enhanced antitumor immunity through antibody blockade of PD-1 or its ligands (see above). Furthermore, the relatively mild phenotypes of PD-1, PD-L1, and PD-L2 KO mice suggest that blockade of this pathway would result in less collateral immune toxicity than CTLA-4 blockade, a finding that appears to be the case in clinical trials.

While the clinical experience with anti–PD-1 antibodies is less extensive than with anti-CTLA antibodies at this time, results look extremely promising. In the first phase 1 clinical trial with mixed responses, partial responses, as well as a complete response. 80 Tumor regressions were observed in four of the five patients with no membrane PD-L1 displayed either an objective or mixed response. 83 The lack of response in patients whose tumors exclusively expressed cytosolic PD-L1 was also notable, as cytosolic PD-L1 would fail to activate the PD-1 pathway. A recent clinical trial of the PD-1 ligand 1 (PD-L1) induc-

It is logical to imagine that the enhancement of antitumor immune responses upon blockade of this pathway would depend, in significant part, on expression of a ligand for PD-1 within the tumor. Analysis of 42 patients treated with anti–PD-1 in the trial described above demonstrated a strong correlation between PD-L1 expression and response. None of the 17 patients with no membrane PD-L1 expression on pretreatment biopsies responded to anti–PD-1, whereas 44% patients with >5% of tumor cells expressing membrane PD-L1 displayed either an objective or mixed response. 85 The lack of response in patients whose tumors exclusively expressed cytosolic PD-L1 was also notable, as cytosolic PD-L1 would fail to activate the PD-1 pathway.

If validated in a larger series, this finding sets the stage for a broader assessment of immune checkpoint ligands and receptors as targets for antibody blockade, as well as assessment of ligand expression in the tumor as a biomarker for success in blockade of a specific checkpoint pathway.

**Figure 3.** Two mechanisms for programmed cell death 1 ligand 1 (PD-L1) induction on tumors: innate and adaptive.

**Oncology** Immunomodulation and cancer

While the clinical experience with anti–PD-1 antibodies is less extensive than with anti-CTLA antibodies at this time, results look extremely promising. In the first phase 1 clinical trial with a fully human immunoglobulin G4 (IgG4) anti–PD-1 antibody, there were a number of cases of tumor regression, including mixed responses, partial responses, as well as a complete response. 80 Tumor regressions were observed in four of the five histologies examined (melanoma and colon, renal, and lung cancer) and were associated with significant increases in lymphocyte infiltration into metastatic tumor deposits. Results from a second, larger clinical trial, sponsored by Bristol-Myers Squibb (BMS) and extending the treatment with anti–PD-1 (named nivolumab) to 2 years, demonstrated objective responses observed in 31% of patients with advanced melanoma, with an additional 7% achieving disease stabilization for >6 months. Similar response rates were observed in renal cancer, with an additional 27% with disease stabilization for >6 months. Most surprisingly, there was an 18% response rate in non–small cell lung cancer (NSCLC), with additional 7% disease stabilization >6 months. Efficacy against melanoma, renal cancer, and lung cancer was also observed with an anti–PD-L1 antibody. 87 Among 270 nivolumab-treated patients with lung, melanoma, or kidney cancer, one-/two-year landmark survival rates were 42%/14% for lung cancer, 62%/43% for melanoma, and 70%/50% for kidney cancer. Median overall survival in these heavily-pretreated patients (47% with 3 to 5 prior systemic therapies) was 9.6, 16.8, and >22 months, respectively. Among all responders, median response duration was 74, 104, and 56 weeks, respectively. Among responders who discontinued therapy for reasons other than disease and followed for at least 4 months (range 4-14 months), 70% retained their response. 88

As predicted by the distinct phenotypes of the PD-1 KO vs CTLA-4 KO mice, the frequency of immune-related toxicities from anti–PD-1 treatment appears to be less than with anti–CTLA-4. Grade 3/4 drug-related toxicity was <15% and was also largely immune related. In contrast to anti–CTLA-4, the most significant toxicity was pneumonitis, which produced a 1% mortality rate. Recently instituted protocols to manage pneumonitis with steroids and, when necessary, anti-TNF–blocking antibodies appear to mitigate lung toxicity.
There are a number of companies developing and testing antibodies that block the PD-1 pathway; a recent study with a different anti-PD-1 antibody produced by Merck (named lambrolizumab) demonstrated a 38% response rate in melanoma\(^\text{86}\) and an anti-PD-L1 antibody produced by Genentech gave similar response rates in melanoma and NSCLC (but a somewhat lower response rate in kidney cancer) to the BMS anti-PD-1 antibody.\(^\text{85}\) These results validate the PD-1 pathway as an important target for immunotherapeutic targeting. Based on the known interactions between the PD-1 ligands, it is theoretically possible that a PD-1 antibody would have distinct biologic activity from an anti-PD-L1 antibody; an anti-PD-1 antibody would block PD-1 interaction with both PD-L1 and PD-L2, but not the interaction between PD-L1 and CD80. Most anti-PD-L1 antibodies block the interaction between PD-L1 and CD80 and between PD-L1 and PD-1, but would not block PD-1 interaction with PD-L2. Thus, it is possible that, depending on which interactions dominate in a particular cancer, PD-1 and PD-L1 antibodies might not have redundant activity.

Based on the distinct roles of CTLA-4 and PD-1 in regulating distinct components of the immune response, it was postulated that combined blockade of these pathways might provide an additive or synergistic antitumor effect. Indeed, a recent study demonstrated a 41% response rate in melanoma patients treated concurrently with ipilimumab and nivolumab. A larger proportion of the responses were “deep” (>80%) with ipilimumab and nivolumab than observed with nivolumab alone, and there were ≈20% additional mixed responses and stable disease >6 months. However, toxicity was also greater than with nivolumab alone, with a 53% grade 3/4 toxicity rate.\(^\text{87}\)

While the ultimate long-term clinical benefit of this combination remains to be determined, the study emphasizes the potential for combinatorial blockade of multiple checkpoints.

### Additional checkpoints participate in tumor immune resistance and tolerance

Successful clinical outcomes of CTLA-4 and PD-1 pathway targeting have garnered great interest in a number of additional checkpoints. Basic immunologic studies have demonstrated that a number of checkpoint receptors (Figure 4) are expressed coordinately under circumstances of tolerance to self-antigens and chronic infections, as well as in inflammatory settings.

In addition to defined lymphocyte inhibitory receptors, a number of B7-family inhibitory ligands—in particular B7-H3 (CD276) and B7-H4—do not yet have defined receptors, but murine KO experiments support an inhibitory role for both these molecules.\(^\text{96}\) In addition, they are upregulated on tumor cells or tumor-infiltrating cells.\(^\text{88}\) B7-H3 appears to be upregulated on endothelial cells of the tumor vasculature and B7-H4 has been reported to be expressed on tumor-associated macrophages.\(^\text{89}\)

Preclinical tumor models have been used to demonstrate that blockade of many of these individual immune checkpoint ligands or receptors can enhance antitumor immunity and dual blockade of coordinately expressed receptors can produce additive or synergistic antitumor activity. Inhibitors for a number of these immune checkpoint targets are either entering the clinic or are under active development. Those described below are targets with currently available blocking antibodies or small molecule inhibitors, but do not represent a comprehensive list.
Lymphocyte-activation gene 3 (LAG-3 or CD223), 2B4 (CD244), B and T lymphocyte attenuator (BTLA or CD272), T-cell immunoglobulin domain and mucin domain 3 (Tim-3), A2A adenosine receptor (A2aR), and the family of killer inhibitory receptors have each been associated with inhibition of lymphocyte activity and in some cases induction of lymphocyte anergy. Antibody targeting of these receptors, either alone or in combination with a second immune checkpoint blocker has been shown to enhance antitumor immunity in animal models of cancer. Because many tumors express multiple inhibitory ligands, and TIL express multiple inhibitory receptors, there are many opportunities to enhance antitumor immunity via dual or triple blockade of immune checkpoints. While human blocking antibodies specific for a number of these "second generation" inhibitory receptors are under development, none have entered the clinic at this time. Most of these receptors are induced upon T-cell activation, in keeping with the biologic theme that they play roles in feedback inhibition of T-cell responses when their cognate ligands are present. In addition to providing inhibitory signals to activated effector T cells, some of these receptors, such as LAG-3, are highly expressed on Tregs, where they are important to amplify their inhibitory activity.92 This implies that, as with CTLA-4 and PD-1, these receptors play a dual role in ultimately inhibiting effector immune responses and blocking antibodies, therefore have multiple potential mechanisms of action.

LAG-3 was cloned over 20 years ago as a CD4 homologue,90 but its function in the immune checkpoint was only defined in 2005, when it was shown to play a role in enhancing Treg function.93,94 LAG-3 also inhibits CD8 effector function independently of its role on Tregs.95 The only known ligand for LAG-3 is major histocompatibility complex class II molecule (MHCII), which is upregulated on some epithelial cancers (generally in response to γ-interferon), but is also expressed on tumor-infiltrating macrophages and dendritic cells. The role of the LAG-3/MHCII interaction in LAG-3-mediated inhibition of T-cell responses is unclear, since anti-LAG-3 antibodies that do not block the LAG-3/MHCII interaction nonetheless enhance T-cell proliferation and effector function in vitro and in vivo. The MHCII interaction of LAG-3 may be most important for its role in enhancing Treg function. LAG-3 is one of a number of immune checkpoint receptors coordinately upregulated on both Tregs and anergic T cells, and simultaneous blockade can result in enhanced reversal of this anergic state relative to blockade of either receptor. In particular, PD-1 and LAG-3 are commonly coexpressed on anergic or exhausted T cells.95,96 Dual blockade of LAG-3 and PD-1 provide synergy in reversing anergy among tumor-specific CD8 T cells, as well as virus-specific CD8 T cells, in the setting of chronic infection. Dramatic evidence of the effects of coordinate T-cell inhibition by PD-1 and LAG-3 comes from PD-1/LAG-3 double KO mice, which completely reject even poorly immunogenic tumors in a T-cell-dependent fashion, but also develop autoimmune syndromes much more quickly than PD-1 or LAG-3 single knockouts, which are ultimately fatal (though not as quickly as CTLA-4 KOs).97 These findings emphasize the balance between antitumor effects and autoimmune side effects that must be taken into consideration in all of the immune checkpoint blockade strategies.

Tim-3, the ligand of which is galectin-9 (a galectin reported to be upregulated in a number of cancer types, such as breast cancer) inhibits type 1 T-cell (T1) responses and anti-Tim-3 antibodies enhance antitumor immunity.98 Tim-3 has also been reported to be coexpressed with PD-1 on tumor specific CD8 T cells and dual blockade of both molecules significantly enhances the in vitro proliferation and cytokine production of human T cells when stimulated by the NY-ESO-1 cancer-testis antigen. In animal models, coordinate blockade of PD-1 and Tim-3 was reported to enhance antitumor responses and tumor rejection under circumstances where only modest effects from blockade of each individual molecule were observed.99-100 BTLA was first identified as an inhibitory receptor on T cells based on enhanced T-cell responses observed in the BTLA KO mice.101 Subsequently, herpes virus entry mediator (HVEM), which is expressed on certain tumor cell types (e.g., melanoma), as well as on tumor-associated endothelial cells, was demonstrated to be the BTLA ligand.102 This is a rare case in which a TNF family member interacts with an immunoglobulin supergene family member. BTLA expression on activated virus-specific CD8 T cells is relatively low, but it has been demonstrated to be much more highly expressed on tumor-infiltrating lymphocytes from melanoma patients. BTLA T cells are inhibited in the presence of its ligand, HVEM. Thus, BTLA may also be a relevant inhibitory receptor for T cells in the tumor microenvironment.103 The system of HVEM interacting molecules is complex; two additional interacting molecules, CD160 (an immunoglobulin superfamily member) and LIGHT (a TNF family member) appear to mediate inhibitory and costimulatory activity respectively. It also appears that signaling can be bidirectional, depending on the specific combination of interactions. The complexity of this system makes therapeutic inhibition strategies less straightforward than other inhibitory receptors or ligands, though dual blockade of BTLA and PD-1 clearly enhances antitumor immunity.104

A2aR inhibits T-cell responses, in part by driving CD4 T cells to express Foxp3 and develop into Tregs.105 KO of this receptor results in enhanced and sometimes pathologic inflammatory responses to infection. This receptor is particularly relevant in tumor immunity, because the rate of cell death in tumors from cell turnover is high and dying cells release adenosine. In addition, Tregs express high levels of the exoenzymes CD39, which converts extracellular adenosine triphosphate (ATP) to adenosine monophosphate (AMP), and CD73, which converts AMP to adenosine.106 Given that A2aR engagement by adenosine drives T cells to become Tregs, this can produce a self-amplifying loop within the tumor. Indeed, tumors grow more...
slowly in A2aR KO mice, and tumor vaccines are much more effective against established tumors in these mice. A2aR can be inhibited either by antibodies that block adenosine binding or by adenosine analogues, some of which are fairly specific for A2aR. While these drugs have been used in clinical trials for Parkinson’s disease, they have not yet been tested clinically in cancer patients.

Killer inhibitory receptors are a broad category of inhibitory receptors that can be divided into two classes based on structure: killer immunoglobulin receptors (KIR) and C-type lectin receptors, which are type II membrane receptors. These receptors were originally described as critical regulators of the killing activity of NK cells, though many are expressed on T cells and APCs. The importance of their inhibitory role on T cells and APCs (ie, dendritic cells) is less well studied, but the resulting activation of NK cells can provide potent antitumor activity. Many of the killer inhibitory receptors are specific for subsets of human leukocyte antigen (HLA) molecules and possess allele-specificity. However, other receptors recognize broadly expressed molecules, for example, the C-type killer cell lectin-like receptor G1 (KLRG1) recognizes e-cadherin. The potential value of NK cells in antitumor responses when their inhibitory receptors are not appropriately engaged is best exemplified by the significantly enhanced graft-vs-tumor effects in allogeneic bone marrow transplants, elicited by mismatches between donor NK inhibitory receptors and recipient HLA alleles. The big question in therapeutic blockade of NK inhibitory receptors is this: among >20 receptors, which should be targeted?

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Après des décennies de recherche et d’études cliniques visant à exploiter le système immunitaire du patient cancéreux pour attaquer son propre cancer, l’efficacité clinique des vaccins et des inhibiteurs de points de contrôle immuns est désormais démontrée. Ces toutes dernières années ont donc été un tournant dans le traitement du cancer en confirmant la viabilité de l’immunothérapie active. Les découvertes cellulaires et moléculaires fondamentales liées à l’activation du système immunitaire tout comme l’étude du micro-environnement de la tumeur pour identifier des mécanismes de résistance attaquables directement, ont entretenu ces avancées. Les travaux à venir cibleront les nombreuses voies de la régulation immunitaire et développeront des associations conçues de façon rationnelle.

**Immunothérapie cancéreuse par blocage des points de contrôle : le traitement anti-cancéreux d’avenir**

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Targeting B7-H3 in cancer

by S. Koenig, USA

B7-H3 is a phylogenetically conserved protein with varied biological functions. In cancer biology, it appears to promote tumor cell invasion and metastasis and may modulate normal immune cell function. Expression of B7-H3 is pervasive in many solid tumors and its expression appears to be correlated with poorer clinical outcomes in some tumor types. Within both primary and metastatic tumors, B7-H3 may be expressed on multiple cell types, including the differentiated tumor cells, the tumor-initiating or cancer stem cells, and cells of the tumor vasculature. With promising clinical results using monoclonal antibodies directed to other members of the B7 family and their associated immune checkpoint coreceptors expressed on T cells, MacroGenics and Servier are pursuing the clinical development of an Fc-modified monoclonal antibody to B7-H3, called MGA271, which may impede tumor cell growth by various mechanisms. Phase 1 clinical studies are under way with the anticipated start of phase 2 development in 2015.

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B7-H3 was first identified in 2001 from an analysis of a human expression database as a protein that was initially thought to function as a stimulator of T cells. Shortly thereafter, the naturally expressed form of human B7-H3 was revealed to encode a longer protein. Since the initial reports, B7-H3 has emerged as an important regulatory molecule for both normal and pathological conditions. The interest in B7-H3 is derived from its pleotropic effects in vertebrates spanning teleost fish to man with evidence of important biological functions in the emerging embryo through the aging adult. These include effects on fecundity and maturation of the embryo, skeletal system development, immune system function and modulation, and tumor cell invasion and metastasis. How a single protein could have such diverse functional roles is still an evolving question, but in large part is likely to be determined by mechanisms that regulate its expression, the sites in which it appears, and its putative receptors.

The B7 family and immune checkpoint inhibition

B7-H3 shares structural homology (approximately 20% to 30%) with other members of the B7 family, which are expressed to varying degrees on the cell surface of antigen-presenting cells of the immune system, but differ in their distribution on other cell types. A growing interest in B7-H3 has coincided with a large body of data published during the last few years in the rapidly developing field of immuno-oncology. Inhibition of immune checkpoints by monoclonal antibodies bound to inhibitory
coreceptors expressed by T cells (eg, cytotoxic T-lymphocyte antigen 4 [CTLA-4] or programmed death-1 [PD-1]) or directed to their ligands (eg, PD-1 ligand [PD-L1]) within the B7 family resulted in powerful antitumor effects in several solid cancers in human clinical studies (Table I). 5,8

<table>
<thead>
<tr>
<th>Antigen-presenting cell or tumor</th>
<th>Activity on T cell</th>
<th>T-cell coreceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80 (B7-1)</td>
<td>(+)</td>
<td>CTLA-4</td>
</tr>
<tr>
<td>CD80 (B7-2)</td>
<td>(-)</td>
<td>CD28</td>
</tr>
<tr>
<td>CD86 (B7-1)</td>
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<td>CD86</td>
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<td>CD86 (B7-2)</td>
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<td>CD80</td>
</tr>
<tr>
<td>PD-L1 (B7-H1)</td>
<td>(-)</td>
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</tr>
<tr>
<td>PD-L2 (B7-DC)</td>
<td>(+)</td>
<td>ICOS</td>
</tr>
<tr>
<td>B7RP1 (B7-H2)</td>
<td>(+)</td>
<td>B7-H3</td>
</tr>
<tr>
<td>B7-H4</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td>B7-H5 (VISTA)</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td>B7-H6</td>
<td>(Nonreactive)</td>
<td>(NK cell receptor:p30)</td>
</tr>
</tbody>
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Table I. Members of the B7 family of immune regulators.
Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen 4; ICOS, inducible costimulator; NK, natural killer; PD-1, programmed death-1; VISTA, V-domain Ig superfamily member 6.

Significant numbers of patients with usually fatal tumors, such as melanoma, were apparently cured of their cancers.9 Recent data have demonstrated even more profound clinical effects when such therapeutics are combined.10 Thus, there is overwhelming interest not only to understand the best circumstances in which current clinical candidates directed to immune checkpoint molecules or their ligands can be exploited for therapeutic use, but to identify additional members of the B7 family and their receptors that could be targeted for clinical translational purposes.

B7-H3 and adaptive immunity
The initial focus on B7-H3 was prompted by its expression on cells of the immune system, particularly activated dendritic cells (DCs),11 and the manner in which it could modulate an endogenous adaptive immune response. Shortly after the report of the costimulatory properties of B7-H3 in enhancing T-cell proliferation, interferon (IFN)-γ induction, and cytotoxic T-lymphocyte responses, contradictory data began to emerge indicating that B7-H3 expression could inhibit T-cell–dependent responses, including studies performed in mice with knockouts of the B7-H3 gene.12 In particular, B7-H3–deficient mice developed more severe airway inflammation, earlier onset of experimental autoimmune allergic encephalomyelitis, and higher concentrations of anti-DNA autoantibodies compared with B7-H3–bearing animals.13 T-cell stimulation elicited with either anti-CD3 or allogeneic cells was inhibited by the presence of B7-H3, but this suppression could be compensated by costimulation of CD28 expressed on T cells. Similarly, in carefully conducted studies with human cells, B7-H3 suppressed T-cell proliferation and activation, especially in CD4+ T cells; however, attenuation of the inhibition was observed by the addition of interleukin 2 at the inception of the T-cell cultures.14 Beyond its effects on T cells, B7-H3 has been reported to inhibit natural killer (NK)-cell cytotoxic function, possibly by binding to an unidentified inhibitory receptor on NK cells.15

Various explanations could account for the observed disparity in the reported functional roles of B7-H3. It could be ascribed in part to structural differences within the molecule in humans compared with mice, the focus of most investigation of functional studies. Mouse B7-H3 is a type 1 transmembrane protein containing 316 amino acids encoded by 2 immunoglobulin (Ig)-like exons; in humans, a duplication of the B7-H3 exons resulted in a larger 534-amino-acid molecule with 4 Ig-like domains (2 pairs of IgV-IgC), which in theory also could be alternatively spliced and expressed as a 2Ig-like protein, although evidence for significant expression of the latter is not well supported.16 Thus, the size, conformation, and avidity of human B7-H3 compared with the smaller murine version of this protein could account for differences in modulating immune responses. Indeed, in 1 report, cells constructed to express the 2Ig form of B7-H3 led to immune stimulation of human and mouse T cells, while other cells bearing 4Ig-B7-H3 molecules suppressed human T-cell responses.2 However, in a recent report describing the X-ray crystal structure of the murine B7-H3, inhibition of T-cell proliferation was observed with the protein used for crystallization and the functional inhibitory activity was mapped to a particular domain (ie, FG loop) of the B7-H3 molecule.17

B7-H3 is highly glycosylated with 4 predicted N-linked glycans increasing the size of the extracellular domain in the case of murine B7-H3 from 24 kDa to 40 kDa and in humans, up to about 100 kDa.17 It is possible that modifications in carbohydrate form or content among various tissue types, or with-

**Selected abbreviations and acronyms**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>Jak2</td>
<td>Janus kinase 2</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>MGA271</td>
<td>Fc-optimized, humanized, monoclonal antibody to B7-H3</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
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<td>programmed death-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>PD-1 ligand</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
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in pathological tissues (such as tumors), or in different species could affect how B7-H3 engages other molecules and receptors. Most importantly, however, biological responses to B7-H3 could be significantly influenced by the particular receptors bound and engaged. Such receptors could program either activating or inhibitory responses and the outcomes could vary depending on receptor density and cell distribution. Although 1 putative receptor in mouse, called triggering receptor expressed on myeloid cell (TREM)-like transcript 2, has been reported,13 subsequent studies could not substantiate these findings either in mouse or human systems.14 In the absence of a confirmed receptor, it is therefore difficult to speculate on the absolute functional role for B7-H3 in normal and pathological conditions, especially when studied in isolation from other B7 family members and their coreceptors, which also participate in regulating human responses.

**B7-H3 in cancer**

In the context of cancer biology, a substantial literature has appeared over the past 5 years correlating the broad overexpression of B7-H3 on many solid cancers either with poorer clinical outcome or more advanced disease in these patients. These studies conducted by clinicians and pathologists include investigations of patients with prostate, ovarian, breast, colon, renal, non-small cell lung, pancreatic, and head and neck cancers, as well as melanoma, glioblastoma, and neuroblastoma and other small round blue cell tumors of childhood.10-13 In some of these studies, the poorer prognosis inversely correlated with immune infiltrates in the tumor, implying an untoward effect of B7-H3 expression with the generation of antitumor immune responses or migration or proliferation of inflammatory cells to the sites of tumors expressing B7-H3. Rarer studies (eg, those involving gastric and pancreatic cancer) have described improved outcome in association with B7-H3 expression. A notable observation is that for some solid tumors (eg, glioblastoma, renal cell, and ovarian carcinoma), B7-H3 is highly expressed within the tumor vasculature or in tumor-initiating cells of the central nervous system, and in these studies, patients were likely to have worse outcomes.32

**Expression of B7-H3 in normal vs cancerous tissues**

B7-H3 mRNA can be detected in various normal tissues, but the expression of B7-H3 protein in the healthy adult is very restricted. The disparity of ubiquitous detection of B7-H3 mRNA in normal and tumor tissues with the preferential expression of B7-H3 protein only in tumors may in large part be related to the levels of microRNA (miRNA)-29 in these tumors. An inverse relationship between miRNA-29 levels and B7-H3 protein was observed in normal and tumor tissues and cancer cell lines32: knock-in and knockdown experiments of miRNA-29 also led to downregulation and upregulation of B7-H3 protein, respectively, in tumor cell lines. In patients with melanoma, mRNA levels of B7-H3 were relatively increased in later-staged disease and there was an associated inverse expression of miRNA-29c, suggesting that the reduced miRNA-29 may be largely responsible for promoting B7-H3 expression in cancer cells.32 Furthermore, the increased levels of B7-H3 in melanoma correlated with phosphorylated signal transducer and activator of transcription 3 (STAT3) activity. An additional study linking B7-H3 with the STAT3 signaling pathway indicated that chemoresistance to paclitaxel in breast cancer was associated with B7-H3 expression and phosphorylated STAT formation; further analysis also showed dependency on upstream signaling through phosphorylated Janus kinase 2 (Jak2) and the downstream expression of myeloid leukemia-1 (Mcl-1) and survivin, which affect apoptosis in the cancer cells.34 Similarly, increased sensitivity of a pancreatic cell line to gemcitabine was linked to reduced B7-H3 expression in vitro and in vivo and this was associated with higher levels of survivin, which promoted tumor cell death by apoptosis.35

**B7-H3 as a targeted therapeutic in cancer**

In selecting and exploiting a particular cancer-associated protein, like B7-H3, for the purpose of developing targeted therapeutics, different parameters should be considered. Some of these criteria may include whether or not a protein target of interest increases tumor growth, avoids triggering pathways which promote apoptosis or cell death, enhances tumor cell migration or metastasis, fosters new vessel formation, or evades immune surveillance mechanisms. In this context, B7-H3 may contribute to the formation of a cancer by: (i) altering a signaling pathway within cancer cells (and possibly within tumor-initiating stem cells), rendering them insensitive to intracellular molecules that promote cell death, partly through the JAK2/STAT3/survivin-dependent pathway; (ii) enhancing cancer cell migration and invasion of underlying tissues or stromal components, participating in the Jak2/STAT3/survivin-dependent pathway; (iii) fostering neovascularization; (iv) promoting metastasis as a consequence of enhanced mobility and vessel formation; and (v) modulating the adaptive and innate immune responses during the evolution of the cancer from a primary lesion through its dissemination.

Given these varied properties, B7-H3 may be ideal for designing targeted therapeutics with a range of modalities. Furthermore, since B7-H3 expression appears to be limited in its distribution in normal tissues compared with tumors, this may contribute to a more favorable therapeutic window, with diminished potential for triggering of immune responses to normal tissues in conjunction with antibody-mediated blockade of B7-H3. Similarly, a cytotoxic therapeutic approach could also be contemplated as it can, in theory, more effectively avoid damage to the body's normal physiology and function, a safety issue for many cancer treatments.

Which targeted therapeutic approaches might have promise in treating B7-H3–positive malignancies? Independent of its effects on antitumor immune responses, the majority of the published clinical literature indicates that reducing B7-H3 expression on the tumor cells and tumor vasculature should be beneficial. Current genetic methods using short-hairpin RNA
(shRNA) and RNA interference (RNAi) to inhibit B7-H3, while useful tools in the laboratory, are currently impractical for targeting the widely distributed intracellular B7-H3 transcripts due to the inefficiency of these agents and the large copy numbers of inhibitors that would be required.

Another tactic could involve modulating B7-H3 molecules on the surface of cancer cells by an antibody, ligand, or even a small molecule, either by promoting the shedding of B7-H3, or through its reuptake and destruction within the tumor’s phagolysosomes. The loss of membrane-associated B7-H3 would abrogate any receptor-dependent signaling (of both the tumor cell itself and the putative coreceptors for B7-H3 expressed on immune cells). This latter approach assumes that there would be saturation of membrane-associated B7-H3 and its loss in expression would not be compensated by redistribution of molecules from the intracellular pool (often present in B7-H3-positive tumors) to the cell surface. It also presumes that only the membrane pool of B7-H3 promotes its tumorigenic effects, while any intracellular pool is inactive. A variation of this approach would be the blockade of stably

Table II. Expression of B7-H3 in different types of cancer. B7-H3 expression defined by evidence of specific staining with murine monoclonal BRCA69D in ≥10% of tumor cells and/or ≥25% of associated vasculature, with intensity criteria as follows: Neg (negative); 1+ (weak); 2+ (moderate); 3+ (strong). The B7-H3 positive rate = 1+ sample numbers/total tested sample numbers x100%; 2+ or above positive rate = ≥2+ sample numbers/total tested sample numbers x100%.

*Also target expression in tumor vasculature
† Triple negative: 8/17 positive, 2+ or above (47%)
expressed, membrane-bound B7-H3 molecules. Such an approach could mimic the therapeutic successes achieved with an antibody for PD-L1, the B7 ligand for PD-1, the inhibitory coreceptor on T cells.

Antibody-based treatments could be adopted for targeting B7-H3–expressing tumors by building on the recent clinical successes of coupling antibodies to cytotoxic agents for the treatment of lymphoma (ie, brentuximab vedotin) and breast cancer (ie, trastuzumab emtansine) or antibodies linked to radioisotopes, a method used for diagnostics as well as therapeutics for cancers. In fact, a cytotoxin conjugate has shown promise in treating glioblastoma, breast cancer, and osteosarcoma in small animal models, where a single-chain antibody to B7-H3 linked to a recombinant Pseudomonas immunotoxin had antitumor effects and survival benefit. Likewise, an 131I-labeled monoclonal antibody to B7-H3 was used to treat patients with intracerebral metastatic neuroblastoma with evidence of objective responses in some patients.

Antibodies or antibody fragments that amplify their inherent immunological properties could be exploited for the killing of B7-H3–positive cancers. These could include complement-mediated cytolysis and antibody-dependent cellular cytotoxicity (ADCC). In fact, a monoclonal antibody to CD20 (ie, obinutuzumab) with modifications of its Fc domain to enhance effector function was recently approved for the treatment of mantle cell lymphoma. Other more experimental approaches, such as cell-based immunizations, antibodies to B7-H3 generated by cell-based immunizations, and an antibody for PD-L1, the B7 ligand for PD-1, the inhibitory coreceptor on T cells.

In devising our lead therapeutic targeting B7-H3, we chose an initial approach that in principle could impede the suppressive effects on the adaptive (ie, T-cell mediated) immune response. In our characterization of about 50 monoclonal antibodies to B7-H3 generated by cell-based immunizations, we identified an antibody to a particular epitope that was found on most solid tumors, but had extremely limited expression on the 33 normal tissues examined by immunohistochemistry (Figure 1 and Table II).

Given the selective tumor-binding specificity of this antibody, it gave us an opportunity to combine its potential to mediate antibody-dependent cellular cytotoxicity. Such cytotoxic activity could be directed toward the cancer cells, a pool of tumor-initiating or cancer stem-like cells, and any newly formed vessels within the tumor (Figure 2).

A chimeric version of this monoclonal with a human Fc domain was shown to mediate ADCC against tumor cell lines with human peripheral blood mononuclear cells from healthy donors as a source of effector cells. Subsequently, the variable domains of this monoclonal were humanized and we substituted 5 amino acids in non-surface-exposed positions within the Fc domain, which had been shown previously to enhance ADCC activity with other antibody specificities (eg, human epidermal growth factor receptor 2 [HER2]). This modified Fc domain substantially increases binding to an activating Fc receptor expressed by effector cells such as NK cells and macrophages (ie, CD16) and reduces binding to an inhibitory Fc receptor (ie, CD32B). This resulted in enhancement of ADCC activity in vitro and was particularly pronounced when effector cells were obtained from subjects with an allele of CD16 that shows diminished binding to and affinity for the native IgG1 Fc wild-type sequence (Figure 3, page 290). Moreover, the magnitude of ADCC activity was comparably effective against many different cancer cell lines, relatively independent of the density of B7-H3 expression, consistent with our previous reported experiences with other Fc-modified antibodies.

To determine whether this enhanced ADCC function translated into improved in vivo activity, immunodeficient mice engineered to be deficient in murine CD16 and expressing a transgene of the human CD16 low-binding allele were engrafted with 7 different human tumor cell lines and then treated with varying concentrations of MGA271, the humanized, Fc-optimized, monoclonal antibody to B7-H3; NK, natural killer.
Toxicology studies in cynomolgus monkeys treated either with a single administration or 4 once-weekly doses of MGA271 showed no significant adverse effects. Currently, a multicenter phase 1 dose-escalation clinical study in the United States is being conducted with MGA271 in patients who have failed standard therapies and whose tumors express B7-H3. In the first segment of the study, 26 patients with 15 different tumor types were treated with doses of MGA271 from 0.15 mg/kg to 15 mg/kg intravenously with up to 4 weekly doses during the initial cycle of therapy and no dose-limiting toxicity was observed. In the current phase 1B segment of the study, an increased number of patients (ie, 45) with particular tumor types are being enrolled and treated with 15 mg/kg of MGA271 weekly until progression of tumor is observed. It is anticipated that phase 2 development will begin in 2015 in multiple tumor types and in combination with other agents which may complement the biological activity of MGA271.

In conclusion, there is strong scientific and clinical rationale for pursuing directed therapeutics to B7-H3. As in most successful treatments of cancers, a combination of strategies, including immune-based and cytotoxic agents directed to B7-H3–expressing tumors, may result in the most favorable outcomes for patients.

Acknowledgments: I would like to thank the team at MacroGenics for the development of MGA271 (including the enclosed figures) and Drs Ezio Bonvini, Paul Moore, and Jon Wigginton for reviewing the manuscript, and Ms Melinda Hanson for her assistance in preparing this submission.

Keywords: B7-H3; cancer; checkpoint inhibitors; immunotherapy; monoclonal antibody
Targeting B7-H3 in cancer – Koenig

References


LE CIBLAGE DE LA PROTÉINE B7-H3 DANS LE CANCER

B7-H3 est une protéine phylogénétiquement conservée, aux fonctions biologiques variées. En oncologie, elle semble favoriser l’invasion des cellules tumorales et les métastases et pourrait moduler la fonction cellulaire immunitaire normale. B7-H3 s’exprime dans de nombreuses tumeurs solides et son expression est corrélée à des évolutions cliniques plus défavorables dans certains types de tumeurs. Que ce soit dans des tumeurs primaires ou métastatiques, B7-H3 s’exprime sur de nombreux types cellulaires, comme les cellules tumorales différenciées, les cellules souches cancéreuses ou à l’origine d’une tumeur et les cellules du système vasculaire tumoral. Compte tenu des résultats cliniques prometteurs réalisés avec des anticorps monoclonaux dirigés vers d’autres membres de la famille B7 et leurs corécepteurs des points de contrôle (checkpoints) immunitaires associés, exprimés sur les cellules T, MacroGenics et Servier poursuivent le développement clinique d’un anticorps monoclonal à fragment Fc modifié (fragment constant cristallisable) ciblant B7-H3, appelé MGA271, qui pourrait empêcher la croissance de la cellule tumorale par divers mécanismes. Des études cliniques de phase 1 sont en cours, avec un début prévu du développement de phase 2 en 2015.
Epigenetics and cancer

Epigenetic defects in cancer

by R. Chaligné and E. Heard, France

Epigenetics concerns heritable changes in gene expression that are not linked to changes in the DNA sequence. Potential epigenetic regulators range from chromatin-associated proteins to DNA methylation and non-coding RNAs. Groundbreaking research over the last half century has revealed the importance of epigenetic mechanisms in development and disease. The investigation of normal processes such as X-chromosome inactivation and genomic imprinting has enabled a deeper understanding of the molecular basis of epigenetic mechanisms, and this knowledge can now be used to explore disease. Indeed, disruption of epigenetic control is frequent in cancer and the potential for reversal of epigenetic changes, contrary to genetic changes, means that epigenetic-based therapies are increasingly being considered in the treatment of cancer. Here, we provide an overview of some of the links between epigenetics and cancer.

Epigenetics was first coined by Conrad Waddington and, in one of its more recent definitions, concerns heritable changes in gene expression or gene function that are not due to changes in DNA sequence. Our understanding of epigenetic mechanisms included in this definition, has radically increased in the last few years with the arrival of breakthrough technologies. Although all cells within an organism contain the same genomic DNA, numerous epigenetic regulators and transcription factors organize the genome into accessible and closed chromatin, to ensure a correct and specific transcriptional program in a given cell type. Chromatin is a macromolecular complex of DNA and proteins, which not only provides a scaffold for the packaging of our genome into the cell nucleus, but also influences gene expression and genome functions, such as DNA replication and DNA repair. It can also act as a scaffold for epigenetic information that is heritable through cell divisions, both at the level of the DNA (eg, DNA methylation), or at the level of histones and other proteins associated with them. The nucleosome is defined as the basic functional unit of this macromolecular complex. Basically, 147 base pairs of DNA are wrapped around 8 histone proteins, composed of two of histone H2A, H2B, H3, and H4. We generally divide chromatin into two different states, heterochromatin that is highly condensed, late replicating, and prin-
The components of the different types of heterochromatin and euchromatin are subject to covalent modifications that are thought to contribute to their specificities. Today, at least four different DNA modifications, numerous histone variants, and several tens of different classes of histone modifications have been identified (Figure 2).\(^1\)\(^-\)\(^4\) Such variants and modifications can influence chromatin organization and DNA accessibility to transcription factors, by changing noncovalent interactions within and between nucleosomes. They can also act as docking sites for "reader" proteins that bind to them, either alone or in combination, and such "reader" proteins can recruit further chromatin modifiers and remodeling chromatin complexes. The ensemble of DNA-based processes, including transcription, DNA replication, and DNA repair, can be influenced by such chromatin states. Mutations or abnormal expression level of chromatin regulators can have a major impact on the regulation of epigenetic mechanisms, leading in some case to abnormal development or to disease. Indeed, there is an increasing realization that epigenetic changes may be tightly linked to cancer. On the one hand, epigenetic modifications such as global changes in DNA methylation and chromatin structure clearly accompany tumorigenesis. This correlates with aberrant gene expression...
Epigenetics and cancer

Epigenetic regulators of DNA methylation

- DNA methyltransferases (DNMTs)

- DNA hydroxylases (e.g. TET proteins)

Epigenetic regulators involved in histone acetylation

- Deacetylases (HDACs)

- Acetyltransferases (HATs)

Epigenetic regulators involved in histone methylation

- Demethylases (HDMs)

- Methyltransferases (HMTs)

Epigenetic regulators involved in histone phosphorylation

- Phosphatases

- Kinases

Epigenetic regulators involved in chromatin remodeling

- SWI/SNF complex

Chromatin remodeling complex

Examples of mutated genes in cancer

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<th>Tumor</th>
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as well as genetic instability, although whether such epigenetic changes are cause or consequence is not always clear. On the other hand, epigenetic changes may underlie tumor initiation, through the appearance of epimutations that, for example, result in aberrant repression of tumor suppressor genes or expression of oncogenes. The degree to which epigenetic changes are involved in cancer and can be used to assess the tumor state are active areas of research.

Epigenetics and epigenomics of cancer

The recent decrease in next generation DNA sequencing costs, coupled with the improvement in techniques, such as chromatin immunoprecipitation, have provided an unprecedented view of the genomes and epigenomes of normal and cancer cells.\(^5\) We can now readily analyze DNA modifications,\(^5\) histone variants and posttranslational modifications,\(^5\) transcription factor binding sites,\(^5\) and chromosome conformation,\(^5\) and have a more comprehensive view of nucleosome positioning.\(^10\) Transcriptome analyses, using RNaseq, have revealed that a large portion of our genome is transcribed, and several non–protein-coding RNAs have been identified. Given work on processes such as X inactivation and imprinting, where long noncoding RNA (lncRNA) play key roles,\(^11\) the discovery of numerous other lncRNAs suggests that some of them may have an important function in epigenetic regulation.\(^12\) The first suggestion that epigenetic instability might be involved in cancer dates from the 1970s and 1980s.\(^13\) Subsequent analyses of gene expression and DNA methylation patterns revived interest in epigenetic misregulation in cancers, as they revealed both broad and more punctual alterations in epigenomic landscapes (see reference 14 for review). Nevertheless, these studies were mainly correlative up until recently. Insights into a direct role of epigenetic processes in cancer came from recent whole genome sequencing studies of numerous cancer types, notably through The Cancer Genome Atlas (TCGA) consortium, where sets of somatic mutations in epigenetic regulators were identified.\(^15,16\) A high frequency of somatic mutations in genes coding for chromatin-associated proteins that are known to regulate DNA methylation patterns, histone posttranslation modifications, and chromatin remodeling have been found. Such changes might be “driver” mutations in the process of carcinogenesis. For example, \textit{KDM6A}, an X-linked histone demethylase, is mutated in up to 12 distinct cancers (Figure 2).\(^17\) Another example is the histone methyltransferase, \textit{KMT2B} which has been found to be mutated in almost 90% of non–Hodgkin lymphoma patients.\(^18\) In glioma, the histone variant H3.3 has been found mutated with single codon changes within its N-terminal tail, which is the target for posttranslational modifications.\(^19\) Another important recent discovery is that patients with leukemia often have mutations in genes such as \textit{TET2}, \textit{IDH1}, \textit{IDH2}, and \textit{DNMT3A}, which are all involved in regulating DNA methylation patterns. This has provided insight into why patients with leukemia show a significant response to DNA methylation inhibitors (see below), and represents a promising avenue for future patient stratification strategies. Polycomb group proteins, such as the EZH2 histone methyltransferase, together with the histone H3K27 methylation modification it lays down, have been frequently correlated with cancer, although their exact roles are still not clear. Indeed, EZH2 is thought to promote or inhibit tumorigenesis in a context-dependent manner. This may also be the case for several epigenetic modifiers that may have rather different influences on gene expression and cell proliferation depending on their exact partners, protein complex stoichiometry, as well as their target chromatin landscape.

In addition to the identification of epigenetic modifiers as potential drivers in cancer, the genome-wide mapping of chromatin modifications, thanks to highly specific antibodies, has provided further insights into the nature and extent of epigenetic abnormalities in cancer. For example, comparisons of DNA methylation profiles, and the binding regions of chromatin regulators and histone modifications in human cancers has revealed a link between hypermethylated gene promoters and genes with a particular “bivalent” status for histone modifications in cancer cells.\(^20,21\) Indeed, bivalent genes show an enrichment of both the H3K4me3 mark (usually associated with euchromatin) and the H3K27me3 mark (associated with facultative heterochromatin; Figure 1).\(^22\) This bivalent chromatin state had been previously associated with genes in embryonic stem cells that are poised to show lineage specific expression patterns during differentiation and opened up the possibility that in cancer, the bivalent state represents a similar poised and possibly dedifferentiated state. Furthermore, by comparing normal and tumor tissue from the same individual, epigenomic studies have discovered intriguing profiles showing altered DNA methylation and H3K9 methylation profiles, in large regions spanning several hundreds of kilobases, termed large organized chromatin K-modifications (LADs), often corresponding to lamin-associated domains (LADs).\(^23,25\) During normal development or cellular stress processes, such as following injury, changes in LADs are thought to reflect or even contribute to cellular plasticity. It has been proposed that such changes in cancer cells may reflect or contribute to their increased plasticity. Furthermore, large domains of H3K27me3, H3K9me2/3, and DNA methylation, identified in bladder and other cancers,\(^23,26,27\) have been associated with coordinated repression of the genes within them, and this has been correlated with particular types of tumor prognosis in some cases.\(^28,29\) Why and how these particular genomic regions are more vulnerable to epigenomic perturbations is still a mystery, but could be due to aberrant targeting of epigenetic complexes to regions that are clustered in the nucleus, such as topologically-associated domains, or to perturbation of long-range regulatory sequences (see reference 30 for review).

Epigenetics and nuclear disorganization in cancer

Global perturbations in heterochromatin and unusual nuclear architecture are hallmarks of cancer that have been tradition-
ally used by pathologists in tumor classification (Figure 1). An example of this concerns the disappearance or disruption of the heterochromatic Barr body, or inactive X chromosome, that has long been associated with the most aggressive breast tumors by clinicians. The extent to which the disappearance of the Barr body is linked to more general nuclear disorganization, chromatin disruption as opposed to physical loss of the inactive X chromosome, remains to be found. Although some studies reported that in certain types of tumor (eg, basal-like molecular subtype breast tumors), the Barr body loses association with the non-coding X-inactive specific transcript (XIST; responsible for triggering its inactivation) and becomes euchromatic, others have reported that the Barr body remains inactive and XIST RNA coating can still be found, even in basal-like tumors. Nevertheless, the epigenetic instability and degree of gene reactivation from the inactive X chromosome and other types of facultative heterochromatin in cancer have not been systematically investigated to date.

Epigenetic therapy in cancer

Given the evidence that epigenetic changes are a frequent feature in cancer, and that in some cases the misregulation of cancer-related genes may be at the epigenetic rather than at the genetic level (epimutation vs DNA sequence mutation), this provides great promise for cancer treatment. Indeed, epimutations have the potential to be reversed via chemical agents, known as “epidrugs”, unlike genetic mutations, which are essentially irreversible. Such epidrugs are at various stages of development and some are already being used as therapeutic agents in cancer treatment. The first epidrugs that were approved by the US Food and Drug Administration (FDA) for cancer therapy target DNA methylation: azacitidine (5-aza-2’-deoxycytidine) and decitabine (5-aza-2’-deoxycytidine), both being nucleoside analogues and irreversible inhibitors of the DNA methyltransferase enzymes DNMT1 and DNMT3. These drugs are being used as first-line treatments for patients with myelodysplastic syndrome (see reference 37 for review). Subsequently, chemical inhibitors targeting histone deacetylases, HDACs (eg, SAHA and romidepsin or FK228), have been FDA-approved for treatment of refractory cutaneous T-cell lymphomas (see reference 38 for review). Such drugs are clearly successful in the clinic, although it is still not entirely clear what their relevant target genes are. For example, HDACs show poor enzyme specificity, their mechanism of action is still not fully understood, and so far there is no striking gene expression signature or profile that can predict whether a patient will benefit from the use of HDAC inhibitors. The situation is very similar for DNMT inhibitors, which have been shown to lead to global hypomethylation, although we still do not know their precise mechanism of action in a clinical context. For both types of epidrug, the lack of reliable molecular biomarkers for predicting either clinical activity or resistance is a serious drawback, limiting clinicians’ ability to achieve the vision of “personalized medicine.” Furthermore, so far these drugs have only been used with success in specific hematological cancers and their use for treatment of solid tumors has had limited success. Several pharmaceutical and biotech company research groups have also developed highly potent, selective, small molecule inhibitors of the H3K27me3 histone methyltransferase, EZH2, although there are potential drawbacks in inhibiting this enzyme given its context-specific action (see above). Thus, a major challenge for the use of such inhibitors in cancer will be to gain a better understanding of their mechanisms of action, and the biology of their target proteins.

Nevertheless, the investment so far in developing epidrugs has clearly paid off, as exemplified by the recent preclinical success of small molecule inhibitors of bromodomain-containing protein 4 (BRD4), an acetyl-lysine chromatin-binding protein. Recent studies revealed that several such bromodomain and extraterminal (BET) proteins are involved in cancer, and that they can be targeted by small molecule antagonists that directly bind to them and prevent the interaction of the bromodomain “reader” module to acetylated histones, thus preventing assembly of active gene transcriptional complexes at genes. Insights into the mechanism underlying the efficiency of inhibiting the BRD4 protein came from studies on the fusion of the bromodomain protein BRD4 with a nuclear protein in testis (NUT). This fusion-protein leads to the development of aggressive NUT midline carcinoma. Aberrant regulation of BRD4 has also been reported in other cancers, such as colon and breast tumors. The use of cell-based, high throughput screening of chemical libraries has led to the development of compounds that can selectively inhibit each of the four known BET proteins. The development of selective inhibitors that target such epigenetic “reader” proteins thus represents a very promising horizon for cancer therapy.

Concluding remarks

Epigenetic mechanisms are essential for normal development and are often perturbed in the context of cancer. The advent of new technologies has enabled hypotheses concerning the molecular origins of cancer to be confirmed, but has also opened up a new era of research. The belief that cancer is driven by genetic abnormalities remains true, however it is clear now that epigenetic pathways play an important function in cancer development. The hallmarks of cancer, such as self-renewal, differentiation blockade, escape from cell death, and invasiveness, may all be influenced by epigenetic processes in tumor cells. The development of genomic techniques, single-cell profiling, and highly specific tools for exploring epigenomic changes, as well as the use of specific inhibitors of epigenetic modifiers, opens up new horizons for an understanding of the molecular mechanisms underlying carcinogenesis and exciting perspectives for cancer treatment.

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Professor Edith Heard is a Professor at the Collège de France, and holds the chair of Epigenetic and Cellular Memory. She is noted for her studies of X chromosome inactivation. Since 2010, she has been the Director of the Genetics and Development Biology Unit at Institut Curie, Paris. In 2013, she was made Fellow of the Royal Society in recognition for her discoveries in epigenetics. She received the Prix Jean Hamburger in 2009 and the Grand Prix de la FRM in 2011. Professor Heard graduated from Cambridge University, UK, with a BA in Natural Sciences, and completed her PhD in cancer research at the Imperial Cancer Research Fund Laboratory in London, UK.

References

Keywords: Barr body; cancer; epigenetic; epigenomic; epimutation
ANOMALIES ÉPIGÉNÉTIQUES DANS LE CANCER

L’épigénétique relève des modifications transmissibles de l’expression génique qui ne sont pas liées aux changements de la séquence ADN. Les régulateurs épigénétiques potentiels vont des protéines associées à la chromatine à la méthylation de l’ADN en passant par les ARN non codants. La recherche innovante de ces cinquante dernières années a montré l’importance des mécanismes épigénétiques dans le développement et la pathologie. L’observation de processus normaux comme l’inactivation du chromosome X et l’empreinte génomique a permis de mieux comprendre les bases moléculaires des mécanismes épigénétiques et d’utiliser cette connaissance pour explorer les pathologies. En effet, les perturbations du contrôle épigénétique sont fréquentes au cours du cancer et la possibilité d’inverser les modifications épigénétiques, contrairement aux modifications génétiques, rend les traitements basés sur l’épigénétique de plus en plus intéressants dans le cancer. Nous donnons dans cet article un aperçu de certains liens entre épigénétique et cancer.
The genome exists naturally in a repressed state. Most of the DNA is occluded by nucleosomes assembled into highly condensed structures inaccessible to regulatory and functional proteins required for gene transcription initiation. Transient opening of chromatin regions is driven by chromatin remodeling factors and histone-acetyltransferases (HAT) associated with the polymerase complex. HAT action is reversed by histone deacetylases (HDAC). HDAC expression is modified in many tumors and their overexpression has been correlated with poor clinical outcome in certain cancers. In the past few years, HDAC inhibitors (HDACi) have been extensively studied as antitumor drugs. The HDACi vorinostat and romidepsine are approved in the US for the treatment of cutaneous T-cell lymphoma. Romidepsine has been approved for the treatment of peripheral T-cell lymphoma and belinostat is currently under priority review at the US Food and Drug Administration for the same indication. Vorinostat has also been registered in Japan. Therapeutic benefit of HDACi as monotherapy in solid tumors has not yet been demonstrated. Multiple clinical trials are currently under way to evaluate these agents alone or in combination with other antitumor strategies in hematological and solid tumors. However, better understanding of the pleiotropic antitumor effects of these molecules, as well as the identification of accurate predictive biomarkers, is still required to further develop these drugs in the future.

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www.medicographia.com

The major part of the genome exists as nucleosomes comprising ≈146 bp of DNA coiled 1.7 times around an octamer of histone proteins (H2A, H2B, H3, and H4 histones; two of each). Nucleosome stability is maintained by multiple contacts along the entire length of the nucleosomal DNA. The histone N-terminal tails protrude from the nucleosome particle, allowing availability of these sites for histone modifications that regulate the structure and function of chromatin. The genome is, by default, in a repressed state due to these highly condensed and inaccessible nucleosome structures.

Depending on stimuli received by the cell during cell differentiation, a concerted action of sets of transcription factors specific to each cell type and to their state of differentiation and activation, drives gene expression and thus protein synthesis. This becomes possible via the chromatin structure regulation driven by remodeling factors and histone-acetyltransferases (HAT) associated with the polymerase complex, which is reversed by histone deacetylase (HDAC) recruited to chromatin.
that has just been transcribed. This chromatin regulation mediated by the HAT and the HDAC, together with DNA methylation, constitute the major mechanisms of functionally relevant genome changes not linked to nucleotide sequence alterations, called epigenetics.1

Many disease stages have been found to involve epigenetic deregulation. Diseases of blood cells have constituted an invaluable field of investigation that has allowed a better understanding of epigenetic failures, leading to aberrant growth and differentiation and, ultimately, to malignant development. They have also provided insight into autoimmune or inflammatory diseases where the immune system is chronically active.

The main types of drugs that have been developed to target epigenetic deregulation in recent years are molecules interfering with methylation and acetylation enzymes. Even though reversible histone acetylation was discovered in 1964,2 it was only until 1996 that the first bona fide histone deacetylase, HDAC1, was isolated and cloned.3 Since then, structure and function of HDAC have been widely explored, and HDACi started to be developed and studied to treat a variety of conditions, such as neurological disorders, immune disorders, hematological diseases, HIV and other viral, bacterial, and parasite infections, graft vs host disease, and mainly cancer.4

Although inhibition of HDAC would be expected to result in a global increase in gene transcription, only 20% of all known genes are affected by HDACi. About 10% of them are down-regulated and the others are upregulated.5 It is now clear that HDACi antitumor activity is not limited to inhibition of histone deacetylation by HDACi, but also to inhibition of HDAC-mediated deacetylation of other proteins. This HDACi action leads to interference with HDAC-concerted regulation and interaction with transcription factors, signal-transduction molecules, DNA-repair proteins, chaperone proteins, and corepressors. The result of these HDACi pleiotropic effects is a multitude of antitumor activities, with exquisite tumor specificity. However, mechanisms and specificity of these activities are not fully understood. Indeed, HDAC isotype function does not seem to be redundant and differs depending on cell types.6

The latest efforts in HDACi development have focused on the design of molecules with improved HDAC specificity and pharmacokinetic profile, hoping to reduce their toxicity and increase their therapeutic benefit. Preclinical and clinical studies combining HDACi with other anticancer drugs have multiplied in the recent years. Whether these improvements will have an impact on clinical effect remains to be demonstrated. This review will attempt to give an overview of current HDACi clinical development strategies, as well as unsolved questions and future directions in this field.

**HDAC family**

HDAC are a family of enzymes found in numerous organisms, including bacteria, fungi, plants, and animals, that catalyze the removal of acetyl groups from ε-N-acetylated lysine residues of various proteins substrates, including histones, transcription factors, α-tubulin, and nuclear importers.7 In humans, 18 HDAC genes have been identified. Eleven of these HDAC contain highly conserved deacetylase domains and are zinc (Zn2+) dependent enzymes, while seven are nicotinamide adenine dinucleotide (NAD+) dependent proteases.8 Based on sequence phylogeny and their homology to yeast HDAC, their subcellular localization, and enzymatic activity, they are divided as follows.8

**Figure 1. Chemical reaction for lysine acetylation and deacetylation.** (A) The classical family of histone deacetylase (HDAC) removes the acetyl group from acetyl lysine, releasing acetate and the reaction requires zinc (Zn2+), while (B) Sirtuins remove the acetyl group using a completely different mechanism, releasing products that are different from acetate in a nicotinamide adenine dinucleotide (NAD+) dependent reaction.

**Abbreviations:** ADP, adenosine diphosphate; HAT, histone-acetyltransferases.

**SELECTED ABBREVIATIONS AND ACRONYMS**

- **CTCL**: cutaneous T-cell lymphoma
- **HAT**: histone-acetyltransferases
- **HDAC**: histone deacetylase
- **HDACi**: histone deacetylase inhibitor
- **PTCL**: peripheral T-cell lymphoma
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◆ Zn⁺-dependent HDAC

Class I (HDAC1, 2, 3, and 8). These HDAC are homologous to the yeast Rpd3 protein, mainly found in the nucleus, although HDAC8 is also found in the cytoplasm or associated to the cell membrane. Their main targets are histones. They are small and expressed ubiquitously. Knockout animal studies have shown that they are involved in cell survival, proliferation, and differentiation.

Class II (HDAC4, 5, 6, 7, 9, and 10). These HDAC are homologous to the yeast Hda1 protein, are large, act in association with tissue specific transcription factors, and have both histone and nonhistone protein targets. They have a more tissue-specific regulatory function than class I HDAC. Class II HDAC are further divided in two subclasses: class IIA (HDAC4, 5, 7, and 9) and class IIB (HDAC6 and 10). They are expressed in a limited number of cell types, and either shuttle between the nucleus and cytoplasm (ie, class Ila), or are mainly cytoplasmic (ie, class Ilb).

Class IV (HDAC11). This HDAC shares sequence similarity with the catalytic core regions of both class I and II enzymes, but does not have a strong enough sequence identity to be placed in their class.

◆ NAD⁺-dependent HDAC

Class III, also called sirtuins (SIRT). There are seven human SIRTs (SIRT1 to 7) and they are homologous to the yeast protein Sir2. They regulate gene expression in response to changes in the cellular redox status, cell proliferation, differentiation, genome stability, cell survival, metabolism, energy homeostasis, organ development, aging, and cancer. SIRT1, 2, 3, 5, 6, and 7 have lysine deacetylase activity while SIRT4 and 6 display monoribosyltransferase activity. SIRT5 has also been shown to have protein lysine demalonylase and desuccinylation activity. They shuttle between the nucleus and the cytoplasm. ²

**HDAC inhibitors**

In 1977, Fliggs and colleagues described the induction of acetylated histone accumulation upon exposure to n-butyrate, ¹⁰ and soon after, Candido and colleagues published that n-butyrate inhibited deacetylation. ¹¹ However, specificity of this inhibition was not fully demonstrated. In 1987, trichostatin A (TSA), isolated from a Streptomyces strain as an antifungal compound, was described as an inducer of murine erythroid differentiation, ¹² whereas romidepsin is an antitumor cyclic depsipeptide isolated from Chromobacterium violaceum. ¹⁶

Zn⁺-dependent HDAC inhibitors may come from natural or synthetic sources. Examples of natural sources include Chromobacterium violaceum (depsipeptide), sponge association between Poeciliastra sp and Jaspis sp (Psammaplin A), Streptomyces (TSA), dietary fibers (butyric acid), garlic (Diallyl disulfide), and cruciferous vegetables such as broccoli (Sulphorophane). Synthetic and natural HDAC inhibitors have a common pharmacophoric model characterized by a zinc-binding moiety in the catalytic pocket, a capping group (Cap), and a straight-chain alkyl, vinyl, or aryl linker connecting the two parts (hydrophobic linker). A kink atom (connecting unit, CU) connects the linker to the Cap.

**Figure 2. Pharmacophore model for zinc (Zn⁺)-dependent histone deacetylase inhibitors (HDAC).**

A common model generally accepted for the HDACi is depicted in this figure. All molecules share a zinc-binding moiety (ZBG) in the catalytic pocket, a capping group (Cap), and a straight-chain alkyl, vinyl, or aryl linker connecting the two parts (hydrophobic linker). A kink atom (connecting unit, CU) connects the linker to the Cap. After reference 17: Giannini G et al. Future Med Chem. 2012;4:1439-1460. © Futurescience.

In 1998, two HDAC inhibitors that later became clinically relevant were reported: suberoylanilide hydroxamic acid (SAHA, vorinostat) and FK228 (romidepsin). Vorinostat was designed and synthesized as a hybrid polar compound that strongly induced erythroid differentiation, ¹⁵ whereas romidepsin is an antitumor cyclic depsipeptide isolated from Chromobacterium violaceum. ¹⁶

They can be classified according to their chemical structure into the following groups: (i) carboxic acids; (ii) benzamide derivatives; (iii) cyclic tetrapeptides (epoxyketones and nonepoxyketones); (iv) hydroxamic acids; (v) ketones; and (vi) miscellaneous structures.

NAD⁺-dependent HDAC inhibitors target either their substrate-binding cleft or the NAD⁺-binding domain according to docking studies. ⁸ They may be classified according to their chemical structure as follows: (i) NAD derivatives; (ii) naphthopyranone inhibitors; (iii) dihydrocoumarin derivatives; and (iv) 2-hydroxynaphtaldehyde derivatives.

Based on in vitro biochemical assays using purified HDAC isoenzymes, HDAC inhibitors such as vorinostat, panobinostat, belinostat, or abexinostat have been identified as pan-HDACi, since they inhibit both class I and class II HDAC. Other inhibitors
Clinical development of histone deacetylase inhibitors – Salcedo Magguilli

Epigenetics and cancer

Oncology

Cloning and cancer

HDACi have been described to regulate the expression of both proapoptotic and antiangiogenic proteins. This regulation results in the activation of both extrinsic and intrinsic apoptotic pathways shifting the balance away from cell survival and toward cell death. Downregulation of prosurvival proteins such as BCL-2, BCL-XL, MCL-1, FLIP, and X-linked inhibitor of apoptosis protein (XIAP) have been described in different models. They have also been shown to upregulate proapoptotic proteins, such as BIM, BAK, NOXA, BMF, and BAX, by acetylation and stabilization of specific promoters or p53 protein. Death receptors and ligands that mediate extrinsic pathway apoptosis are upregulated upon treatment with HDACi. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) sensitivity may be restored in TRAIL-resistant malignant cells. In addition, BH3 interacting domain death agonist (BID), the only member of the BCL-2 family that is primarily activated through the extrinsic apoptotic pathway, has been shown to be activated after cell exposure to HDACi, leading to cell death.

Angiogenesis

HDACi have been shown to trigger early double-stranded DNA breaks in cancer cells probably associated with ROS generation measured by the induction of phosphorylated histone H2AX. Furthermore, HDACi may disrupt DNA repair by favoring acetylation of DNA repair proteins and/or downregulating them. Examples of these HDACi-targeted proteins are Ku70, Ku 86, RAD50, RAD51, BRCA1, and MRE11. Interestingly, disruption of DNA repair by HDACi may be more pronounced in transformed vs normal cells, contributing to their selectivity.

Interference with DNA repair

HDACi can act at multiple points in the cell cycle progression at different phases of the cell cycle to block progression, notably in G1, G2, and mitosis. Each cell-phase block is associated with a different outcome. HDACi block progression from G1 into S phase by upregulating genes that negatively control this process, notably CDK inhibitor p21. This can deliver a cytostatic effect that is reversed with the removal of the drug, and is seen on both normal and tumor cells. HDACi also induce G2/M arrest by downregulation of G2/M phase specific cyclins, however, this results in cell protection from HDACi. The HDACi-sensitive G2 phase arrest is defective in the majority of immortalized, virally transformed, or tumor cell lines. In these cells, HDACi treatment leads to aberrant mitosis by several mechanisms that can trigger cell death, and therefore represents a selective cytotoxicity in comparison with normal cells, which are protected by a normal G2 phase arrest.
Other effects

Other HDACi-mediated mechanisms that contribute to their antitumor effect comprise: (i) proteotoxic and endoplasmic reticulum stress through disruption of aggresome and accumulation of misfolded proteins; (ii) interference with the function of corepressors such as Bcl-6, a corepressor involved in lymphomagenesis; (iii) interference with signaling pathways by regulation of protein kinases including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), glycogen synthase kinase (GSK)-3β, p38, and STAT-5; (iv) proteasome inhibition and alteration of the expression of the ubiquitinated-protein shuttle HR23B; (v) interruption of cytoprotective autophagy; (vi) repression of metastasis-related genes; (vii) immune regulation either by inhibiting inflammation mediators or by inducing immune responses against tumors; (viii) interference with chaperone function, in particular hyperacetylation of Hsp90, reducing its association with cancer-related proteins, which results in their proteosomal degradation; and (ix) elimination of cancer stem cells (CSC).

Late clinical development and approval of HDACi

The hydroxamic acid vorinostat (SAHA, Zolinza® from Merck Sharp & Dohme Corporation) and the cyclic peptide romidepsin (FK228/despispeptide, Istodax® from Celgene) are approved in the US for the treatment of relapsed cutaneous T-cell lymphoma (CTCL). Romidepsin obtained fast-track approval for the treatment of peripheral T-cell lymphoma (PTCL) and the hydroxamic acid belinostat (PXD101, Beleodaq® from Topo-target/Spectrum Pharmaceuticals) is currently under priority review at the US Food and Drug Administration (FDA) for the same indication.

In addition, the Merck licensee, Taiho, has registered vorinostat for the treatment of CTCL in Japan. The benzamide chidamide is an HDAC10 inhibitor (Epizasa® from Shenzhen Chipscreen Bioscience) that has been submitted for registration in China for the treatment of PTCL, and has been licenced to Huya bioscience in the US.

Vorinostat was approved in 2006 by the FDA on the basis of two phase 2 studies: one pivotal and one supportive. In the pivotal study, 74 patients with CTCL at stage 2B or higher were treated in an open-label, single-arm study. Response was assessed by the severity-weighted assessment tool (SWAT), and it was concluded that 30.5% of the patients had clinically important pruritus relief and 13.6% had complete resolution of their pruritus. This effect was maintained for at least 4 weeks. The main observed adverse events were nausea, diarrhea, thrombocytopenia, and anemia. However, the European Medicines Agency (EMA) considered that the clinical benefit was not compelling and was lacking overall survival assessment. In addition, reported responses were considered by the agency as partial responses. As a consequence, Merck withdrew its European marketing application in 2009 (EMEA/90664/2009).

Romidepsin was approved in 2009 by the FDA on the basis of two open-label, single-arm trials. The overall response rate of CTCL patients was similar in both studies (34% and 35%) based on investigator assessments. Serious adverse events included infections, arrhythmia, edema, leukopenia, and thrombocytopenia. In 2011, the FDA granted accelerated approval for this drug for the treatment of PTCL on the basis of a phase 2 single-arm trial, where objective response rate (ORR) was 25%, including 15% complete response. The median response was 17 months. Adverse events in these patients included thrombocytopenia, neutropenia, and infections. In 2012, the EMA confirmed the refusal of the approval of this drug due to a lack of an established benefit as a result of a noncomparative nature of the pivotal efficacy data submitted (EMA/CHMP/27767/2013).

A summary of currently registered phase 3 studies is shown in Table I.

HDACi in early clinical development

More than 390 clinical trials using HDACi are registered at the National Institutes of Health clinical trials registry. Most of them study the therapeutic action of HDACi in cancer indications. Of interest, the pan-HDACi abexinostat have shown promising durable responses in early clinical trials in lymphoma patients (ORR 30%). In particular, follicular lymphoma patients have shown an ORR of 46%. Evaluable patients who achieved objective responses included 3 complete responses and 13 partial responses. The safety profile was manageable. Enrollment in the phase 2 part of the study of this investigational HDACi drug is currently ongoing (personal communication).

The development of new generation HDACi has focussed on designing molecules with better pharmacokinetic profiles and/or potency, and/or narrower HDAC target specificity. In addition, several novel HDACi have been molecularly designed as hybrid molecules in order to either concomitantly inhibit another tumor-related target such as PI3K and topoisomerase I, or to promote tumor-specific localization. An interesting exercise of HDACi-related toxicity improvement has been pioneered by the group developing abexinostat. An administration schedule has been conceived on the basis of pharmacokinetic/pharmacodynamic modeling to diminish the thrombocytopenia toxic effect of this HDACi. This schedule has been successfully applied to patients.

Given the relatively restricted spectrum of activity of HDACi when administered as single agents, the development of combination therapies has multiplied. Numerous closed or ongoing trials study the effect of various HDACi in combination with standard cytotoxic agents based on the hypothesis that HDACi would lower the survival threshold of tumor cells. In addition, potentiation of antitumor efficacy by combining HDACi with radiotherapy has been shown in preclinical studies and has been well tolerated in metastatic patients.
Combination of HDACi with targeted therapies has also been widely explored. Considerable evidence has shown that proteasome inhibitors act synergistically with HDACi. In the absence of normal proteasome function, misfolded proteins are degraded via formation of aggresome, and this pathway depends on HDAC6. When HDAC6 is inhibited, aggresomes cannot function, and this results in proteotoxic-induced apoptosis. Indeed, several clinical trials have shown encouraging results in multiple myeloma when combining the proteasome inhibitor bortezomib with romidepsin, vorinostat, and panobinostat. Another therapeutic combination studied in clinical trials is the synergy observed with inhibitors of DNA methyl transferase (DNMT), which enhances reactivation of tumor suppressor genes. Notably two studies combining vorinostat or belinostat with DNMT inhibitors (DNMTi) indicated that the combination may result in clinical efficacy and showed that the treatment was well tolerated in patients with myeloid malignancies. Multiple other combination approaches have been explored in preclinical studies based on scientifically-based synergistic combinations or counteraction of HDACi-resistance mechanisms, and which have started to translate in clinical settings. These include combinations with agents targeting several tyrosine kinases, Hsp90, apoptotic proteins, CDKs, NF-κB, autophagy, or the immune system. A summary of HDACi in early clinical phases is given in Table II (page 306).

**Table I. Histone deacetylase inhibitors (HDACi) in phase 2/3 or phase 3 clinical trials.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Targeted HDAC class (potency)</th>
<th>Indication</th>
<th>Co-treatment</th>
<th>Primary outcome</th>
<th>Sponsor</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydroxamic acids</strong></td>
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<tr>
<td>Vorinostat (MK-0693, Zolinza®)</td>
<td>I, II, IV (μM)</td>
<td>MM</td>
<td>Bortezomb</td>
<td>PFS</td>
<td>Merck Sharp &amp; Dohme</td>
<td>Merck Sharp &amp; Dohme</td>
<td>Recruiting</td>
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<td></td>
<td></td>
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<td>Paclitaxel, Carboplatin</td>
<td>Merck Sharp &amp; Dohme</td>
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<td></td>
<td></td>
<td>High grade glioma</td>
<td>Temozolomide or Bevacizumab</td>
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<td>Merck Sharp &amp; Dohme</td>
<td>Suspended</td>
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<td></td>
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<td>Merck Sharp &amp; Dohme</td>
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<td></td>
<td>AML (children)</td>
<td>Cytarabine, Daunorubicin, Idarubicin</td>
<td>EPS, rate of allogenic HCT</td>
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<td>Merck Sharp &amp; Dohme</td>
<td>Recruiting</td>
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<td>Bortezomb</td>
<td>PFS</td>
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<td>OS</td>
<td>University of Leeds</td>
<td>Celgene, Merck Sharp &amp; Dohme</td>
<td>Recruiting</td>
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<td>ORR</td>
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<td></td>
<td>MM</td>
<td>Bortezomb and Dexamethasone</td>
<td>PFS</td>
<td>Novartis Pharmaceuticals</td>
<td>Novartis Pharmaceuticals</td>
<td>Active not recruiting</td>
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<td><strong>Benzamides</strong></td>
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<tr>
<td>Entinostat (SNDX-275/MS-275)</td>
<td>I, II, IV (μM)</td>
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<td>Endocrine therapy</td>
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<td>Bayer, Syndax</td>
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<tr>
<td>Valproate</td>
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</table>
### Table II. Histone deacetylase inhibitors (HDACi) in early clinical trials.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Targeted HDAC class (potency)</th>
<th>Studied indications</th>
<th>Studied combinations</th>
<th>Clinical phases</th>
<th>Company (originator)</th>
<th>Licensee</th>
</tr>
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<tbody>
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<td></td>
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<tr>
<td>Hydroxamic acids</td>
<td></td>
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</tr>
<tr>
<td>Vorinostat (MK-0683, Zolinza®)</td>
<td>I, II, IV (µM)</td>
<td>Pancreatic, NHL, AML, MDS, head and neck cancer, squamous cell cancer</td>
<td>PI, CT, IM</td>
<td>Phase 1, Phase 2</td>
<td>Merck Sharp &amp; Dohme Corp, USA</td>
<td>Taiho, Japan</td>
</tr>
<tr>
<td>Belinostat (PDX101, Beleodaq™)</td>
<td>I, II, IV (µM)</td>
<td>HL, ALL, CML, MDS, HCC, NSCLC, sarcoma, CTCL, ovarian, mesothelioma</td>
<td>PI, TKi, CT, DNMTi</td>
<td>Phase 1, Phase 2</td>
<td>TopoTarget, Denmark</td>
<td>Spectrum Pharmaceuticals, USA</td>
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<tr>
<td>Panobinostat (LBH589, Faradak)</td>
<td>I, II, IV (µM)</td>
<td>Breast, NHL, CLL, ALL, AML, MDS, MM, other solid tumors</td>
<td>TKi, HoT, CT, DNMTi, PI, IM, RT</td>
<td>Phase 1, Phase 2</td>
<td>Novartis, Switzerland</td>
<td></td>
</tr>
<tr>
<td>Givinostat (ITF-2357)</td>
<td>I, II (nM)</td>
<td>cMPN</td>
<td>None</td>
<td>Phase 1, Phase 2</td>
<td>Il Italiafarmaco, Italy</td>
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</tr>
<tr>
<td>Abexinostat (CG-781, PCI-24781, S-78454)</td>
<td>I, II, IV (nM)</td>
<td>CTCL, PCTL, NHL, CLL, AML, sarcoma, other solid tumors</td>
<td>CT, RT</td>
<td>Phase 2</td>
<td>Celera, USA (discontinued)</td>
<td>Pharmacies, USA; Servier, France</td>
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<tr>
<td>CHR-3996</td>
<td>I (nM)</td>
<td>MM</td>
<td>API</td>
<td>Phase 1/2a</td>
<td>Chroma Therapeutics, UK</td>
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<tr>
<td>Tefinostat (CHR-2845)</td>
<td>I (nM) Monocyte, Macrophage-targeted</td>
<td>HCC</td>
<td>None</td>
<td>Phase 1/2a</td>
<td>Chroma Therapeutics, UK</td>
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<tr>
<td>Pracinostat (SB-939)</td>
<td>I, II, IV (nM)</td>
<td>AML, MDS, sarcoma, gastrointestinal, endometrial, CRC, thyroid, prostate</td>
<td>DNMTi</td>
<td>Phase 1, Phase 2</td>
<td>SBIO, Singapore</td>
<td>MEI Pharma, USA</td>
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<tr>
<td>AR-42</td>
<td>Not found (µM)</td>
<td>Various hematological and solid tumors</td>
<td>CT</td>
<td>Phase 2</td>
<td>Arno Therapeutics, USA</td>
<td></td>
</tr>
<tr>
<td>Quinestinat (UNJ-28481585)</td>
<td>I, II, IV (µM)</td>
<td>Melanoma, MM, MDS, CLL, AML, CML, NHL, CTCL, various solid tumors</td>
<td>PI, IM</td>
<td>Phase 1, Phase 2</td>
<td>Il Johnson &amp; Johnson, USA</td>
<td></td>
</tr>
<tr>
<td>Resminostat (4SC-201, BYK-106740)</td>
<td>I, II, IV (µM)</td>
<td>NSCLC, CRC, HL, HCC, various solid tumors</td>
<td>CT, TKi</td>
<td>Phase 1, Phase 2</td>
<td>Takeda, Japan (discontinued)</td>
<td>4SC, Germany; Yakult Honsha, Japan</td>
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<tr>
<td>Roclozinostat (ACY-1215)</td>
<td></td>
<td>HDAC6</td>
<td>MM, NHL</td>
<td>Phase 1, Phase 2</td>
<td>Acetylron, USA</td>
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<tr>
<td>SHP-141</td>
<td>I, II, IV (µM) Topical gel</td>
<td>CTCL</td>
<td>None</td>
<td>Phase 1</td>
<td>Shape Pharmaceuticals, USA</td>
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<td>Cyclic peptides</td>
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<tr>
<td>Romidepsin (FK228, Istodax®)</td>
<td>I (nM)</td>
<td>Various solid tumors, NHL, CTCL</td>
<td>PI, CT</td>
<td>Phase 1, Phase 2</td>
<td>Astellas, Japan (discontinued)</td>
<td>Celgene, USA</td>
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<td>Benzamides</td>
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<td>Chidamide (CS-055)</td>
<td>I, II (nM)</td>
<td>NHL, CRC, NSCLC, prostate, breast</td>
<td>CT</td>
<td>Phase 1, Phase 2</td>
<td>Shenzhen Chip-screen Biosciences, China</td>
<td>Hoya Bioscience, USA</td>
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<tr>
<td>Entinostat (MS-275, SNX-275)</td>
<td>I (µM)</td>
<td>Melanoma, prostate, NSCLC, AML, CML, MDS, HL, CRC</td>
<td>TKi, HoT, DNMTi</td>
<td>Phase 2</td>
<td>Bayer, Germany</td>
<td>Syndax, USA</td>
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<tr>
<td>4SC-202</td>
<td>I (µM)</td>
<td>MDS, MM, CLL, AML</td>
<td>None</td>
<td>Phase 1</td>
<td>Takeda, Japan (discontinued)</td>
<td>4SC, Germany</td>
</tr>
<tr>
<td>Mocetinostat (MGCD0103)</td>
<td>I, IV (µM)</td>
<td>MM, MDS, DLBCL, HL</td>
<td>DNMTi</td>
<td>Phase 1, Phase 2</td>
<td>Mirati Therapeutics, USA</td>
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<tr>
<td>Hybrid molecules</td>
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<tr>
<td>CUDC-907</td>
<td></td>
<td>NHL, MM, various solid tumors</td>
<td>None</td>
<td>Phase 1</td>
<td>Curis, USA</td>
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</tbody>
</table>

**Abbreiations:** ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; API, aminopeptidase inhibitor; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; cMPN, chronic myeloproliferative neoplasms; CRC, colorectal cancer; CT, chemotherapy; CTCL, cutaneous T-cell lymphoma; DNMTi, DNA methyltransferase inhibitor; DLBCL, diffuse large B-cell lymphoma; HoT, hormone therapy; HCC, hepatocellular carcinoma; HL, Hodgkin’s lymphoma; IM, immunomodulators; MDS, myelodisplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin’s lymphoma; NSCLC, Non-small cell lung cancer; PI, protease inhibitor; RT, radiotherapy; TKI, tyrosine kinase inhibitor.

Pending questions and future directions

Despite the fact that HDACi represent attractive antitumor drugs due to a minimal effect on normal tissues and relatively low toxicity, clinical evidence of efficacy remains limited, in particular in solid tumors.\(^1\,^{13}\)

The clinical impact of the improvement introduced in new generation HDACi and/or of various combination therapy strategies is not yet well established. None of them are approved yet, nor adopted as therapeutic options. However, it is hoped that continued translational research efforts will enable better identification of resistant vs susceptible patient populations. For example, ZFP64, a member of the C2H2 type zinc-finger family, has been identified as a possible predictive biomarker of response to the HDACi resminostat of patients with hepatocellular carcinoma, as published in the 4SC product web site information.\(^{114}\) Predictive biomarkers of response to HDACi include STAT1 and high phosphorylation of STAT3 in B- and T-cell lymphoma cell lines,\(^{58}\) and genes involved in antioxidation,\(^{115}\) as well as HR23B in CTCL patients treated with vorinostat.\(^{51}\) Nevertheless, a recent study in malignant pleural mesothelioma cell lines showed that this marker did not predict vorinostat sensitivity in vitro,\(^{116}\) indicating that efforts to find relevant predictive biomarkers taking into account tumor types should continue.

A more recent approach to improve understanding of how HDACi could be developed and used more effectively is the use of cell-based screening assays. These assays are anticipated to better reflect physiological conditions in which HDACi exert their antitumor activity, i.e., in concert with other proteins that impinge on gene transcription. In addition, they could allow a better grasp of mechanisms by which, in certain conditions, HDACi may actually promote tumor growth and metastasis.\(^{117}\)

Continued monitoring of efficacy in ongoing clinical studies linked to the application of pertinent translational research approaches are anticipated to lead to the identification of smarter clinical strategies, in terms of sensitive patient selection, HDAC tumor type specificity, and combination strategies in the near future.

Acknowledgments: I thank Jean-Pierre Abastado, Ioana Kloos, and Michael Bunbridge for critical reading of this article as well as Isabelle Sanson, Anne Trincot, and Stephanie Lemen for technical assistance.

Keywords: cancer; clinical trial; epigenetics; gene expression; HDAC; HDAC inhibitor; histone deacetylase

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18. Thaler F, Minucci S. Next generation histone deacetylase inhibitors: The answer...


Clinical development of histone deacetylase inhibitors – Salcedo Magguilli
DÉVELOPPEMENT CLINIQUE DES INHIBITEURS DES HDAC

L’état naturel du génome est réprimé. La majeure partie de l’ADN est cachée par des nucléosomes assemblés en structures très condensées inaccessible aux protéines fonctionnelles et régulatrices nécessaires au démarrage de la transcription génique. Les facteurs de remodelage de la chromatine et les histone-acétyltransférases (HAT) associées au complexe de la polymérase commandent l’ouverture transitoire des régions chromatiques. Les histone-désacétylases (HDAC) inhibent l’action de l’HAT. L’expression de l’HDAC est modifiée dans de nombreuses tumeurs et dans certains cancers, sa surexpression est corrélée à une évolution défavorable. Ces dernières années, les inhibiteurs de l’HDAC (HDACi) ont été très étudiés en tant que médicaments antitumoraux. Deux HDACi, le vorinostat et la romidepsine, sont autorisés aux États-Unis dans le traitement du lymphome T cutané. La romidepsine a été autorisée dans le traitement du lymphome T périphérique et le belinostat est actuellement en cours d’analyse prioritaire à la FDA pour la même indication. Le vorinostat est également enregistré au Japon. Le bénéfice thérapeutique des HDACi en monothérapie dans les tumeurs solides n’a pas encore été démontré mais de nombreuses études cliniques sont en cours pour les évaluer seuls ou en association avec d’autres stratégies antitumorales dans les tumeurs solides et hématologiques. Cependant, optimiser le développement futur de ces molécules nécessite une meilleure compréhension de leurs effets pléiotropes antitumoraux et une identification de biomarqueurs prédictifs plus fiables.
Apoptosis is a process of programmed cell death responsible for the removal of no-longer-needed, damaged, or potentially dangerous cells. Apoptosis is controlled by the B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL2) family of proteins, which can be divided into 3 subgroups according to amino acid sequence, structure, and function: the BCL2-like pro-survival proteins required for cell survival, the pro-apoptotic BCL2-associated X (BAX)/BCL2-antagonist/killer (BAK) proteins essential for unleashing cellular destruction via the caspase cascade, and the pro-apoptotic BCL2 homology domain 3 (BH3)-only proteins, which are critical to initiate apoptosis signaling. It is now firmly established that mutations or other defects that cause abnormalities in the expression of pro-apoptotic or anti-apoptotic BCL2 family members promote tumorigenesis and render cancer cells refractory to diverse chemotherapeutic drugs. This review describes current understanding of the molecular regulation of apoptosis by the BCL2 protein family, the impact of defects in this process on cancer, and finally discusses currently evolving strategies for direct therapeutic modulation of the BCL2-family-regulated apoptotic pathway for treatment of cancer.
proteins to cause the morphological and biochemical characteristics of apoptosis (eg, plasma membrane blebbing, chromatin condensation, internucleosomal DNA fragmentation) that precipitate cellular demolition.4

In the death-receptor pathway, members of the tumor necrosis factor receptor (TNFR) family with an intracellular death domain (eg, TNF type 1 receptor [TNFR1]; FAS, also called apoptosis antigen 1 [APO-1] and cluster of differentiation 95 [CD95]) trigger Fas-associated death domain (FADD) adapter-protein–mediated activation of the "initiator caspase," caspase 8 (in humans, also caspase 10), which then proteolytically activates the effector caspases (Figure 1). In the BCL2-regulated pathway, cell death is triggered by developmental cues or cellular stressors (eg, growth factor deprivation, DNA damage; Figure 1). This causes transcriptional and/or post-transcriptional activation of BCL2 homology domain 3 (BH3)-only proteins, the proapoptotic subgroup of the BCL2 family, which initiate apoptosis by activating BCL2-associated X protein (BAX)/BCL2 antagonist/killer (BAK) (the second proapoptotic subgroup of the BCL2 family) through 2 processes (Figure 1). Activated BAX/BAK proteins oligomerize, causing mitochondrial outer membrane permeabilization (MOMP). This leads to the release of apoptogenic proteins, such as cytochrome c and second mitochondria-derived activator of caspases (Smac; also referred to as direct inhibitor of apoptosis binding protein with low pI [Diablo]), which promote apoptotic protease-activating factor 1 (Apaf-1) adaptor–protein–mediated activation of the initiator caspase, caspase 9, and amplification of effector caspase activation (Figure 1). The death-receptor and the BCL2-regulated apoptotic pathways are connected through caspase 8–mediated proteolytic activation of the BH3-only protein BH3-interacting-domain death agonist (BID) (Figure 1).

**Figure 1.** Two distinct, but ultimately converging, pathways for induction of apoptosis. Diagram showing the mediators of the BCL2-regulated and the death receptor–initiated apoptotic pathways and their connection through caspase 8–mediated proteolytic activation of the proapoptotic BCL2-family member BID. Abbreviations: APAF-1, apoptotic protease activating factor 1; BAK, BCL2 antagonist/killer; BAX, BCL2-associated X protein; BCL2, B-cell chronic lymphocytic leukemia/lymphoma 2 protein; BH3, BCL2 homology domain 3; BID, BH3-interacting-domain death agonist; cyt c, cytochrome c; DIABLO, direct inhibitor of apoptosis binding protein with low pl; FADD, Fas-associated death domain; FAS, Fas ligand; IAP, inhibitor of apoptosis binding protein; SMAC, second mitochondria-derived activator of caspases; tBID, truncated BID; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

**Selected abbreviations and acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>A1</td>
<td>BCL2-related protein A1</td>
</tr>
<tr>
<td>B-RAF</td>
<td>serine/threonine-protein kinase B-Raf</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated death promoter (protein)</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL2 antagonist/killer</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell chronic lymphocytic leukemia/lymphoma 2 (protein)</td>
</tr>
<tr>
<td>BCL-W</td>
<td>BCL2-like protein 2</td>
</tr>
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<td>BCL-XL</td>
<td>BCL2-like protein 1</td>
</tr>
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<td>BH</td>
<td>BCL2 homology</td>
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<tr>
<td>BH3</td>
<td>BCL2 homology domain 3</td>
</tr>
<tr>
<td>BID</td>
<td>BH3-interacting-domain death agonist</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL2-interacting mediator of cell death</td>
</tr>
<tr>
<td>BMF</td>
<td>BCL2-modifying factor</td>
</tr>
<tr>
<td>BOK</td>
<td>BCL2-related ovarian killer</td>
</tr>
<tr>
<td>BOO</td>
<td>BCL2 homolog of ovari</td>
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<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelocytic leukemia</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>MCL-1</td>
<td>myeloid-cell leukemia sequence 1</td>
</tr>
<tr>
<td>MOMP</td>
<td>mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>NOXA</td>
<td>phorbol-12-myristate-13-acetate-induced protein 1</td>
</tr>
<tr>
<td>p53</td>
<td>protein p53</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>tBID</td>
<td>truncated BID</td>
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<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
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<tr>
<td>TNFR1</td>
<td>tumor necrosis factor type 1 receptor</td>
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</table>
Structures and functions of the members of the 3 major subgroups of the BCL2 family of proteins

There are 3 major subgroups of the BCL2 family of proteins: (i) the prosurvival BCL2-like proteins (BCL2, BCL2-like protein 1 [BCL-XL], BCL2-like protein 2 [BCL-W], myeloid-cell leukemia sequence 1 [MCL-1]), BCL2-related protein A1 [A1, also known as BFL1], BCL2-like homolog of ovary [BOO, also known as DIVA]); (ii) the BAX/BAK proteins (BAX, BAK, BCL2-related ovarian killer [BOK]); and (iii) the BH3-only proteins (BCL2-interacting mediator of cell death [BIM], BID, p53-upregulated modulator of apoptosis [PUMA], BCL2-modifying factor [BMF], BCL2-associated death promoter [BAD], BCL2-interacting killer [BIK], phorbol-12-myristate-13-acetate-induced protein 1 [NOXA], harakiri [HRK]) (Figure 2). These proteins are related to each other by the presence of at least 1 of 4 recognized BCL2 homology (BH) domains; the prosurvival members and the BAX/BAK proteins have 4 BH domains, whereas the BH3-only proteins only have the BH3 domain. In addition, there are some proteins that contain 1 or more BH domains, but do not readily fit into any of the 3 aforementioned subgroups. For most of these “odd BH-domain–containing proteins” the functions are unknown and they will not be dealt with further in this article.

◆ Essential functions of the different prosurvival BCL2 family members

The prosurvival BCL2 family members all promote cell survival when overexpressed (such as in transgenic mice) and studies with gene-targeted mice have revealed their essential functions. BCL2 is required for the survival of renal epithelial progenitor cells, mature lymphocytes, and melanocyte progenitors. Consequently, Bcl-2−/− mice die from polycystic kidney disease around 30 to 40 days, are lymphopenic, and turn prematurely gray.5

BCL-XL is needed for the survival of erythroid progenitors, platelets, certain neuronal populations, and developing sperm cells.7 Accordingly, Bcl-x−/− mice die around embryonic day 14 (E14) and Bcl-2−/− mice have abnormally low platelet levels and males are subfertile. Bcl-w−/− mice develop normally and have no overt defects in adult life with the exception of male infertility, which was ascribed to abnormal death of the supporting Sertoli cells. MCL-1 is essential for survival of undifferentiated cells in the early embryo (Mcl-1−/− embryos die prior to implantation) and for survival of cardiomyocytes, certain neuronal populations, and a broad range of hematopoietic cells, including the stem/progenitor cells.8 The critical functions of A1/BFL1 are not fully resolved because the presence of 4 closely related, often co-expressed A1 genes makes gene targeting difficult. Studies with mice lacking 1 A1 gene, A1α, or transgenic mice expressing a short hairpin RNA (shRNA) that downregulates all A1 genes indicated that A1 plays a role in the survival of granulocytes as well as of immature B- and T-lymphoid progenitors.9 The mouse Boo/Diva gene has mutations that are predicted to render the protein nonfunctional, but it remains possible that the human protein does play a role in cell survival. There is currently very little information (eg, from analysis of double-knockout mice) on the overlapping functions of prosurvival BCL2 family members.

◆ Essential functions of the BAX/BAK proteins

Mice lacking either BAX or BAK alone are largely normal, with the exception of male infertility in the former and increased platelet numbers in the latter. The generation of BAX/BAK doubly deficient mice revealed remarkable functional overlap be-

![Diagram showing the 3 major subgroups of the BCL2 protein family: the BCL2-like prosurvival proteins that are essential for cell survival, the proapoptotic BAX/BAK-like proteins that are essential for unleashing the effector phase of apoptosis and the proapoptotic BH3-only proteins that are critical for the initiation of the BCL2-regulated apoptotic pathway. The asterisk above the TM (transmembrane domain) of the stick model of a BH3-only protein indicated that only some [eg, BIM], but not all [eg, BAD], BH3-only proteins have a transmembrane domain.](https://example.com/diagram)

**Figure 2.** The members of the 3 major subgroups of the BCL2 protein family. Diagram showing the 3 major subgroups of the BCL2 protein family: the BCL2-like prosurvival proteins that are essential for cell survival, the proapoptotic BAX/BAK-like proteins that are essential for unleashing the effector phase of apoptosis and the proapoptotic BH3-only proteins that are critical for the initiation of the BCL2-regulated apoptotic pathway. The asterisk above the TM (transmembrane domain) of the stick model of a BH3-only protein indicated that only some [eg, BIM], but not all [eg, BAD], BH3-only proteins have a transmembrane domain.

Abbreviations: A1, BCL2-related protein A1; A1α, BCL2-related death promoter; A1α, BCL2 antigenic killer; A1β, BCL2-associated X protein; A1γ, BCL2; BCL2-like protein 1; BCL2, BCL2-related ovarian killer; BAD, BCL2-related ovarian killer; BAX, BCL2-interacting mediator of cell death; BMF, BCL2-modifying factor; BOK, BCL2-related ovarian killer; BIM, BCL2-interacting killer; BIM, BCL2-interacting killer; BMF, BCL2-modifying factor; BOK, BCL2-related ovarian killer; BOK, BCL2-related ovarian killer; HRK, harakiri; MCL-1, myeloid-cell leukemia sequence 1; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; PUMA, p53-upregulated modulator of apoptosis; TM, transmembrane domain.
Essential functions of the different proapoptotic BH3-only proteins

Biochemical studies have shown that BH3-only proteins differ markedly in their affinities for binding to the various prosurvival BCL2 family members (Figure 3). BIM, PUMA, and BID (after caspase-mediated processing to the so-called truncated BID [tBID] form) bind with high (low nM or even sub-nM) affinity to all prosurvival BCL2-like proteins and are therefore sometimes referred to as “promiscuous binders” (Figure 3). All other BH3-only proteins are “selective binders”; for example, NOXA binds to MCL-1 and A1/BFL1, whereas BAD binds to BCL2, BCL-XL, and BCL-W (Figure 3). Moreover, only some BH3-only proteins (BIM, PUMA, tBID) appear able to bind the multi-BH-domain prosurvival BAX/BAK proteins although there is disagreement in the literature on whether some others, such as BMF or NOXA, can also do this.

The “promiscuous” BH3-only proteins can all elicit apoptosis when overexpressed by themselves. Conversely, enforced expression of combinations of complimentary “select binders,” such as NOXA plus BAD, is needed to induce efficient cell killing.12 This indicates that all prosurvival BCL2-like proteins present in a cell must be neutralized by BH3-only proteins to initiate apoptosis, presumably by liberating primed BAX/BAK (this has been dubbed the “indirect activation” model). The “direct activation” model postulates that some BH3-only proteins (called “direct activators,” eg, BAD, PUMA, tBID) can bind and thereby activate BAX/BAK and that these BH3-only proteins are kept in check by binding to the prosurvival BCL2-like proteins until displaced by the “indirect activators” (eg, BAD, NOXA). Experiments with gene-targeted mice have shown that loss of the “promiscuous binders,” BIM, PUMA, and BID, causes profound abnormalities, whereas loss of most other BH3-only proteins on their own has only minor impact. BIM is critical for apoptosis induced by growth factor withdrawal, deregulated calcium flux, or endoplasmic reticulum (ER) stress, and also contributes to apoptosis triggered by DNA damage. BIM-deficient mice have defects in the deletion of autoreactive T- and B-lymphoid cells and removal of activated lymphocytes during shutdown of acute as well as chronic immune responses. This causes abnormal accumulation of lymphoid cells and plasma cells with a predisposition to autoimmune disease and lymphoid malignancy.14 PUMA is directly transcriptionally activated by the tumor suppressor protein p53 and essential for apoptosis triggered by DNA damage.15 PUMA also contributes substantially to apoptosis triggered by certain p53-independent stimuli, such as cytokine deprivation, ER stress, and treatment with glucocorticoids or phorbol ester.16 Activation of BID by caspase 8, leading to amplification of the caspase cascade via BAX/BAK and activation of caspase 9, is critical for FAS- or TNFR1-induced killing of certain cell types (called type 2; eg, hepatocytes, pancreatic β cells), but dispensable in others (called type 1; eg, thymocytes).16 BH3-only proteins have overlapping functions; this is best demonstrated by the profound resistance of cells from BIM/PUMA–doubly deficient mice and BIM/PUMA/BID–triply deficient mice to a broad range of apoptotic stimuli and the remarkable lymphadenopathy and autoimmune disease predisposition of these animals.17

Antagonistic interactions between proapoptotic and prosurvival BCL2 family members

Genetic and crystallographic studies have illuminated the functional and structural interactions between the members of the 3 subgroups of the BCL2 protein family. Remarkably, loss of BIM (even loss of 1 Bim allele) can overcome all abnormalities caused by loss of BCL2 and some of the defects (eg, failure of fetal erythropoiesis) elicited by loss of BCL-XL.18 Moreover, loss of BAK rescues the abnormal drop in platelets that is caused by BCL-XL deficiency. These functional antagonisms are mirrored by strong physical interactions of these proteins, which involve insertion of the BH3 domain of either the BH3-only or the BAX/BAK protein into the groove on the surface of the prosurvival BCL2-like protein that is formed by their BH1, BH2, and BH3 domains.19

During their activation (ie, through direct binding by BH3-only proteins or after release from the prosurvival BCL2-like proteins), BAX/BAK undergo dramatic structural changes.20 This leads to dimerization and ultimately multimerization of BAX/BAK, which causes MOMP in a manner that is still not resolved. This constitutes the point of “no return” in the control of apoptosis signaling. Hence, getting tumor cells to this point is what needs to be achieved by anticancer drugs that directly modulate the apoptotic machinery.
The role of BCL2 family members in cancer

The study of cell death is tightly interwoven with cancer research. The first cell death regulatory gene from any species, Bcl-2, was discovered because of its recurrent chromosomal translocation (t14;18) in human follicular center B-cell lymphoma.1

- Abnormalities in the expression of BCL2 family members can promote tumor development

Although chromosomal translocations involving genes for prosurvival BCL2 family members are rare in cancers other than follicular lymphoma, it has become clear that somatically acquired copy number amplifications of the genomic regions harboring the Mcl-1 and Bcl-x genes are present at relatively high frequencies in diverse human cancers.2 The amplified genomic regions in these cancers are relatively large and therefore contain additional (non–cell-death regulatory genes), but initial functional studies using RNA interference (RNAi)-mediated knockdown indicated that excess MCL-1 or BCL-XL may indeed be critical for the sustained growth of these transformed cells.22

Finally, gene expression profiling studies (using microarray or RNA-Seq technology) and protein analysis have found abnormally high levels of prosurvival BCL2-like proteins (or their messenger RNAs [mRNAs]) in a substantial number of human cancers, even though many of those are likely not to have overt chromosomal alterations of the corresponding genes. This may be due to epigenetic modifications or the fact that oncogenic pathways that are activated in these tumors cause their transcriptional induction, translational increase, or posttranslational stabilization.

Abnormalities in genes encoding proapoptotic BCL2 family members have also been found in human cancers. Loss of both alleles of Bim was reported in nearly 20% of human mantle cell B lymphoma.23 Moreover, abnormally low levels of BIM and PUMA, in part ascribed to hypermethylation of the genes for these BH3-only proteins, has been observed in several human cancers, including renal cell carcinoma or Burkitt Lymphoma.24 Loss of Bax or Bak genes appear to be rare in human cancers because mutations in 4 alleles, which would be needed to obliterate the BAX/BAK checkpoint,10 is an unlikely event. Curiously, however, loss of the region harboring the Bok gene was found in several human cancers.25

Studies with transgenic and gene-targeted (knockout) mice have confirmed and extended the findings from the studies of human cancers. Overexpression of BCL2 or its prosurvival relatives in lymphoid cells promotes lymphomagenesis, particularly in combination with oncogenic lesions that deregulate the control of cell proliferation, such as by c-MYC overexpression.26 Similarly, loss of the BH3-only proteins BIM, PUMA, or multi–BH1-domain proapoptotic BAX also promote tumorigenesis.27

- The role of prosurvival BCL2 family members expressed under endogenous control in the development and sustained growth of tumors

Although it is well established that overexpression of prosurvival BCL2 family members can promote tumorigenesis, there are still only few reports from studies on the importance of these proteins, expressed under endogenous control, for the development and sustained growth of cancers. Interestingly, BCL-XL and MCL-1, but not BCL2 were found to be critical for c-MYC–driven pre-B/B lymphoma and acute myelocytic leukemia (AML).27 A likely explanation for this may be that BCL-XL and MCL-1, but not BCL2, are expressed in the cell populations from which these hematological malignancies emerge (leukemia/lymphoma-initiating stem cells). MCL-1, under endogenous control, appears to play a particularly prominent role in tumor development, since it was also found to be essential for the induction and sustained growth of AML driven by diverse oncogenes, such as MLL-ENL.28

- The role of the proapoptotic BCL2 family members in anticancer drug–induced killing of tumor cells

It has long been known that chemotherapeutic drugs and γ-radiation can induce apoptosis in cancer cells. Studies with transgenic mice or tumor-derived cell lines transduced with expression vectors revealed that abnormally increased levels of prosurvival BCL2 family members can render nontransformed as well as cancerous cells resistant to a broad range of anticancer therapeutics.2 Studies using gene-targeted mice or shRNA expression vectors have revealed the roles of the BH3-only proteins and BAX/BAK in the killing of cancer cells by chemotherapeutic drugs. Combined loss of BAX and BAK rendered experimentally transformed cells resistant to a broad range of chemotherapeutic drugs and γ-radiation (Figure 4). The BH3-only proteins PUMA and, to a lesser extent, NOXA, which are directly transcriptionally regulated by the tumor suppressor p53, were shown to be critical for the killing of lymphoma and certain other neoplastic cells by DNA damage–inducing anticancer therapies29 (Figure 4). Unexpectedly, BIM, which does not appear to be directly regulated by p53, also contributes to this response29 (Figure 4). PUMA and BIM were shown to mediate glucocorticoid-induced killing of lymphomas and leukemias, and BIM as well as BMF were critical for the response of tumor cells to paclitaxel or histone deacetylase (HDAC) inhibitors (Figure 4). How BIM, PUMA, and BMF are induced in response to these nongenotoxic drugs is not clear. BIM and, to a lesser extent, BAD are essential for the killing of diverse cancer cells by inhibitors of oncogenic kinases, such as imatinib for inhibition of the breakpoint cluster region (BCR)-Abelson tyrosine kinase (ABL) fusion protein (BCR-ABL) in chronic myelocytic leukemia (CML), erlotinib/gefitinib for the inhibition of mutant epidermal growth factor receptor (EGFR) in lung cancer, and serine/threonine–protein kinase B-Raf (B-RAF) or mitogen-activated extracellular signal–regulated kinase (MEK) inhibitors for the treatment of melanoma or colon carcinomas bearing B-Raf mutations30.
Interestingly, a polymorphism in Bim that attenuates its expression is enriched among East Asian patients with CML or lung cancer, who show poor de novo responses to inhibitors of oncogenic kinases (imatinib or erlotinib/gefitinib, respectively).\(^3\) Flipping the BCL2-regulated apoptotic switch for cancer therapy

Since efficient induction of apoptosis appears critical for the responses of many cancers to a broad range of therapeutics and since many cancers bear mutations that impair efficient induction of apoptosis (eg, mutations in p53, disabling efficient induction of PUMA, or overexpression of prosurvival BCL2 family members), substantial efforts are being undertaken to develop drugs that can directly flip the BCL2-regulated apoptotic switch (Figure 5).

**Direct inhibition of prosurvival BCL2 family members by BH3 mimetics**

One approach involves the generation of small molecular weight compounds that mimic the action of the BH3-only proteins, binding in the groove on the surface of prosurvival BCL2-like proteins, thereby inhibiting their antiapoptotic activity (Figure 5). ABT-737 and the structurally closely related, orally available ABT-263 bind to BCL2, BCL-XL, and BCL-W; ABT-199 binds only to BCL2;\(^3\) and WEHI-539 selectively inhibits BCL-XL.\(^3\) These BH3 mimetics can kill certain tumor cells (eg, CLL) as single agents, but in many other cancers they need to be combined with standard anticancer therapies or inhibitors of oncogenic kinases for efficient killing. Functional and biochemical studies have revealed that ABT-737 initiates apoptosis mostly by displacing BIM (and possibly other BH3-only proteins) from BCL2, thereby allowing it to bind and neutralize the other prosurvival BCL2-like proteins that are present in those cancer cells.\(^3\) Excitingly, both ABT-263 and ABT-199 are currently showing early promise in clinical trials for CLL and certain other cancers.\(^3\) Of course, not only tumor cells, but also nontransformed cells in healthy tissues are affected by BH3 mimetics and their response will be dose limiting. ABT-263 and ABT-737 cause a drop in platelets, consistent with their ability to bind BCL-XL.

![Figure 4. Different anticancer agents activate distinct BH3-only proteins to initiate apoptosis in tumor cells. Diagram showing which currently used anticancer agents activate which BH3-only proteins to initiate apoptosis in tumor cells. Abbreviations: BAD, BCL2-associated death promoter; BH, BCL2 homology domain; BIK, BCL2-interacting killer; BIM, BCL2-interacting mediator of cell death; BMF, BCL2-modifying factor; HDAC, histone deacetylase; HRK, harakiri; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; p53, protein 53; PUMA, p53-upregulated modulator of apoptosis; tBID, truncated BID (BCL2-interacting-domain death agonist).](image)

![Figure 5. Apoptosis regulators targeted for cancer therapy. Diagram showing which components of the BCL2-regulated and the death-receptor-initiated apoptotic pathways are being targeted by anticancer therapeutics that are currently being developed (indicated with a red circle). Abbreviations: APAF-1, apoptotic protease activating factor 1; BAK, BCL2 antagonist/killer; BAX, BCL2-associated X protein; BCL2, B-cell chronic lymphocytic leukemia/lymphoma 2 protein; BH, BCL2 homology domain; cyt C, cytochrome C; DAXLB, direct inhibitor of apoptosis binding protein with low pl; FADD, Fas-associated death domain; FASL, Fas ligand; FLIP, FADD-like interleukin 1β-converting enzyme (FLICE)-inhibitory protein; IAP, inhibitor of apoptosis; tBID, truncated BID (BCL2-interacting-domain death agonist); TNF-α, tumor necrosis factor α; TNFR1, tumor necrosis factor type 1 receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor.](image)
which is critical for platelet survival. It will of course be important to determine to what extent BH3 mimetics will augment the toxicity to healthy tissues that is caused by the anticancer therapeutics that they are likely to be combined with in the clinic. I predict that combinations of BH3 mimetics with inhibitors of oncogenic kinases could be particularly promising, because the latter should only affect the cancerous, but not the healthy cells. Since MCL-1 and A1/BFL1 are abnormally highly expressed in certain cancers, it may also be of interest to develop BH3 mimetics that target these prosurvival BCL2 family members. Since MCL-1 is critical for the survival of several essential and hard-to-replace cell types, such as cardiomyocytes, it has been argued that targeting MCL-1 will not be a viable option for cancer therapy. It is, however, important to remember that irreversible loss of a protein does not equate to transient inhibition of a protein. Hence, targeting MCL-1 with BH3 mimetics may still be a viable strategy for cancer therapy, particularly in tumor cells exquisitely dependent on this prosurvival protein.

Finally, certain cancers, such as B-cell lymphomas in immunosuppressed transplant recipients and Kaposis sarcoma, are associated with infection by viruses that carry BCL2-like prosurvival proteins (eg, the Epstein-Barr virus BCL2 homolog, BHRF1, and the Kaposis sarcoma-associated virus BCL2 homolog, Ks-BCL2). If these proteins are critical for the sustained survival of these tumor cells, targeting them with BH3 mimetics may be an attractive strategy. Since it should be possible to engineer such compounds so that they do not bind to the human BCL2-like prosurvival proteins, they would be expected to cause minimal collateral damage. Thus it might even be possible to combine them with chemotherapeutic drugs that act nonspecifically, for example, by causing DNA damage (eg, cyclophosphamide, etoposide).

◆ Other approaches to activate the BCL2-regulated pathway for cancer therapy

In addition to targeting prosurvival BCL2 family members directly, it may also be possible to act upon them indirectly by targeting one of their regulators. In the case of MCL-1, which has a short half-life due in part to ubiquitin-dependent protranslational degradation, indirect targeting might be achieved by inhibiting a deubiquitinase that delays this degradation. Of course, since such regulators act not only upon MCL-1, but many other proteins as well, unwanted side effects may arise from effects on other client proteins.

Conclusions

In conclusion, the first cell death regulator, BCL2,21 and its functions22 were discovered nearly 30 and 25 years ago, respectively. Since then, the field has made enormous progress, defining the components of the cell death pathways, their functions in health and disease (particularly cancer), and their structures. This has culminated in the development of the first 2 drugs that directly flip the apoptotic switch and they are showing early promise in clinical trials for treatment of CLL and certain other cancers. Having been involved in cell death research for nearly 25 years, I hope that these drugs and future compounds will contribute to the armamentarium for treating cancer and possibly other diseases. ■

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LE RÔLE DE LA MORT CELLULAIRE PROGRAMMÉE DANS LE DÉVELOPPEMENT DES TUMEURS ET LE TRAITEMENT DU CANCER

L’apoptose est un processus de mort cellulaire programmée responsable de l’élimination des cellules potentiellement dangereuses, abîmées, ou devenues inutiles. L’apoptose est contrôlée par la famille des protéines BCL2/féœcumé lymphoïde chronique à cellules B/lymphome 2), qui peuvent être divisées en 3 sous-groupes selon la séquence, la structure et la fonction des acides aminés : les protéines prosurvie de type BCL2 nécessaires à la survie cellulaire, les protéines proapoptotiques BAX (X associé à BCL2) (BAK [antagoniste de BCL2/tueuses ou killer]) essentielles au déclenchement de la destruction cellulaire par la voie de la chaîne des caspases et les protéines proapoptotiques BCL2 du domaine d’homologie 3 seulement (BH3), déterminantes pour déclencher le signal de l’apoptose. Ces mutations ou autres dêfts responsables d’anomalies dans l’expression des membres de la famille BCL2 pro- ou antiapoptotiques sont maintenant connus pour favoriser la formation des tumeurs et rendre les cellules cancéreuses réfractaires aux différentes chimiothérapies. Cet article décrit les connaissances actuelles sur la régulation moléculaire de l’apoptose par la famille des protéines BCL2, l’impact des anomalies de cette régulation dans le cancer et analyse les stratégies existantes de modulation thérapeutique directe de la voie apoptotique régulée par la famille BCL2 dans le traitement du cancer.

Keywords: apoptosis; BCL2 family; cancer; caspase; intrinsic pathway; p53
Acting on the BCL2 dependence of cancer cells

by O. Geneste, France

Apoptosis is a form of programmed cell death that is essential for development and tissue homeostasis, but is almost systematically impaired in tumor cells, allowing their survival despite subjecting to many apoptotic stimuli. Deregulation of the B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL2)-family proteins, which represent a key point of control in apoptosis, clearly plays a major role in the aberrant survival of tumor cells. This protein family functions by engaging a network of interactions. Notably, the prosurvival BCL2 members prevent apoptosis by binding to and, in effect, sequestering the proapoptotic members of this family. Therefore, such interactions with the prosurvival BCL2 members in tumor cells are attractive targets for new cancer treatments. Despite the challenging nature of this class of target, structurally guided drug discovery efforts have seen the emergence of compounds that have very promising results in early clinical trials. This review focuses on these strategies to directly target members of the prosurvival BCL2 family, with particular emphasis placed on the yet “undrugged” member myeloid-cell leukemia sequence 1 (MCL-1).

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Acting on the BCL2 dependence of cancer cells – Geneste

MEDICOGRAPHIA, Vol 36, No. 3, 2014 319
Prosurvival
BCL2, BCL-XL, BCL-W, MCL-1, A1

BH1 BH2 BH3 BH4 TM

Proapoptotic
BAX, BAK, BOK

BH3-only: BIM, BAD, PUMA, BID, NOXA, BMF, BIK, HFk

BH3 TM

Figure 1. Schematic representation of the BCL2 family of proteins.

Proteins of the BCL2 family can be divided into 3 major subgroups: the prosurvival BCL2-like proteins, the proapoptotic multidomain BAX-like proteins, and the proapoptotic BH3-only proteins. The prosurvival members are playing an essential role by sequestering the proapoptotic members. The proapoptotic BAX-like proteins are necessary for the activation of the mitochondrial apoptotic cell death pathway and the BH3-only proteins are crucial for the initiation of this death pathway in response to various stressful conditions. Some of these BH3-only proteins contain a transmembrane domain.

Abbreviations: BAD, BCL2-associated death promoter; BAK, BCL2 antagonist/killer; BAX, BCL2-associated X protein; BCL2, B-cell chronic lymphocytic leukemia/lymphoma 2; A1, BCL2-related protein A1; BCL-XL, BCL2-like protein 1; BCL-W, BCL2-like protein 2; BH, BCL2 homology (domain); BID, BH3-interacting-domain death agonist; BIK, BCL2-interacting killer; BIM, BCL2-interacting mediator of cell death; BMF, BCL2-modifying factor; BOK, BCL2-related ovarian killer; HFk, harakiri; MCL-1, myeloid-cell leukemia sequence 1; NOXA, phorbol-12-myristate-13-acetate–induced protein 1; PUMA, p53-upregulated modulator of apoptosis; TM, transmembrane domain.

These proteins share 4 BCL2-homology domains (BH1-4) and preferentially localize at the mitochondrial outer membrane due to the presence of a hydrophobic domain at their carboxyl-terminal ends. (ii) The multidomain proapoptotic proteins BCL2-associated X protein (BAX), BCL2 antagonist/killer (BAK), and BCL2-related ovarian killer (BOK). These proteins have only 3 BH domains (BH1-3) even though their amino-terminal ends contain a helix resembling a BH4 domain. When synthesized, BAX and BAK are essentially inactive and upon activation, they acquire the ability to translocate to and insert into mitochondrial membranes, oligomerize, and trigger MOMP (Figure 2). The absence of both BAX and BAK renders cells resistant to MOMP, highlighting an essential role of these proteins in apoptosis. BOK has a more restricted expression pattern than BAX and BAK and its precise function is much less understood. For instance, it remains unclear whether BOK can functionally substitute for BAX or BAK.5

(iii) The proapoptotic BH3-only proteins. These proteins function upstream of BAX and BAK and are activated by various stress stimuli by transcriptional, translational, and posttranslational mechanisms. The proapoptotic activity of BH3-only proteins requires their interaction with the prosurvival proteins. BH3-only proteins show clear binding affinity differences for the various prosurvival proteins. BCL2-interacting mediator of cell death (BIM), p53-upregulated modulator of apoptosis (PUMA), and a truncated form of BH3-interacting-domain death agonist (tBID; formed by caspase-8-mediated cleavage) binds with high affinity to all prosurvival proteins, whereas BCL2-associated death promoter (BAD) binds to BCL2, BCL-XL, and BCL-W, and phorbol-12-myristate-13-acetate–induced protein 1 (NOXA) binds to MCL-1 and A1 specifically.6 Moreover, a subset of the BH3-only proteins (BIM, PUMA, and tBID), called activator BH3-only proteins, are able to bind and activate the multi-BH-domain proapoptotic BAX or BAK proteins.6,8 Precise structural changes occurring during BAX activation have recently been resolved.8

The prosurvival BCL2 members exert their function by making high-affinity interactions with proapoptotic members. These interactions rely on the binding of the BH3 domain (about 25 amino-acid-long α-helix structure) of proapoptotic members to a hydrophobic groove at the surface of the prosurvival proteins that is formed by their BH1, BH2, and BH3 domains.9 The activation of proapoptotic family members, such as BAX and BAK, leads to exposure of their BH3 domain, which increases their interaction with prosurvival BCL2 proteins.

In many cancers, the balance between the proapoptotic and antiapoptotic (ie, prosurvival) BCL2 members is tipped toward survival by genetic or epigenetic changes, signaling pathway alterations, and posttranslational modifications. For example, increased expression of BCL2, BCL-XL, and MCL-1 has been reported both in hematological malignancies and solid tumors.10-12 Numerous mechanistic studies support the notion that prosurvival BCL2 members maintain survival of cancer cells by sequestering either the activator BH3-only proteins (such as BIM) or BAX and BAK themselves. Therefore,
What we have learned from currently available rationally design inhibitors of the BCL2 prosurvival proteins.

Structural information about BH3-mediated interactions has provided the necessary insights to bind to various BH3 peptides have been solved (Protein Data Bank entries 1PQ1, 1PQ0, 2NL9, 1WSX, and 1BXL).13-16 The promiscuous binding of the BH3-only proteins BIM, PUMA, and tBID with strong affinity to all prosurvival BCL2 members, BAD binds specifically to BCL2, BCL-XL, and BCL-W, and NOXA binds specifically to MCL-1 and A1. In addition, some BH3-only proteins (BIM, tBID, and PUMA) can directly activate the BAX-like proteins. Abbreviations: BAX, BCL2-associated death promoter; BAX, BCL2-associated X protein; BH3, BCL2 homology (domain); BIM, BCL2-interacting mediator of cell death; MCL-1, myeloid-cell leukemia sequence 1; PUMA, p53-upregulated modulator of apoptosis; tBID, truncated BCL2-interacting-domain death agonist; MOMP, mitochondrial outer membrane permeabilization; NOXA, phorbol-12-myristate-13-acetate-induced protein 1.

Targeting prosurvival BCL2 family members

Binding of the BH3 domains to the prosurvival BCL2 family members involves 4 conserved hydrophobic residues on one face of the BH3 α-helix. These residues form electrostatic interactions with 4 hydrophobic pockets (P1-P4) in the binding grooves of the prosurvival proteins. A number of either crystal or solution structures of prosurvival BCL2 family members bound to various BH3 peptides have been solved (Protein Data Bank entries 1PQ1, 1PQ0, 2NL9, 1WSX, and 1BXL).13-16 The promiscuous binding of the BH3-only proteins BIM, PUMA, and tBID to all prosurvival BCL2 proteins can be explained by the fact that the BH3-binding grooves of these proteins share many features due to sequence similarities in their BH1, BH2, and BH3 domains and by mutual conformational adjustments of both partners upon binding. However, the selectivity of some of these interactions relies on subtle differences in a few key residues within both the BH3 domain of the proapoptotic proteins and the BH3-binding grooves of the prosurvival proteins. Structural information about BH3-mediated interactions has provided the necessary insights to rationally design inhibitors of the BCL2 prosurvival proteins.

How structural data concerning BH3-domain binding modes and selectivity have been used to discover prosurvival–BCL2-protein inhibitors, so called BH3 mimetics, is well described in reference 17.

◆ What we have learned from currently available prosurvival–BCL2-protein inhibitors

Targeting the BCL2 family members directly with small molecules is a highly challenging task for several reasons. First of all, these intracellular protein-protein interactions are of very high affinity (subnanomolar range for some of them), and the BH3 binding site on the prosurvival BCL2 proteins is rather shallow, flexible, and does not form an easily tractable pocket. It is therefore a major challenge to discover small-molecule inhibitors with suitable drug-like properties for clinical development.

A number of small-molecule inhibitors of prosurvival BCL2 proteins have been characterized (Table I), but most of them bind their target with too poor affinity to be able to functionally inhibit BCL2 proteins in cells and actually trigger cell death independently of the mitochondrial apoptotic pathway, which strongly suggests that most of their biological activity is not due to their inhibition of prosurvival BCL2 proteins. From published work, 4 compounds with sufficient affinity for their target appear to convincingly trigger the mitochondrial apoptotic pathway through inhibition of prosurvival BCL2 proteins. These compounds are ABT-737,15 which binds to BCL2, BCL-XL, and BCL-W; ABT-26319 (called navitoclax), an orally active clinical derivative of ABT-737; ABT-199,20 which selectively binds BCL2; and WEHI-539, which selectively binds BCL-XL.21

Table I. Inhibitors of prosurvival BCL2 proteins.

<table>
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<tr>
<th>Molecules</th>
<th>Targets</th>
<th>Affinity range</th>
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<tr>
<td>BH3Is</td>
<td>BCL2, BCL-W, MCL-1</td>
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<tr>
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<td>BCL2, BCL-XL, MCL-1</td>
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<td>WEHI-539</td>
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<td>YC137</td>
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Abbreviations: BAX, B-cell chronic lymphocytic leukemia/lymphoma 2; A1, BCL2-related protein A1; BCL-W, BCL2-like protein 2; BCL-XL, BCL2-like protein 1; MCL-1, myeloid-cell leukemia sequence 1.
ABT-737 exhibits single-agent activity, especially on blood cancer models such as B-cell lymphomas, CLLs, acute myeloid leukemias (AMLs), acute lymphoblastic leukemias, a subgroup of multiple myelomas, and some small cell lung carcinomas. In lymphoid malignancies, the crucial target of ABT-737 has been shown to be the BCL2-BIM complexes. The single-agent activity of ABT-737 may be the consequence of constitutive death signals that are induced by some oncogenes. For example, deregulated MYC oncogene can trigger proapoptotic signals and favors MOMP. Thus, cancer cells with such MYC alterations require a compensatory survival signal that can be provided by overexpression of the prosurvival BCL2 family members. In agreement with this notion are the facts that some highly aggressive lymphomas harbor both a MYC (t8;14) and a BCL2 (t14;18) translocation, and two-thirds of cancers with MCL1 or BCL2L1 (coding for BCL-XL) amplifications also have amplifications in the chromosomal region carrying MYC.

There is rationale for combining BH3 mimetics with cytotoxic drugs (eg, genotoxic drugs, which can increase PUMA and NOXA expression; histone deacetylase inhibitors, which can increase BIM expression), but there is also a risk that such a combination would increase the toxicity of the chemotherapy for the healthy tissues. More promising combinations would be to use BH3-mimetic drugs with targeted therapies such as inhibitors of oncogenic kinases, which have been shown to induce BIM expression (or other BH3-only proteins). Such combinations should be specifically synergistic in cancer cells versus healthy cells, as only the cancer cells have deregulated kinase activity due to oncogenic mutations. Consistent with this is the recent finding that tumor cells carrying mutations in RAS (one of the most aggressive oncogenes) are efficiently killed by the combined inhibition of BCL-XL (by short hairpin RNA [shRNA] or ABT-737) and mitogen-activated extracellular signal–regulated kinase (MEK). This suggests that inhibitors specifically targeting BCL-XL, such as WEHI-539 (or derivative) could be of particular interest in the context of RAS-mutant tumors. More generally, it is believed that in many solid tumors, BCL-XL plays a more important prosurvival role than BCL2.

In the clinic, early trials of ABT-263 have focused on lymphoid malignancies and small cell lung cancer. A clinical trial focusing on CLL patients showed promising results for ABT-263 used as a single agent, where a partial response was observed in 31% (9/29) of patients. Results obtained with ABT-263 in small cell lung cancer patients were disappointing, with only 1 partial response out of 39 patients. The dose-limiting toxicity of ABT-263 is a transient acute thrombocytopenia resulting from the inhibition of BCL-XL, which controls platelet lifespan. This finding led to the discovery and development of ABT-199, which is specific for BCL2. A recent phase 1 trial of ABT-199 showed highly promising results in CLL patients (55 evaluable patients) with 84% overall response rate, 65% with a partial response, and 18% with a complete response. The response in CLL patients was independent of the 17p-deletion (missing short arm of chromosome 17) high-risk marker. Preliminary results of the ABT-199 phase 1 trial in non-Hodgkin lymphoma (especially mantle-cell lymphoma) patients were also very encouraging. As expected from its binding selectivity for BCL2, ABT-199 did not show platelet toxicity; instead, tumor lysis syndrome (reflecting rapid apoptosis of tumor cells) was a dose-limiting toxicity.

**Targeting the yet “undrugged” prosurvival protein MCL-1**

MCL-1 is overexpressed in multiple types of cancer and participates in tumor development and resistance to anticancer therapies. Moreover, a recent study reported that the MCL-1 gene is one of the most frequently amplified genes in a large number of tumor types in human and that MCL-1 is required for survival of breast cancer and non-small cell lung carcinoma (NSCLC) cell lines. Therefore, MCL-1 is recognized as an important potential therapeutic target in cancer. So far, no compound that directly targets MCL-1 convincingly has entered clinical development (ie, MCL-1 is as yet “undrugged”), and MCL-1 has clearly been shown to be a resistance mechanism to the compound ABT-737, which targets BCL2/BCL-XL/BCL-W, and to other anticancer drugs. Studies using either small interfering RNA (siRNA) or indirect approaches to downregulate protein level have shown that MCL-1 is a major survival factor in tumor cells.

MCL-1 has specific features compared with other prosurvival BCL2 members regarding the regulation of its expression. Indeed, MCL-1 expression is remarkably tightly controlled by multiple mechanisms. These include transcriptional, posttranscriptional, and posttranslational levels of regulation (Figure 3). At the transcriptional level, MCL-1 is regulated by various transcription factors, including signal transducer and activator of transcription (STAT), E2F transcription factor 1 (E2F1), STAT3, PU.1, hypoxia-inducible factor-1 (HIF-1), and ternary complex factor (TCF)–serum response factor (SRF), which are themselves downstream targets of key oncogenic pathways. At the posttranscriptional level, MCL-1 messenger RNA (mRNA) is subjected to regulation by microRNAs (miRNAs), most notably by miR-29b. Loss of miR-29b has been shown to be a mechanism involved in MCL-1 overexpression, especially in AML, and restoration of miR-29b in AML cells induces apoptosis and reduces tumorigenicity. On the other hand, the phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B)/mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway promotes tumor-cell survival in a mouse model by stimulating the translation of Mcl-1. At the posttranslational level, MCL-1, in contrast to other prosurvival BCL2 family members, is a very short-lived protein with a half-life of 20 minutes to a few hours depending on cell type. MCL-1 protein stability is controlled by the ubiquitin/proteasome pathway and 3 distinct E3 ubiquitin ligases (MCL-1 ubiquitin ligase [MULE], β-transducin repeat-containing protein [TBC1D3], and MBD4).
Altogether, the multiple mechanisms controlling MCL-1 expression and stability could lead to various potential approaches to downregulate MCL-1 for anticancer therapy. A number of compounds globally inhibiting either transcription (eg, CDK9 inhibitors) or translation have been shown to exert their cytotoxic effects by downregulating MCL-1.48 Indeed, because MCL-1 is a very short-lived protein, tumor cells that are dependent on MCL-1 for survival die rapidly in response to global transcription or translation inhibitors. In theory, this finding points to several possible opportunities to indirectly target MCL-1, but these approaches present several issues. The lack of specificity for compounds such as global transcription blockers is almost certain to cause significant toxicity and an acceptable therapeutic window for such treatment might be difficult to achieve. Another important limitation for compounds that downregulate MCL-1 by globally inhibiting transcription (or translation) regards their use in combination with other anticancer drugs. For instance, transcription or translation blockers have, in fact, been shown to counteract the effects of anticancer drugs such as the proteasome inhibitor bortezomib or the histone deacetylase inhibitor vorinostat, which need to induce expression of proapoptotic proteins such as NOXA, BIM, or BCL2-modifying factor (BMF) to exert their cytotoxicity.45 Rather than blocking its synthesis, accelerating degradation of the MCL-1 protein could also be an interesting approach to target MCL-1. In that respect, USP9X, which has been shown to stabilize MCL-1 by catalyzing its deubiquitination, is an attractive target.41 Targeting USP9X could be a more specific approach than the ones discussed above, but one open question is whether accelerating MCL-1 degradation will lead to sufficient MCL-1 downregulation to induce apoptosis. So, even if, due to its complex regulation, MCL-1 can be targeted by multiple mechanisms, the best strategy to target MCL-1 is most probably the direct inhibition by BH3-mimetic drugs, which is more specific and more likely to induce a quicker apoptotic response in tumor cells. From work published so far, compounds with only moderate affinity for MCL-1 (<100nM) have been described.46

Conditional knockout experiments in mice have shown that Mcl-1 is an essential survival protein in hematopoietic stem cells and cardiomyocytes.47,48 Therefore, the question of therapeutic window is of particular importance when targeting MCL-1. This point was nicely addressed in a recent work using models of AML in mice where the Mcl-1 gene can be selectively target-ed in an inducible fashion.49,50 Importantly, this study showed that leukemic cells were significantly more sensitive to Mcl-1 loss than normal

**Figure 3.** MCL-1 expression and stability are controlled by multiple mechanisms. MCL-1 gene transcription is regulated by multiple transcription factors (such as STAT5, HIF-1, and TCF-SRF) that are downstream targets of key oncogenic pathways. MCL-1 messenger RNA (mRNA) is regulated by the microRNA miR-29b. The PI3K-AKT-mTORC1 pathway stabilizes MCL-1 transcription. Stability of the MCL-1 protein is controlled by the ubiquitin-proteasome pathway and 3 E3 ubiquitin ligases (MULE, β-TrCP, and FBW7) have been shown to contribute to MCL-1 ubiquitination and subsequent degradation. On the contrary, USP9X stabilizes MCL-1 by catalyzing its deubiquitination. Phosphorylation of MCL-1 on threonine residue (Thr) 163 by extracellular signal-regulated kinase (ERK) increases its half-life, whereas phosphorylation of serine residue (Ser) 159 by glycogen synthase kinase 3 (GSK3) promotes its ubiquitination and degradation.43 Proteasomal degradation of MCL-1 has also been shown to be enhanced by phosphorylation of Thr92 by the cyclin-dependent kinase 1 (CDK1)/cyclin B1 complex during mitosis.44

**Abbreviations:** β-TrCP, β-truncadin repeat-containing protein; AKT, AKT or protein kinase B; CDK, cyclin-dependent kinase; E2F1, E2F transcription factor 1; ERK, extracellular signal-regulated kinase; FBW7, F-box and WD repeat domain containing 7; GSK3, glycogen synthase kinase 3; HIF-1, hypoxia-inducible factor 1; MCL-1, myeloid-cell leukemia sequence 1; miR-29b, microRNA 29b; MULE, MCL-1 ubiquitin ligase; mTORC1, mammalian target of rapamycin (mTOR) complex 1; PI3K, phosphatidylinositol 3-kinase; PU.1, Ezb transformation-specific-unique sequence (ETS)-family transcription factor PU.1; STAT, signal transducer and activator of transcription; TCF-SRF, ternary complex factor (TCF)-sequestration response factor (SRF); USP9X, ubiquitin-specific peptidase 9, X-linked.
hematopoietic stem cells and progenitor cells. The difference in sensitivity to Mcl-1 depletion between AML cells and normal hematopoietic cells was measurable in terms of percentage of remaining viable cells, but perhaps more clearly in terms of kinetics of cell death. These data suggest that there should be a therapeutic margin with anti-MCL-1 drugs for AML treatment. In addition, it is important to realize that the irreversible loss of MCL-1, such as that resulting from gene-knockout experiments, is not equivalent to its pharmacological inhibition in a timely fashion. While long-term inhibition of MCL-1 may not be tolerated, there should be a therapeutic window due to a higher level of MCL-1 dependency in tumor cells versus normal cells resulting in a more rapid death of tumor cells. So, in the clinic, a therapeutic window could be achievable using an intermittent schedule combined with strategies to increase the level of MCL-1 dependency in tumor cells. Such a strategy could be combinations with treatments that prime tumor cells to MCL-1-dependent cell death, especially in terms of kinetics of cell death. One complicating factor for the BH3-mimetic approach is the high MCL-1 turnover, meaning that a stock of intact MCL-1 protein will be rapidly renewed once the inhibitory drug has disappeared from the tumor cells. So, the therapeutic window is likely to result from a “fine tuning” between drug pharmacokinetics, schedule of administration, kinetics of tumor cell death, and MCL-1 turnover, which could turn out to be challenging for drug discovery and clinical development. Nevertheless, the BH3-mimetic approach, because of its specificity and rapid mechanism of action, probably represents the best opportunity to target MCL-1 for therapeutic benefit.

Conclusions
Acting on the BCL2 dependency of tumor cells is a highly innovative and attractive mode of intervention for cancer treatment. No drugs directly targeting prosurvival BCL2 family members have yet been approved, but the very promising results obtained in early clinical trials, especially for the BCL2-specific inhibitor ABT-199, strongly indicate that such BH3-mimetic compounds will soon be part of anticancer therapy. However, for such compounds to become efficient therapeutic agents, it will be necessary to better understand the BCL2-like dependency of tumor cells at the molecular and cellular levels so that relevant diagnostic biomarkers could be identified. The discovery of BH3-mimetic compounds specifically targeting each prosurvival BCL2 family member will clearly be helpful to characterize this phenotype of BCL2-like dependency and therefore to design better treatments.

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Olivier Geneste obtained his PhD in 1995 at the Université Claude Bernard Lyon 1, France, where he worked on mechanisms of induction of metabolizing enzymes (P450 cytochromes) in response to carcinogens. His first postdoctoral position was in a Centre National de la Recherche Scientifique (CNRS) laboratory in Lyon, France, and involved studying the functional consequences of germine mutations in the RET gene in the group directed by Marc Billaud. In 1996, he moved to the former Imperial Cancer Research Fund, London, UK, where he worked in the laboratory of Richard Traisman on chromatin modifications that modulate transcriptional activation and the control of the serum response factor (SRF) transcription factor by actin dynamics. He moved to the Servier Research Institute, Croissy, France, in 2001 and is now director of a drug discovery program that focuses on targeting prosurvival BCL2 family members.

References
AGIR SUR LA DÉPENDANCE BCL2 DES CELULLES CANCÉREUSES

L’apoptose est une forme de mort cellulaire programmée, essentielle au développement et à l’hémostasie tissulaire mais elle est presque systématiquement altérée dans les cellules tumorales qui survivent malgré les nombreux stimuli apoptotiques. Une dérégulation des protéines de la famille BCL2 (lecumie lymphoïde chronique/lymphome 2 à cellules B), point clé du contrôle de l’apoptose, joue à l’évidence un rôle majeur dans la survie aberrante des cellules tumorales. Cette famille de protéines fonctionne par un réseau d’interactions, en particulier les membres anti-apoptotiques BCL2 qui préviennent l’apoptose en se liant aux membres pro-apoptotiques de cette famille et, de ce fait, en les séquestrant. De telles interactions avec les membres anti-apoptotiques BCL2 dans les cellules tumorales représentent des cibles intéressantes pour de nouveaux traitements anticancéreux. En dépit de la nature difficile de ce type de cible, des efforts de recherche orientés sur la structure moléculaire ont permis l’émergence de composés dont les résultats des premières études cliniques sont très prometteurs. Cet article s’intéresse à ces stratégies de ciblage direct des membres de la famille anti-apoptotiques BCL2 et particulièrement au membre MCL-1 pour lequel il n’existe pas encore d’inhibiteur en développement clinique.
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RNA interference and cardiovascular disease

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The heart is the first organ to form and function in the embryo, and all subsequent events in the life of the organism depend on its uninterrupted, second-to-second contractility. Heart development is a highly complex process involving the integration of multiple cell types into the three-dimensional organ and its seamless connection to the blood vessels of the vascular system. Even subtle abnormalities in the events of heart formation can cause congenital heart disease, the most common human birth defect. Acquired and inherited disorders of the adult heart also lead to a loss of contractility and heart failure, the most common cause of morbidity and mortality worldwide. Despite the therapeutic benefits of numerous treatment options, including cholesterol-lowering drugs, blood pressure medications, and other drugs, as well as mechanical devices and surgical techniques to sustain cardiac function, the prevalence of cardiovascular disease continues to increase, highlighting the need for new therapeutic approaches. Recent studies have revealed pivotal roles for a class of small RNA molecules, known as microRNAs, in the control of heart development, disease, and regeneration. MicroRNAs also serve as sensitive biomarkers for cardiovascular disease, and can be targeted by specific microRNA-based inhibitors and mimics, creating new therapeutic opportunities for disease intervention. This review provides an overview of this rapidly evolving area of research and considers important questions for the future.

MicroRNA control of heart development, disease, and regeneration

by E. N. Olson, USA
trial of a miRNA therapeutic for suppression of hepatitis C virus (HCV) replication has raised possibilities for a new class of disease-modifying therapeutics based on miRNA biology.\(^7\)

This article provides a general overview of the rapidly moving field of miRNA biology and considers how miRNAs have revealed new facets of cardiovascular disease processes and suggested revolutionary ideas for therapeutic development.

**The biology and functions of miRNAs**

miRNAs are short, noncoding RNA molecules that act as negative regulators of gene expression by inhibiting the expression of specific proteins encoded by messenger RNAs (mRNAs) (Figure 1). Generally about 22 nucleotides in length, miRNAs anneal through complementary base pairing with sequences in protein-coding mRNAs and act through various mechanisms to diminish protein translation. miRNAs associate with mRNA targets within a multiprotein complex known as the RNA-induced silencing complex (RISC) in the cytoplasm of cells (Figure 2).

The human genome has been estimated to encode approximately 1000 miRNAs that are predicted to regulate a majority of protein-coding genes. Indeed, virtually all normal cellular processes and diseases appear to be subject to miRNA control. Many miRNAs are conserved across a wide range of organisms, indicating the evolutionary pressure to maintain their functions.

An especially powerful, and vexing, feature of miRNA-based regulation is the ability of single miRNAs to regulate vast numbers of mRNAs. Many individual miRNAs, for example, are predicted to target hundreds of mRNAs with varying efficiencies. Moreover, individual miRNAs can be targeted simultaneously by many miRNAs. Thus, there is enormous regulatory complexity in miRNA control of gene expression. Rather than functioning as “on-off” switches, miRNAs generally exert relatively modest effects on individual targets, acting as rheostats to fine-tune protein expression; it is the aggregate effect of

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**Selected abbreviations and acronyms**

- anti-miR: microRNA inhibitor
- HCV: hepatitis C virus
- MI: myocardial infarction
- miRNA: microRNA
- mRNA: messenger RNA
- RISC: RNA-induced silencing complex

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**Figure 1. How miRNAs work.**

miRNAs anneal to mRNAs and inhibit the production of proteins. miRNAs are regulated in response to stress signals. Their inhibitory activity is also influenced by stress. The stress-dependent regulation of miRNA expression and activity plays a key role in disease modulation.

**Abbreviations:** miRNA, microRNA; mRNA, messenger RNA.

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**Figure 2. Therapeutic manipulation of miRNAs.**

miRNAs associate with mRNAs in a multiprotein complex known as the RISC complex. Inhibitors (anti-miRs) of pathological miRNAs block miRNA function, leading to an increase in expression of miRNA targets, and beneficial effects. Conversely, miRNA mimics increase the repressive influence of beneficial miRNAs on their targets.

**Abbreviations:** anti-miR, microRNA inhibitor; miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex.
partial inhibition of many targets that leads to their potent biological effects. A major challenge in the field is to understand how miRNAs evoke their biological actions and to identify the cellular targets that mediate their effects. This ambiguity in mechanism of action poses particular challenges in the development of miRNA-based drugs, since it is difficult to ascribe therapeutic efficacy to the engagement of a miRNA-based drug with a specific cellular target.

**Therapeutic modulation of miRNAs**

miRNAs play especially important roles in disease. Genetic deletion of miRNAs in mice and other organisms often has little or no effect, whereas in response to stress or injury, the biological actions of miRNAs become magnified. There are numerous examples in which genetic deletion of a miRNA enhances the response of an organism to disease, and others in which genetic deletion of a miRNA blocks disease pathogenesis. Their stress-sensitive actions make miRNAs particularly attractive targets for therapeutic modulation. As schematized in Figure 1, stress signals can modulate the expression of miRNAs or can enhance their repressive influence on downstream protein targets.

While traditional drug targets such as cell surface receptors, enzymes, and ion channels are effectively inhibited by specific small molecules, there are a variety of mechanisms whereby cells can develop resistance to drugs directed against single cellular targets. Mutations in drug targets within cells, for example, can confer drug resistance, as demonstrated for the anticancer drug imatinib. Desensitization of cell surface receptors or downregulation of enzymes can also render cells or tissues insensitive to the effects of classical drugs. In contrast, the ability of miRNAs to target multiple components of complex biological pathways avoids the requirement of single cellular proteins for their activity. Instead, miRNAs often regulate collections of genes that function at different steps in complex biological pathways. Therein lies their ability to control processes that might be resistant to inhibition of a single cellular component.

The ability of miRNAs to modulate important biological pathways offers opportunities for the regulation of miRNA function using oligonucleotide inhibitors (anti-miRs) and miRNA mimics (Figure 2). Oligonucleotides directed against specific miRNA sequences are efficiently taken up by a variety of tissues following systemic delivery and are well tolerated without toxicity. Inhibition of a miRNA with an anti-miR results in the elevated expression of its targets. Such inhibitors are being developed against pathological miRNAs that promote disease. Conversely, miRNA mimics enhance the repressive influence of miRNAs on their targets, resulting in repression of gene expression. In this case, miRNA mimics that enhance the activity of beneficial miRNAs would be expected to diminish disease progression. A more in-depth discussion of therapeutic strategies to modulate miRNA function is presented in the accompanying article by Dr Eva van Rooij.

**Roles of miRNAs in cardiovascular development**

miRNAs have been shown to play essential roles in development of the heart. Formation of the heart requires precise and complex interactions among diverse cell types, including cardiomyocytes and cells that form blood vessels, connective tissue, endocardium, and the cardiac conduction system. Initial evidence for the involvement of miRNAs in heart development came from studies in which the enzyme Dicer, which controls the biosynthesis of miRNAs, was genetically deleted specifically in the developing mouse heart. Cardiac deletion of Dicer in early cardiac precursor cells resulted in embryonic lethality due to a block in heart growth and development.

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**Figure 3.** Roles for miRNAs in vascular development.

Various miRNAs with roles in blood vessels and vascular disease are shown.

illary, deletion of Dicer from neural crest cells, which contribute to the formation of the cardiac outflow tract, results in severe abnormalities in heart development that include ventricular septal defects, double outlet right ventricle, and interrupted aortic arch. Subsequent studies revealed miRNAs that are expressed specifically in different cell types of the heart, and deletion of these miRNAs in mice causes a spectrum of heart abnormalities, including ventricular septal defects, thin-walled myocardium, and cardiac rhythm disturbances. Considering the prevalence of these types of developmental cardiac abnormalities in humans, it is likely that miRNAs modulate aspects of human congenital heart disease.

Many miRNAs have been implicated in blood vessel formation and in maintenance of blood vessel integrity. Specific miRNAs are expressed in endothelial cells that form the inner lining of blood vessels, as well as in smooth muscle cells that confer structure and contractility to arteries and veins. Deletion of specific miRNAs in animal models of disease has been shown to cause rupture of blood vessels, failure in vascular smooth muscle differentiation, as well as abnormal vessel patterning.

There are numerous diseases associated with excessive or insufficient blood vessel numbers, and manipulating miRNAs in these disease settings has shown benefit in animal models of vascular disease. Some of the roles of miRNAs in blood vessel function and dysfunction are shown in Figure 3. Diseases of the eye that arise from abnormalities in heart development that include ventricular septal defects, double outlet right ventricle, and interrupted aortic arch. Subsequent studies revealed miRNAs that are expressed specifically in different cell types of the heart, and deletion of these miRNAs in mice causes a spectrum of heart abnormalities, including ventricular septal defects, thin-walled myocardium, and cardiac rhythm disturbances. Considering the prevalence of these types of developmental cardiac abnormalities in humans, it is likely that miRNAs modulate aspects of human congenital heart disease.

Roles of miRNAs in heart disease

The first evidence suggesting the involvement of miRNAs in heart disease came from studies demonstrating changes in expression of specific miRNAs in diseased hearts from mice and humans. The adult heart responds to injury or stress by increasing miR-29 expression in response to pressure overload. Ongoing studies are aimed at determining whether inhibition of miR-29 in larger animals confers similar benefit.

Prolonged hypertrophy and other forms of cardiac stress, such as myocardial infarction (MI), result in fibrosis in which fibroblasts within the heart produce excessive extracellular matrix proteins, such as collagens. Fibrosis impedes cardiac contractility and also disturbs electrical conduction within the heart. A family of miRNAs known as the miR-29 family plays a critical role in suppressing fibrosis in the heart and other organs. Members of the miR-29 family inhibit expression of collagens and other proteins involved in fibrosis. Under conditions of heart disease, miR-29 expression declines, which unleashes the fibrotic process. In light of these findings, there has been intense effort toward the development of therapeutic strategies to elevate miR-29 production as a strategy to prevent fibrosis. Conversely, during the formation of aortic aneurysms, in which blood vessels become pathologically dilated and susceptible to rupture, miR-29 is upregulated, preventing the production of extracellular matrix proteins and leading to aortic dilation. In this setting, therapeutic strategies to diminish hypertrophic growth in which cardiac muscle cells increase in size, but not in number. Cardiac hypertrophy initially serves as a compensatory mechanism to enhance cardiac output, but hypertrophy ultimately becomes deleterious by causing stiffness of the ventricular walls of the heart and is a major predictor for mortality. Numerous miRNAs have been shown to promote and inhibit cardiac hypertrophy, as schematized in Figure 4.

Other miRNAs regulate cardiomyocyte survival, changes in cardiac metabolism, and other processes associated with the progression of heart disease.

A particularly dramatic example of miRNA modulation of cardiac disease comes from studies of the cardiac-specific miRNA, miR-208a. This miRNA is encoded by an intervening sequence within the gene encoding alpha-myosin heavy chain, the major contractile protein of the heart. In response to cardiac stress, the adult heart changes the pattern of myosin expression from the alpha isoform toward the beta isoform, which is thought to contribute to a diminution of cardiac contractility. Genetic deletion of miR-208a in mice or inhibition with an anti-miR prevents myosin switching from alpha to beta and improves cardiac function in response to pressure overload. Prolonged hypertrophy and other forms of cardiac stress, such as myocardial infarction (MI), result in fibrosis in which fibroblasts within the heart produce excessive extracellular matrix proteins, such as collagens. Fibrosis impedes cardiac contractility and also disturbs electrical conduction within the heart. A family of miRNAs known as the miR-29 family plays a critical role in suppressing fibrosis in the heart and other organs. Members of the miR-29 family inhibit expression of collagens and other proteins involved in fibrosis. Under conditions of heart disease, miR-29 expression declines, which unleashes the fibrotic process. In light of these findings, there has been intense effort toward the development of therapeutic strategies to elevate miR-29 production as a strategy to prevent fibrosis. Conversely, during the formation of aortic aneurysms, in which blood vessels become pathologically dilated and susceptible to rupture, miR-29 is upregulated, preventing the production of extracellular matrix proteins and leading to aortic dilation. In this setting, therapeutic strategies to diminish
miR-29 would be beneficial by enhancing the production of extracellular matrix proteins, contributing to greater rigidity of the vessel wall. These opposing effects of miR-29 in different tissues and different disease states highlight the challenges of modulating miRNAs systemically, since there may be some tissues in which miRNAs exert beneficial effects and others in which their effects are deleterious.

Roles of miRNAs in heart regeneration
The adult mammalian heart cannot repair itself following injury.23 The lack of regenerative activity of the heart is due to the inability of adult cardiac muscle cells to divide and replace those that are lost during disease and aging. Identifying the key regulators of cardiomyocyte proliferation and therapeutic manipulation of this process represents one of the central challenges in cardiovascular medicine today.

In an especially exciting series of recent studies, a collection of miRNAs was shown to regulate heart regeneration in mice. One miRNA, called miR-15, is highly expressed in the adult heart and prevents cardiac muscle cells from dividing. Inhibition of miR-15 with an anti-miR allows for cardiomyocyte proliferation and enhances the ability of the adult heart to regenerate following injury.24-26 Two other miRNAs, miR-590 and miR-199a, when overexpressed in the heart through viral delivery, potently stimulate proliferation of cardiomyocytes and sustain cardiac function following myocardial infarction.27 These findings provide a potentially powerful new means of promoting heart repair by preventing myocyte loss through miRNA modulation. Identification of the targets of these miRNAs will also provide new insights into the mysterious process of heart regeneration. There is also evidence that specific miRNAs can activate the expression of cardiac muscle genes in non-muscle cells from mice and humans.28-30 These exciting findings suggest additional opportunities for repair of the injured heart through reprogramming of fibroblasts and other non-muscle cells into cardiomyocytes through manipulation of miRNA expression.

Looking to the future
Our understanding of the mechanisms of action, regulation, and potential therapeutic manipulation of miRNAs has progressed at a remarkably rapid pace in recent years, and it seems a virtual certainty that miRNA-based therapeutics are on the near-term horizon. However, many interesting and important questions remain to be addressed. For example, to what extent can miRNAs serve as reliable biomarkers for disease progression in humans? For those miRNAs that are detected in blood or other body fluids, how do they get there, where are they going, and what are their potential functions outside of cells? Might extracellular miRNAs function in inter-tissue signaling? Can new approaches be developed to selectively target miRNAs in individual tissues in vivo with inhibitors or mimics? Of the ~1000 miRNAs encoded by the human genome,31 only a relatively small fraction has been intensely studied. How many additional miRNAs participate in disease processes?

Given the influence of individual miRNAs on vast numbers of protein-coding miRNAs and the relatively modest effects of miRNAs on individual targets, a complete understanding of the mechanism of miRNA action will require systems biology approaches combined with bioinformatics. A thorough understanding of miRNA functions will also require analysis of the proteome (the complete collection of proteins within a cell) and how this is modulated over time by miRNA manipulation. Considering the pace of research in this area, answers to these and other questions should soon be forthcoming.
**RÔLE DES microARN DANS LE CONTRÔLE DU DÉVELOPPEMENT, DE LA PATHOLOGIE ET DE LA RÉGÉNÉRATION CARDIAQUES**

Le cœur est le premier organe à se former et à fonctionner dans l’embryon et tous les événements ultérieurs dans la vie de l’organisme dépendent de sa contractilité ininterrompue, seconde après seconde. Le développement cardiaque est un processus extrêmement complexe associant l’intégration de nombreux types de cellules dans cet organe tridimensionnel et sa connexion homogène aux vaisseaux sanguins du système vasculaire. Même de légères anomalies pendant la formation du cœur peuvent provoquer une cardiopathie congénitale, malformation de naissance humaine la plus courante. Les maladies héréditaires ou acquises du cœur adulte conduisent aussi à une perte de contractilité et à une insuffisance cardiaque, cause la plus fréquente de morbidité dans le monde. Malgré les bénéfices thérapeutiques de nombreux traitements, comme les hypolipémiants, les antihypertenseurs et d’autres médicaments, ainsi que les dispositifs mécaniques et les techniques chirurgicales qui maintiennent la fonction cardiaque, la prévalence de la maladie cardio-vasculaire continue à augmenter, soulignant le besoin de nouveaux traitements. Dans des études récentes, le rôle clé d’une classe de petites molécules d’ARN, connues sous le nom de microARN, a été démontré dans le contrôle du développement, de la pathologie et de la régénération cardiaques. Ces microARN servent aussi de biomarqueurs sensibles pour la maladie cardio-vasculaire et peuvent être ciblés par des substances mimétiques et inhibitrices basées sur des microARN spécifiques, offrant de nouvelles possibilités thérapeutiques. Cet article passe en revue ce domaine de recherche en évolution rapide et examine les questions importantes pour le futur.

**References**

Despite a detailed understanding of the molecular and cellular processes governing cardiac function and contractility, cardiovascular disease remains the primary cause of morbidity and mortality worldwide. Although numerous treatment options show therapeutic benefit, such as statins, angiotensin-converting enzyme (ACE) inhibitors, and β-blockers, cardiovascular disease continues to increase in prevalence, underscoring the need for new therapeutic strategies. In recent years, prominent roles for microRNAs (miRNAs) have been uncovered in a variety of cardiovascular disorders. miRNAs are short, single-stranded, noncoding RNAs that anneal with complementary sequences in messenger RNAs (mRNAs), thereby suppressing protein expression. Their known sequence and heightened functions under conditions of pathophysiological stress and disease make them attractive candidates for therapeutic manipulation. Lessons learned from antisense technologies catalyzed opportunities to therapeutically regulate miRNAs by using oligonucleotide chemistries, which by now have proven to be efficacious in targeting pathological miRNAs in animals and even humans. The aim of this review is to discuss the current approaches to target miRNAs, summarize potential therapeutic targets for cardiovascular disorders, and consider the challenges associated with this new therapeutic modality.

In the last decade, it has become increasingly clear that microRNAs (miRNAs) are relevant players during disease, including cardiovascular disorders. Because of their known and conserved sequence, antisense chemistries, known as anti-miRs, were rapidly developed to target disease-related miRNAs in vivo. By now, anti-miR chemistries have proven to be efficacious in targeting pathological miRNAs in animals and even humans. Here the current state of miRNA therapeutics is discussed and potential hurdles in developing these novel drugs will be contemplated.

miRNA therapeutics

miRNAs are small, noncoding RNAs that negatively influence gene expression in a sequence-dependent fashion. Since individual miRNAs engage numerous messenger RNA (mRNA) targets that are often encoding multiple components of complex intracellular networks, the manipulation of a miRNA can have a profound impact on cellular phenotypes (Figure 1). In the last few years, genetic gain- and loss-of-function approaches, as well as pharmacological modulation of individual miRNAs or miRNA families in animal disease models, have shown miRNAs to be important reg-
Anti-miR chemistries

such as 2’-O-methyl (2’-OMe), 2’-O-methoxyethyl (2’-MOE), 2’-fluoro (2’-F), or locked nucleic acid (LNA), in which the 2’-O-oxygen is bridged to the 4’ position via a methylene linker to form a rigid bicycle, locked into a C3’-endo (RNA) sugar conformation. Another chemical modification applied to confer oligonucleotide stability by nuclease resistance and facilitate cellular uptake is the balance between phosphodiester (PO) and phosphorothioate (PS) linkages between the nucleotides. PS backbone linkages, whereby sulfur replaces one of the nonbridging oxygen atoms in the phosphate group, are more resistant to nucleases than PO, thereby providing more stability to the oligonucleotide. In addition to increasing stability, the PS backbone modifications promote plasma protein binding, thus reducing clearance by glomerular filtration and urinary excretion, which facilitates tissue delivery of anti-miRs in vivo.

There are several key requirements for anti-miRs to achieve effective pharmacological inhibition of disease-associated miRNAs, namely in vivo stability, specificity, and high binding affinity to the miRNA of interest. The modifications that are most commonly used are high-affinity 2’ sugar modifications, such as 2’-O-methyl (2’-OMe), 2’-O-methoxyethyl (2’-MOE), 2’-fluoro (2’-F), or locked nucleic acid (LNA), in which the 2’-O-oxygen is bridged to the 4’ position via a methylene linker to form a rigid bicycle, locked into a C3’-endo (RNA) sugar conformation. Another chemical modification applied to confer oligonucleotide stability by nuclease resistance and facilitate cellular uptake is the balance between phosphodiester (PO) and phosphorothioate (PS) linkages between the nucleotides. PS backbone linkages, whereby sulfur replaces one of the nonbridging oxygen atoms in the phosphate group, are more resistant to nucleases than PO, thereby providing more stability to the oligonucleotide. In addition to increasing stability, the PS backbone modifications promote plasma protein binding, thus reducing clearance by glomerular filtration and urinary excretion, which facilitates tissue delivery of anti-miRs in vivo.

In 2005, Krutzfeldt et al reported on the first mammalian in vivo study using modified cholesterol-conjugated oligonucleotides complementary to the mature miRNA sequence, called antagomirs, to inhibit miR-122, a liver-specific miRNA. Antagomirs are fully 2’-OMe modified, harbor optimized PS modifications (2 at the 5’ end and 4 toward the 3’ end), and require a >19-nucleotide length for highest efficiency. Their high level of specificity is indicated by the fact that they can discriminate between single nucleotide mismatches of the targeted miRNA. Remarkably, these studies indicated that a single intravenous bolus injection of an antagomir is sufficient to inhibit the function of its target miRNA for weeks, for both specifically expressed miRNAs (like miR-122) and more broadly expressed miRNAs (like miR-16). These experiments also indicated that the anti-miR chemistries do not cross the blood-brain barrier. These lines of evidence validated the efficacy of antagomirs and founded the basis for the use of antisense oligonucleotides to silence miRNAs in vivo.

More recently, striking data demonstrating the therapeutic power of LNA-modified anti-miRs have been reported in rodents (see Table I [page 337] for cardiovascular examples), nonhuman primates, and even humans. Although all of the 2’ modifications improve nuclease resistance and increase duplex melting temperature (Tm), LNA possess the highest affinity with an increase in Tm of +2°C to +8°C per introduced LNA modification against complementary RNA, and leads to the thermodynamically strongest duplex formation with complementary RNA known due to their high affinity. It should be noted that, unlike antagomirs, these molecules are un conjugated, indicating that cholesterol conjugation for this chemistry is not required for functional miRNA inhibition. As a consequence of the high binding affinity, biological activity is often already attained with shorter LNA-modified oligonucleotides. Several studies have reported that subcutaneous delivery of high-affinity 15- to 16-nucleotide LNA/DNA mixmers containing roughly 50% LNA bases targeting the 5’ region of the mature miRNA are sufficient to establish a functional effect in vivo. Even tiny 8-mer LNA anti-miRs with a complete PS backbone directed against the seed region of a miRNA (the region most important for target recognition) silence miRNAs in vivo with-
Pharmacokinetic and -dynamic properties of anti-miRs

Anti-miRs currently use various high-affinity 2'-sugar modifications such as 2'-O-methyl (2'-OMe), 2'-O-Methoxyethyl (2'-MOE), 2'-fluoro (2'-F), or locked nucleic acid (LNA) conformationally restricted nucleotides. To increase nuclease resistance, most anti-miRs chemistries to date harbor phosphorothioate (PS) backbone linkages, whereby sulfur replaces one of the nonbridging oxygen atoms in the phosphate group. Anti-miRs containing cholesterol, conjugated via a 2'-O-Me linkage, named antagonirs, are fully complementary to the mature miRNA sequence with several PS moieties to increase stability. Several unconjugated phosphorothioated antisense molecules with various high-affinity 2'-sugar modifications such as 2'-MOE, 2'-F, or LNA are currently also being used. While all these modifications improve nuclease resistance and increase duplex melting temperature ($T_m$), the high duplex melting temperature of LNA-modified oligonucleotides enables efficient miRNA inhibition with shorter anti-miRs.

**Abbreviations:**
- A: adenine
- C: cytosine
- G: guanine
- T: thymine
- U: uracil


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Figure 2. Anti-miR chemistries.
Anti-miRs chemistries currently use various high-affinity 2'-sugar modifications such as 2'-O-methyl (2'-OMe), 2'-O-Methoxyethyl (2'-MOE), 2'-fluoro (2'-F), or locked nucleic acid (LNA) conformationally restricted nucleotides. To increase nuclease resistance, most anti-miRs chemistries to date harbor phosphorothioate (PS) backbone linkages, whereby sulfur replaces one of the nonbridging oxygen atoms in the phosphate group. Anti-miRs containing cholesterol, conjugated via a 2'-O-Me linkage, named antagonirs, are fully complementary to the mature miRNA sequence with several PS moieties to increase stability. Several unconjugated phosphorothioated antisense molecules with various high-affinity 2'-sugar modifications such as 2'-MOE, 2'-F, or LNA are currently also being used. While all these modifications improve nuclease resistance and increase duplex melting temperature ($T_m$), the high duplex melting temperature of LNA-modified oligonucleotides enables efficient miRNA inhibition with shorter anti-miRs.

**Pharmacokinetic and -dynamic properties of anti-miRs**

The size and charge of anti-miRs limit intestinal absorption, thereby preventing them from becoming good candidates for oral administration. Although relatively little is known about the mechanisms of cellular uptake, subcutaneous delivery warrants rapid uptake of anti-miR chemistries in many tissues.

Once inside cells, many modified anti-miRs are extremely stable with half-lives in the order of weeks. Additionally, while systemically delivered antagonirs appear to accumulate in a cytoplasmic compartment distinct from processing bodies and induce miRNA degradation by a RNA interference–independent pathway, $4,5$ LNA-modified anti-miRs seem to inhibit miRNA function by sequestration of the mature miRNA, $6,7$ implying different modes of action for the different anti-miR chemistries.

For the heart, it is known that anti-miRs reach cardiomyocytes, fibroblasts, and vascular cell populations, but it will be of interest to determine whether some cell types are more efficiently targeted, and whether stress plays a role in cellular uptake and distribution. Additionally, since the currently used doses provide a vast excess of anti-miR copies relative to the miRNA present within the cell, the duration of effects suggests that there is a cellular reservoir that, over time, enables anti-miR to inhibit newly formed miRNAs. Subcellular sites of anti-miR storage, as well as kinetics of release, may vary depending on chemical modifications of the oligonucleotide. The impact of a miRNA on its target depends on the relative ratio of miRNA to mRNA target. miRNAs display a range of intracellular concentrations, with the most abundant miRNAs being expressed at up to tens of thousands of copies per cell. Strategies to inhibit miRNA function and thereby derepress expression of their targets are based on the presumption that relatively modest increases in targets are sufficient to evoke significant therapeutic benefits, since individual gene target regulation in response to miRNA modulation in general is relatively modest, with the average change being less than twofold following miRNA inhibition. $39,40$ The profound effect of some miRNAs suggest that it is the cumulative impact of small changes in expression of myriad targets versus pronounced changes in single targets that mediate the biological actions of miRNAs.

Although the pharmacological effect of an anti-miR is the summation of the derepression of genes that are related in function or contribute to a joint cellular event, it is thought that miRNA function becomes pronounced under conditions of injury or stress. In line with this statement, genetic analyses of miRNAs in mice have revealed relatively minor functions under conditions of homeostasis in controlled laboratory settings, while pronounced effects can be observed under appropriate stress conditions. $11$ The heightened function of a miRNA during stress can also be explained by changes in abundance of the miRNA, of its miRNA targets, or by differences in miRNA activity under stress, whereby both the severity and the type of cellular stress influence whether an miRNA is regulated by a miRNA (van Rooij, unpublished data).
Common toxicities with anti-miR therapeutics

A common source of toxicities with anti-miR chemistries can arise from the chemistry itself and depends on the chemical modifications that are used. PS oligonucleotides, for example, can inhibit the tenase complex in the intrinsic clotting cascade, 43 activate the alternative pathway of the complement cascade, 44 and activate innate immunity. Additionally, while LNA-containing oligonucleotides have the potential to improve potency for targeting miRNAs, some signs of hepatotoxicity have been observed with LNA-modified antisense oligonucleotides directed against mRNAs measured by serum transaminases, organ weight, and body weight. 45 Further toxicity studies of chemically-modified miRNA inhibitors will be required to establish safety parameters for the different anti-miR chemistries, but will likely be chemistry- and even sequence-dependent.
In contrast to many other therapeutic modalities, anti-miR drugs are designed knowing that they will affect all genes that are under the control of the target miRNA. While miRNAs often target many related genes involved in cellular processes, which are intended to be manipulated by the anti-miR therapeutic, a single miRNA will likely also target unrelated genes and possibly produce unexpected (sometimes undesired) changes in gene expression. The potential pleiotropy of miRNA action contrasts with the mechanistic basis of most classical drugs, which act with maximal specificity against single cellular targets.

Potential sources of toxicity after administration of a miRNA inhibitor can result not only from toxicities induced by the chemistry or unwanted gene changes, but can also arise from effects of the anti-miR on off-target, nondiseased tissues. While some miRNAs have a very cell- or tissue-specific expression pattern, systemic inhibition of more broadly expressed miRNAs may have multiple effects in different tissues, confounding the interpretation of the responses to miRNA-based therapeutics and potentially causing unwanted effects outside the tissue of interest. Caution should be taken when using miRNA therapeutics for more chronic indications and local delivery options should be contemplated.

Potential therapeutic miRNA targets in cardiovascular disease

The rapidly growing knowledge on the functional relevance of miRNAs during heart disease, the shortage of effective therapies, and the ability to potently and specifically regulate miRNAs in vivo has catalyzed efforts to explore pharmacological manipulation of miRNAs for the treatment of heart disease.

By now, many preclinical rodent studies have shown effective cardiac delivery and miRNA inhibition using anti-miR chemistries, and have indicated the potent effects of miRNA inhibition under disease conditions (Table 1). Two examples of miRNA targets that are now seriously being considered as clinical candidates for cardiovascular disease are outlined below.

Control of pathological cardiac remodeling by miR-208a

Myosin heavy chain (MHC) is the major contractile protein of striated muscles and α-MHC is the predominant myosin isoform expressed in the adult rodent heart. An intron of this gene encodes miR-208a which, like the host gene, is expressed specifically in the heart, making miR-208a the only cardiac-specific miRNA that has been annotated so far. Most relevant to cardiac disease is the finding that miR-208a knockout mice display reduced fibrosis and hypertrophy in response to cardiac stress and fail to upregulate β-MHC expression, which is a sensitive marker of pathological cardiac remodeling. Therapeutic inhibition of miR-208a in a hypertension-driven model of heart failure by subcutaneous injection of LNA-modified anti-miR evokes similar benefits in settings of heart disease by potent and functional inhibition of the cardiomyocyte-specific miR-208a. These findings provide proof-of-concept support for the potential therapeutic benefit of anti–miR-208a inhibitors in the setting of heart disease. Whether oligonucleotide inhibition of miR-208a confers cardiac benefits in various forms of heart disease beyond diastolic dysfunction remains to be determined.

Gene profiling studies identified a cohort of predicted mRNA targets that are elevated in expression in response to anti-miR-208a inhibition in vivo, many of which have unknown functions in the heart. While such genes serve as sensitive biomarkers of anti-miR efficacy, their unknown functions underscore the challenges associated with establishing the mechanistic basis of therapeutic efficacy of this anti-miR.

Control of cardiomyocyte apoptosis and regeneration by the miR-15 family

The miR-15 family, which includes miR-15a, 15b, 16-1, 16-2, 195, and 497, is broadly expressed, and members have been implicated in cell-cycle arrest and cell survival in a variety of cell types, by regulating many antiapoptotic and cell-cycle genes. During heart disease, this family is upregulated in response to cardiac stress and myocardial infarction (MI), which cause death of cardiomyocytes and loss of pump function. A miRNA family is characterized by the fact that the members have a comparable seed sequence, the main region responsible for target recognition, but differ in their 3’ end. This implies that miRNA family members, in theory, should be able to target comparable miRNAs. However, the divergence in sequence between different members of miRNA families prevents their collective inhibition by delivery of single antisense oligonucleotide inhibitors. One approach to potentially overcome such miRNA redundancy is through the use of tiny LNA inhibitors (Figure 2), which target the conserved seed regions of miRNAs and thereby enable inhibition of coexpressed miRNA family members that may have redundant biological functions. A recent report showed that an 8-mer directed against the seed region of the miR-15 family was able to target multiple members of the miR-15 family, which in a model of ischemia reperfusion showed that miR-15 family inhibition reduced infarct size and improved cardiac function 2 weeks after ischemic damage.

Whether inhibition of the miR-15 family exerts a cardioprotective effect through enhanced cardiomyocyte survival, or through improvement of the regenerative capacity of the heart, remains to be determined. However, due to the broad expression pattern of the miRNA family and its involvement in cell proliferation and survival, for more chronic treatment regimens, localized delivery strategies, like cardiac catheters or injectable hydrogel techniques, should be contemplated to prevent unwanted side effects. Recent data in a porcine model of ischemic injury additionally showed a potential advantage to deliver anti-miR to the heart using a catheter. miR-92a, a member of the miR-17-92 cluster, has been implicated in neoan-
MicroRNA therapeutics – van Rooij

Dr Eva van Rooij is a group leader at the Hubrecht Institute in the Netherlands. She attended University Hospital Maastricht in the Netherlands where she received a PhD at the Department of Cardiology. She then went on to complete postdoctoral training in Molecular Biology at the University of Texas Southwestern Medical Center in the lab of Dr Eric Olson, where she served as lead scientist in the studies that linked microRNAs to cardiovascular disease. Her work subsequently became the foundation of miRagen Therapeutics Inc, a company focused on the development of microRNA therapeutics, where she served as Senior Director of Biology in overseeing all the preclinical studies for the company’s microRNA programs. She now heads an academic lab to further unveil the molecular signaling pathways that are relevant for cardiovascular biology.

Looking to the future

miRNA research has unveiled an unconventional disease mechanism that provides a unique opportunity to exploit an entirely new area of biology. Obviously, there are several significant advantages to miRNAs becoming a new class of drug target. Their small size, and known and conserved sequence, makes them attractive candidates from a development standpoint. Additionally, the direct downstream targets of a single miRNA are commonly related genes that function in a comparable cellular process or signaling cascade. This implies that targeting of a single miRNA will likely result in a dramatic effect, due to the combinatorial effect of gene expression changes in all these related downstream targets. Based on the obvious relevance and importance of miRNAs during disease, there is great enthusiasm for the continued exploration of miRNAs as new drug targets.

With the advent of miRNAs as viable therapeutic targets for many serious health conditions, many new companies, like miRagen Therapeutics, Regulus Therapeutics, and Mirna Therapeutics, have been established to translate the exciting scientific discoveries into real-world, commercial uses. At the same time, some older companies, which had an initial focus on the development of small interfering RNA (siRNA)-based compounds, have begun to explore their proprietary chemistries and delivery systems for the design of miRNA inhibitors and mimics. So far, Santaris Pharma, the Denmark-originated company that developed the LNA chemistry, has been the only company that has used an miRNA inhibitor in phase 2 clinical trials for the treatment of hepatitis C. Although so far no miRNA-based therapeutics for cardiovascular disorders have reached human trials, the promising results in numerous animal models of diseases, such as heart failure, cardiac hypertrophy, fibrosis, and hyperlipidemias, suggest that human data will soon be forthcoming.

While we are taking important steps forward in developing anti-miR chemistries as a novel therapeutic, numerous challenges and questions remain in the path toward development of miRNA-based therapeutics in general. Currently, there are a lot of unknowns still surrounding the mode of action of the different anti-miR chemistries. Current data suggest that specific chemistries might affect cellular uptake and differ in the degree of blocking of the function of a miRNA. Their long-term effect (up to weeks or even months) indicates a high degree of stability, but also suggests the cell has a reservoir of anti-miR, that with time is slowly released into the cytoplasm to bind to or scavenge the newly formed copies of a miRNA. However, the precise mechanism is still unclear and more in-depth biochemical analysis will be required to gain more insight. Additionally, in most animal studies to date, the doses used are unlikely to be therapeutically feasible. Follow-up preclinical studies will have to guide appropriate dosing regimens to establish the lowest possible efficacious doses, while attempting to prevent unacceptable side effects.

Despite the unknowns, the interest surrounding miRNAs as novel therapeutic entities is tremendous. The anticipated success of these early forerunners will likely trigger the search for additional miRNA therapeutic targets, innovation in the areas of miRNA inhibitors and mimics, and will advance the search for techniques for efficient in vivo delivery of these therapeutics. While we are eagerly awaiting more efficacy data in human subjects, the next few years promise to provide many more insights into miRNA and anti-miR biology, and will hopefully further strengthen the enthusiasm for this new class of drugs.
References


Keywords: anti-miR; cardiovascular disease; microRNA; miR mimic; oligonucleotide; therapeutics
**Développer les traitements microARN dans les maladies cardio-vasculaires**

Les mécanismes moléculaires et cellulaires de la fonction et de la contractilité cardiaques sont bien connus mais la maladie cardio-vasculaire demeure la première cause de morbi-mortalité dans le monde. En dépit d’un bénéfice thérapeutique avéré pour les statines, les IEC (inhibiteurs de l’enzyme de conversion) et les bétabloquants, la prévalence de la maladie cardio-vasculaire continue à augmenter, ce qui souligne la nécessité de nouvelles stratégies thérapeutiques. Ces dernières années, le rôle important des microARN (miARN) a été mis en évidence dans différentes maladies cardio-vasculaires. Les miARN sont des ARN courts, non codants, à simple brin, qui s’apparentent aux séquences complémentaires de l’ARNm (ARN messager), provoquant l’extinction de l’expression de la protéine. La connaissance de leurs fonctions amplifiées et de leurs séquences dans des états de stress physiopathologique en font des candidats séduisants pour une manipulation thérapeutique. Les leçons tirées de la technologie antisens ont accéléré l’exploitation des possibilités de régulation des miARN à des fins thérapeutiques par le biais de la chimie des oligonucléotides, ces derniers s’étant montrés efficaces en ciblant les miARN pathologiques chez l’animal et même chez l’homme. Le but de cet article est d’analyser les approches actuelles pour cibler les miARN, de récapituler les cibles thérapeutiques possibles pour les maladies cardio-vasculaires et d’envisager les défis posés par cette nouvelle modalité thérapeutique.
It is evident that the ryanodine receptor (RyR) channel plays an important role in cardiac physiology and pathophysiology. Limiting the diastolic leak using either genetic manipulation of RyR (for instance, alanine substitution of protein kinase A [PKA] phosphorylation site) or Rycals yields clinical benefit in terms of cardiac function post-myocardial infarction and incidence of arrhythmias (ventricular tachycardia and atrial fibrillation) in mice.

The role of leaky ryanodine receptors in heart disease: a novel therapeutic target

by A. R. Marks, USA

Contraction of the heart and limbs requires activation of muscle by the release of intracellular calcium (Ca\textsuperscript{2+}) from the sarcoplasmic reticulum. Impaired release of Ca\textsuperscript{2+} can reduce muscle contraction and increased Ca\textsuperscript{2+} release can be toxic. If intracellular Ca\textsuperscript{2+} is released at a time when the muscle is supposed to be relaxed, disastrous consequences can occur, such as impaired relaxation, extrusion of Ca\textsuperscript{2+} from the cell leading to decreased Ca\textsuperscript{2+} transients, and weakened contraction, arrhythmias, muscle damage, or even death. Intracellular Ca\textsuperscript{2+} release is regulated by specialized channels known as ryanodine receptors (RyRs). RyR channels undergo stress-induced post-translational modifications (chiefly phosphorylation, nitrosylation, and oxidation) that impair binding of the stabilizing subunit calstabin (CALCium STABilizing protein; also known as FKBP) to the channels. Calstabin stabilizes the RyR channel closed state and prevents a pathological leak of intracellular Ca\textsuperscript{2+}. Leaky cardiac RyR2s exacerbate heart failure progression and arrhythmias, leaky skeletal muscle RyR1s impair muscle function in muscular dystrophy, and leaky neuronal RyR2s play a key role in post-traumatic stress disorder. In animal models, a novel class of small molecules, known as Rycals, inhibit stress-induced dissociation of calstabin from RyR channels, reduce intracellular Ca\textsuperscript{2+} leak, and are potential novel therapeutics.

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Excitation-contraction coupling

With each beat of the heart, calcium (Ca\textsuperscript{2+}) is released from the sarcoplasmic reticulum (SR) via the cardiac type 2 ryanodine receptor (RyR2), raising the cytosolic Ca\textsuperscript{2+} concentration about tenfold (∼1 μM) and activating cardiac muscle contraction (Figure 1). The Ca\textsuperscript{2+} is then pumped back into the SR by the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPase 2a (SERCA2a), lowering the cytosolic Ca\textsuperscript{2+} concentration to baseline levels (∼100 nM), and causing relaxation. The Ca\textsuperscript{2+} release and reuptake cycle is initiated by the action potential, an electrical signal that depolarizes the plasma membrane and the specialized invagination of the plasma membrane called the transverse tubule (T tubule). Voltage-gated Ca\textsuperscript{2+} channels on the T tubule are activated by depolarization and allow a small amount of Ca\textsuperscript{2+} to run down its concentration gradient from μM external ([Ca\textsuperscript{2+}]\textsubscript{ext}) to nM cytosolic ([Ca\textsuperscript{2+}]\textsubscript{cyt}). Ca\textsuperscript{2+} entering via the plasma membrane voltage-gated Ca\textsuperscript{2+} channels binds to and activates RyR2 channels, which then release Ca\textsuperscript{2+} stored at high concentration (∼μM) in the SR. The elevation of [Ca\textsuperscript{2+}]\textsubscript{cyt} results in the binding of Troponin C, allowing actin-myosin cross-bridging such that the thick and
Ryanodine receptors are highly specialized calcium release channels

RyR channels are the largest known ion channels possessing enormous cytoplasmic domains that contain binding sites for regulatory subunits that integrate extracellular signals to modulate the release of intracellular Ca\(^{2+}\). Along with the closely related inositol 1,4,5-trisphosphate receptors, they are the only channels that release Ca\(^{2+}\) from intracellular stores. RyR1 is required for skeletal muscle contraction and RyR2 for cardiac muscle contraction. The cytoplasmic domains of the RyR channels are enormous macromolecular complexes (>3 million daltons) that include kinases, phosphatases, phosphodiesterases, and their targeting proteins, as well as regulatory subunits such as calstabin (CALcium release channel STABilizing proteinN, also known as FK506 binding protein [FKBP]) (Figure 2, page 344).\(^2\)

RyR2 are homotetrameric intracellular Ca\(^{2+}\) release channels on the SR with at least two functional domains: the carboxy-terminus containing the transmembrane segments forming the Ca\(^{2+}\) conducting pore and the large amino-terminus (termed the "foot" structure), which contains modulatory binding sites. Several modulatory elements are bound to it, including FKBP (aka calstabin), a member of the immunophilin family of cis-trans peptidyl-prolyl isomerasers (FKBP12 in skeletal muscle\(^3\) and FKBP12.6 in cardiac muscle)\(^4,5\); protein kinase A (PKA) and its anchoring protein, muscle A kinase anchoring protein (mAKAP); protein phosphatase 1 (PP1); and protein phosphatase 2A (PP2A)\(^6\). FKBP12 (calstabin1) and FKBP12.6 (calstabin2), endogenous modulators of RyR1 and RyR2 respectively, have been shown to stabilize the channel complex, resulting in channels that demonstrate full conductance.\(^4,7\) The recruitment of the kinase(s) and phosphatases into the complex is mediated through specific leucine/isoleucine zippers present on both the targeting/anchoring proteins and RyRs.\(^8\)

Role of calstabin in modulating RyR2 function

We originally reported that calstabin1 is a subunit of RyR1\(^3\) that is required to stabilize the closed state of the channel and prevent a pathological intracellular Ca\(^{2+}\) leak through the

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We subsequently discovered that diastolic SR Ca\(^{2+}\) leak plays a prominent role in heart failure (HF) progression. At the time, this was highly controversial because the dogma was that there was SR Ca\(^{2+}\) overload in HF and we reported the opposite—that leaky RyR2 channels leading to SR Ca\(^{2+}\) depletion accounted for the diminished Ca\(^{2+}\) transient observed in HF and the weakened heart muscle contraction. Subsequently, others have confirmed our work showing that the diastolic SR Ca\(^{2+}\) leak through the RyR channels is indeed pathological and contributes to heart disease, and that SR Ca\(^{2+}\) is depleted in failing hearts. Moreover, calstabin2\(^{4,10}\) plays an important physiological role in stabilizing the closed state of RyR2 and is required to prevent the diastolic SR Ca\(^{2+}\) leak.\(^{6}\)

PKA hyperphosphorylation of RyR2 causes depletion of calstabin2 from the RyR2 complex.\(^{5}\) We found that oxidation and nitrosylation of RyR2 also cause depletion of calstabin2 from the RyR2 macromolecular complex.\(^{11,12}\) Moreover, the combination of oxidation, nitrosylation, and Ser2808 phosphorylation depleted nearly all of the calstabin2 from the channel complex.\(^{11,12}\) These observations have been supported by data from RyR2-S2808A mice, which harbor RyR channels that cannot be PKA phosphorylated and are therefore protected against PKA phosphorylation–induced depletion of calstabin2, and from phosphomimetic RyR2-S2808D mice that exhibit decreased levels of calstabin2 in the RyR2 complex.\(^{11-14}\)

**Regulation of ryanodine receptors: role of phosphorylation**

Activation of β-adrenergic receptors (β-ARs) leads to elevated cyclic adenosine monophosphate (cAMP) and activation of PKA (Figure 1).\(^{1}\) β-AR activation has multiple targets, including voltage-dependent L-type Ca\(^{2+}\) channels (Cav1.2), RyR2, and phospholamban, the regulator of SERCA (Figure 1).\(^{1}\)

PKA and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) regulation of RyR2 channel function plays an important role in modulating cardiac contractility and arrhythmogenesis. We have shown that there is a single functional PKA phosphorylation site, a separate single functional CaMKII phosphorylation site on RyR2, and a single functional PKA phosphorylation site on RyR1\(^{15}\) yielding four sites for each type of phosphorylation on each homotetrameric channel. Confusion has been created in the field because Ser2809 on canine and human RyR2 (Ser2815 in murine RyR2) was originally identified as both a PKA and CaMKII phosphorylation site.\(^{16,17}\) This was based on phosphopeptide mapping,\(^{16}\) which identified a phosphorylated peptide that contained two sites, one for PKA and one for CaMKII, but this was not appreciated until years later when site-directed mutagenesis studies and knock-in mice showed that Ser2808 is exclusively phosphorylated by PKA,\(^{18}\) and Ser2815 (Ser2814 in murine RyR2) is phosphorylated by CaMKII.\(^{15,19,20}\) Using site-directed mutagenesis to ablare...
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Cardiovascular disease

Ryanodine channels and heart failure

the phosphorylation sites by substituting an alanine residue for the target serine residues, we showed that the channels can no longer be phosphorylated (RyR1-S2844A ablates PKA phosphorylation of RyR1, RyR2-S2808A ablates PKA phosphorylation of RyR2, and RyR2-S2814A ablates CaMKII phosphorylation of RyR2).\(^5,15\) Knock-in mice for each of these mutations confirmed that the respective channels have only a single PKA and CaMKII phosphorylation site.\(^6,15\) Mice engineered with a RyR2-S2808A mutation have blunted inotropic and chronotropic responses to catecholamines.\(^11,12,15\) Mice engineered with a RyR2-S2814A mutation have RyR2 channels that cannot be phosphorylated by CaMKII, and exhibit a blunted positive force frequency relationship.\(^15\) Mice engineered with a phosphomimetic mutation (substituting an aspartic acid residue for serine) of the RyR2 PKA phosphorylation site showed an age-dependent cardiomyopathy and arrhythmias.\(^12\) Based on these extensive studies there is no longer any rational basis for referring to the sites for PKA and CaMKII phosphorylation of RyR2 as “controversial.”

The stress of heart failure

A key characteristic of HF is the chronic activation of the sympathetic nervous system (SNS) resulting from a maladaptive physiological response to cardiac dysfunction designed to maintain or improve cardiac function. Acute activation of the SNS maintains cardiac function, but at a high cost. \(\beta\)-Adrenergic agonists or phosphodiesterase inhibitors, which are used to treat acute decompensated HF, increase contractility by increasing cAMP levels and activating PKA, which in turn activates key regulators of excitation-contraction coupling including RyR2, thereby increasing Ca\(^{2+}\) release. However, these treatments also increase mortality, in part because they are proarrhythmic and increase energy consumption. On the other hand, blocking neuroendocrine pathways is the current mainstay of HF therapy. The agents used to block neuroendocrine pathways (angiotensin-converting enzyme [ACE] inhibitors and \(\beta\)-adrenergic receptor blockers [\(\beta\)-blockers]) improve cardiac function and survival, but they do so at a high cost. First, their use is limited due to side effects (side effects of \(\beta\)-blockers include depression, lethargy, impotence, blunting of the physiological response to hypoglycemia [a major problem in diabetic patients taking insulin], and impaired exercise capacity). Moreover, initiation of \(\beta\)-blocker and ACE inhibitor therapy, particularly in the outpatient setting, poses challenges for both the physician and patient. It is necessary to carefully titrate \(\beta\)-blockers and ACE inhibitors, both of which have hemodynamic effects that are particularly problematic in HF patients, gradually increasing the dose while monitoring the heart rate and blood pressure. These side effects and physiological response to \(\beta\)-blockers and ACE inhibitors likely explain why only a relatively small percentage of HF patients receive the recommended doses of \(\beta\)-blockers and ACE inhibitors, and why most HF patients are not physiologically \(\beta\)-blocked. Thus, it is clear that the neuroendocrine response (eg, activation of SNS and renin-angiotensin signal

Another factor confounding the use of \(\beta\)-blockers in HF is that their mechanism of action in HF is uncertain. Is it merely slowing the heart rate that explains the benefit of \(\beta\)-blockers in HF? Slowing the heart rate is likely a component of the beneficial effect of \(\beta\)-blockers in HF, as ivabradine, a blocker of the pacemaker current, decreased hospital visits and adverse cardiac events in the SHIFT (Systolic Heart failure treatment with the IF inhibitor ivabradine Trial) study.\(^2\) We have shown that \(\beta\)-blockers indirectly “fix” the leak in RyR2; this is achieved in both animal models of HF\(^20\) and in patients with HF\(^20\) by blocking the \(\beta\)-AR, resulting in reduced PKA phosphorylation and oxidation of RyR2, and preventing depletion of calstabin2 from the RyR2 macromolecular complex. Thus, it appears that Ryca and \(\beta\)-blockers share a common target—the cardiac RyR2 channel leak. In support of this unexpected finding is the fact that mice expressing the phosphomimetic RyR2-S2808D channel with post-myocardial infarction (MI) HF are resistant to \(\beta\)-blockers, while they respond to Ryca the same as wild-type mice.\(^12\)

Cardiac contractility is determined by the amplitude and kinetics of Ca\(^{2+}\) cycling. We reported that diastolic SR Ca\(^{2+}\) leak via RyR2 channels contributes to HF progression\(^6\) and fatal ventricular arrhythmias.\(^24-27\) Prior to the diastolic SR Ca\(^{2+}\) leak model of HF, the dogma was that HF was associated with SR Ca\(^{2+}\) overload. Subsequent to our discovery of the diastolic SR Ca\(^{2+}\) leak in HF, the presence of both the SR Ca\(^{2+}\) depletion and the diastolic SR Ca\(^{2+}\) leak were confirmed in HF by most of the leading experts in the field.\(^9,13,26-30\)

The defective SR Ca\(^{2+}\) handling is characterized by leaky RyR2 channels due to stress-induced dissociation of the stabilizing RyR2 subunit calstabin2 (principally manifested as PKA phosphorylation, oxidation, and nitrosylation of the channel), resulting in a diastolic SR Ca\(^{2+}\) leak, reduced SR Ca\(^{2+}\) content, and decreased Ca\(^{2+}\) transient.\(^6,12,24,31-33\) Compounding this problem is impaired SR Ca\(^{2+}\) uptake due to reduced activity of SERCA2a as a consequence of reduced SERCA2a expression, increased inhibition of the pump by phospholamban,\(^34\) and enhanced Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) activity (Figure 1).\(^1,25\) Thus, these dysfunctional processes conspire to deplete the SR of Ca\(^{2+}\) and lead to impaired cardiac contractility.\(^25\) Not surprisingly, therefore, both the RyR2 leak and the impaired uptake have been targeted with novel therapeutics, which are now undergoing clinical testing in HF patients.

Multiple animal models have been used to support a role for PKA hyperphosphorylation of RyR2 in HF progression.\(^37\) Genetically altered mice harboring RyR2 that cannot be PKA phosphorylated (RyR2-S2808A), were protected against calstabin2 depletion from the RyR2 complex and HF progres-
shown to promote HF progression, the calstabin2-deficient mice have otherwise normal cardiac function and are able to compensate for the loss of calstabin in the absence of a compromised ventricle (eg, no MI). Calstabin2-deficient mice exhibit delayed afterdepolarizations and exercise-induced ventricular tachycardia, and RyR2 from calstabin2-deficient mice exhibit slightly increased open probability at baseline that increases substantially when the mice are exercised.

Arrhythmias due to abnormal RyR2 function

Increased RyR2 activity has been shown to cause arrhythmias, particularly associated with increased catecholaminergic stimulation. This is best exemplified by catecholaminergic polymorphic ventricular tachycardia (CPVT), a rare inherited form of exercise-induced sudden cardiac death that occurs in individuals with structurally normal hearts and normal electrocardiograms. Mutations in RyR2 have been linked to CPVT. RyR2 with CPVT mutations have reduced affinity for calstabin2, and have increased open probability at diastolic [Ca²⁺]. Calstabin2-deficient mice have an increased incidence of atrial fibrillation. Atrial fibrillation could be induced by intrasophageal burst pacing protocol in 3 CPVT mouse models, RyR2-R2474S(+/–), RyR2-N2386I (+/–), RyR2-L433P(+/–), but not in wild type mice. Consistent with these in vivo results, there was a significant diastolic SR Ca²⁺ leak in atrial myocytes isolated from these CPVT mouse models.

Fixing leaky RyR2 channels: a novel therapeutic approach for heart and muscle diseases

The identification of the diastolic SR Ca²⁺ leak via RyR2 as a mechanism underlying HF progression and cardiac arrhythmias has led to novel therapeutic approaches. Matsuzaki and colleagues reported that JTV-519 (K201), a 1,4-benzothiazepine, improved cardiac function in a canine model of pacing-induced HF. Testing the drug in calstabin2-deficient mice showed that the ability of JTV-519 to prevent HF progression and fatal cardiac arrhythmias requires stabilization of the closed state of RyR2 by calstabin2. Moreover, JTV-519 had no effect on the gating properties of normal RyR channels and no effect in healthy dogs and mice. We generated many derivatives of the 1,4-benzothiazepine JTV-519 and have developed a novel class of Ca²⁺ release channel stabilizers known as Rycals. An orally available Rycal, S107, improves skeletal muscle force generation and exercise capacity, reduces arrhythmias and improves muscle function in mice with Duchenne muscular dystrophy by reducing pathologic SR Ca²⁺ leak in cardiac and skeletal muscle. Rycals are protective against post-MI HF progression, and suppressed ventricular tachycardia/ventricular fibrillation and sudden cardiac death in murine models of human CPVT. S107 also raises the...
seizure threshold in mice with leaky neuronal RyR2 channels and improves exercise capacity in mouse models of sarcopenia (age-related loss of muscle function). Leaky RyR2 channels in hippocampal neurons play a key role in stress-induced cognitive dysfunction. Treatment with S107 prevented stress-induced cognitive dysfunction in a murine model, suggesting a novel mechanism and therapeutic approach to post–traumatic stress disorder.11

Conclusions
It is evident that the RyR channel plays an important role in cardiac physiology and pathophysiology. Modulating its activity is important for flight-or-fight responses. Long-term activation of the channel, however, is detrimental, causing progression of HF and arrhythmogenesis. Limiting the diastolic leak using either genetic manipulation of RyR (for instance, alanine substitution of PKA phosphorylation site) or RyCals yields clinical benefit in terms of cardiac function post-MI and incidence of arrhythmias (ventricular tachycardia and atrial fibrillation) in mice. ■

Conflict of interest: Dr Marks is a consultant for and owns shares in ARMGO Pharma Inc, a startup company developing RyR targeted therapeutics. Acknowledgments: Much of the work referred to in this review has been supported by the National Heart Lung and Blood Institute (NHLBI), the Fondation Leducq, the Doris Duke Charitable Foundation, and the Ellison Foundation.

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Dysfonction des récepteurs de la ryanodine et maladie cardiaque : une nouvelle cible thérapeutique

La contraction musculaire, qu'il s'agisse du cœur ou des membres, requiert une activation des myocytes par libération de calcium intracellulaire (Ca²⁺) à partir du réticulum sarcoplasmique. La libération défectueuse de Ca²⁺ peut réduire la contraction musculaire, tandis que la libération augmentée peut être toxique. Une libération impromptue de Ca²⁺ intracellulaire au moment de la relaxation musculaire peut avoir des conséquences désastreuses : altération de la relaxation, extrusion de Ca²⁺ en dehors de la cellule diminuant le flux transitoire de Ca²⁺ cytosolique, contraction affaiblie, arythmies, lésions musculaires ou même décès. La libération de Ca²⁺ intracellulaire est régulée par des canaux spécialisés appelés récepteurs de la ryanodine (RYR). Ces canaux subissent des modifications post-translationalles induites par le stress (principalement phosphorylation, nitrosylation et oxydation) qui altèrent la fixation de la calstabin, une sous-unité stabilisante (CAlcium release channel STABilizing proteinN ou FKBP). La calstabin stabilise l’état fermé du canal RyR et s’oppose à une fuite pathologique de Ca²⁺ intracellulaire. Les RyR2 dysfonctionnels cardiaques aggravent l’insuffisance cardiaque et les arythmies, les RyR1 dysfonctionnels du muscle squelettique dégradent la contraction musculaire dans la dystrophie musculaire et les RyR2 dysfonctionnels neuronaux jouent un rôle clé dans l’état de stress post-traumatique. Des modèles animaux suggèrent qu’une nouvelle classe de petites molécules appelées Rycal, capables d’inhiber la dissociation de la calstabin des canaux RyR induite par le stress et de diminuer la fuite de Ca²⁺ intracellulaire, est porteuse de promesses sur le plan thérapeutique.
The calcium ion (Ca$^{2+}$) is a quintessential player in all functions of the cardiomyocyte, from excitation-contraction (EC) coupling to growth and survival. Various impairments of intracellular Ca$^{2+}$ metabolism are responsible for mechanical dysfunction and arrhythmias in heart failure. One such impairment is diastolic Ca$^{2+}$ leak through ryanodine receptor 2 (RyR2), which has emerged as an independent therapeutic target in heart failure.

Heart failure (HF) is the leading cause of mortality and morbidity, with much of the mortality attributed to sudden cardiac death, frequently due to ventricular arrhythmias. The young are not exempt, as seen with catecholaminergic polymorphic ventricular tachycardia type 1 (CPVT1), typically associated with sudden death during childhood or adolescence. Success with current therapeutic management, though improved, remains underwhelming. Calcium is a pivotal player in cardiomyocyte function, and aberrant intracellular calcium handling leads to deterioration of myocardial contractility and arrhythmias in HF. A pathologic leak of calcium from the sarcoplasmic reticulum through ryanodine receptor channels during diastole has been targeted for therapeutic development. Stemming from the discovery of Rycals®, small molecules that prevent this calcium leak from ryanodine receptor 2 channels, a collaborative partnership established in 2006 between ARMGO Pharma Inc and Servier has aimed to develop a Rycal for the treatment of HF. Completed phase 1 and 2a studies with the Rycal candidate S 44121/ARM036 provide first evidence for the therapeutic potential of such an approach to target leaky RyR2 channels in such patients. The ongoing goal of the collaboration is to select follow-on compounds that improve upon the profile of first-generation Rycal candidates for further development.

Servier's partnership with ARMGO Pharma: targeting heart failure by inhibiting cardiac RyR2 calcium leak

by N. Villeneuve, E. Canet, and J. P. Vilaine, France

Heart failure and CPVT as causes of mortality and morbidity

Heart failure (HF) is the leading cause of mortality and morbidity in the developed world. Despite improvement in therapeutic management of patients with HF, prognosis remains poor, with 30% mortality 1 year after diagnosis; at least half of this mortality is due to ventricular arrhythmias (sudden cardiac death [SCD]). Chronic symptomatic HF is currently treated with several classes of drugs, some of which have been shown to improve survival in randomized clinical trials. Main treatments include angiotensin-converting enzyme (ACE) inhibitors, β-adrenergic antagonists, diuretics, spironolactone, and angiotensin II receptor blockers (ARBs). Digitalis is still used in a small percentage of patients. Though compliance with these therapies regimens at optimal doses is observed within clinical trials, it is estimated that the majority of HF patients are undertreated, largely due to a combination of side effects and lack of efficacy of current therapies. Ventricular arrhythmias leading to SCD are frequent in patients with cardiac diseases. However, SCD also occurs in young subjects with structurally normal hearts and normal electrocardiograms (ECGs). Familial type 1 catecholaminergic polymorphic ventricular tachy-
cardia (CPVT1) is typically associated with sudden death in childhood or adolescence associated with exercise or emotional stress (30% to 50% experience sudden death by the age of 30 when untreated), and is the most lethal channel-associated cause of SCD.

The current pharmacologic treatment for patients identified with CPVT1 is β-adrenergic antagonists (β-blockers), which provide incomplete protection. Patients who experience arrhythmia even under pharmacologic treatment can be offered implantable cardioverter-defibrillators (ICDs). In fact, despite pharmacologic therapies, 50% of patients require shocks from their devices to stop ventricular tachyarrhythmias.1

**Involvement of calcium leak in HF and CPVT**

The calcium ion (Ca^{2+}) is a quintessential player in all functions of the cardiomyocyte, from excitation-contraction (EC) coupling to growth and survival. Various impairments of intracellular Ca^{2+} metabolism are responsible for mechanical dysfunction and arrhythmias in HF.2

One such impairment is diastolic Ca^{2+} leak through ryanodine receptor 2 (RyR2), which has emerged as an independent pharmacologic target in HF3,4 and a potential target of β-blockers.5 Indeed, it was shown in animal models of HF, as well as in HF patients, that chronic sympathetic hyperactivity in HF causes remodeling of the RyR2 complex, leading to a pathologic diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) through the channel and to depletion of the SR Ca^{2+} stores.5,6 This leads to depressed intracellular Ca^{2+} cycling, decreased SR Ca^{2+} release in response to an action potential, and less force produced from EC coupling (Figure 1).7 The resultant deterioration of cardiac function has been extensively studied.8 Genetic investigations have shown that CPVT disease is most-

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**Figure 1. Abnormal intracellular Ca^{2+} handling in failing cardiomyocytes results in reduced contractile force and prolonged relaxation.**

(A) Reduced SR Ca^{2+} storage and release cause abnormal systolic cardiomyocyte function. Abnormal β-AR and GPCR signaling increase the expression of PKA and CaMKII, which hyperphosphorylate and alter the function of RyR2 and LTCC. FKBP12.6 also contributes to stabilization of the RyR2 open probability. Impaired SERCA2a function decreases SR Ca^{2+} loading, resulting in reduced SR Ca^{2+} content and release and, therefore, reduced contractility. Alterations in LTCC function and STIM1-Oral1-mediated store-operated Ca^{2+} influx can also result in abnormal Ca^{2+} handling during systole.

(B) Reduced SR Ca^{2+} resequestration is a key abnormality in diastole. Decreased SERCA2a activity, downregulation of SERCA2a, a decreased PLB/SERCa2a ratio, or PLB hypophosphorylation cause prolonged intracellular Ca^{2+} transients, reduced SR Ca^{2+} loading, and slowed cardiomyocyte relaxation. An increase in levels of NCX might be a compensatory response to prevent Ca^{2+} overloading. While increased NCX activity would be initially adaptive, excessive or sustained NCX activation could contribute to decreased sarcoplasmic reticulum Ca^{2+} content by removing cytosolic Ca^{2+}, and reducing systolic Ca^{2+} transients and contractile function. SR Ca^{2+} leak resulting from impaired RyR2 function and increased expression of PP1 can reduce SR Ca^{2+} content and increase cytosolic Ca^{2+} concentration during diastole. On activation, the β-AR-AC-GPCR complex synthesizes cAMP, which activates PKA.

Hyperactive PKA decreases the Ca^{2+} sensitivity of the myofilaments and prolongs relaxation. Intracellular Ca^{2+}-overload stimulates CaMKII, which contributes to SR diastolic Ca^{2+} leak by hyperphosphorylating RyR2 and induces the transduction of pathological Ca^{2+} signaling.

**Abbreviations:** AC, adenylate cyclase; ATP, adenosine triphosphate; β-AR, β-adrenergic receptor; CaMKII, calcium-and calmodulin-dependent protein kinase type II; Ca^{2+}, calcium ion; cAMP, cyclic adenosine monophosphate; FKBP12.6, FK506-binding protein; GPCR, G-protein–coupled receptor; LTCC, voltage-dependent L-type Ca^{2+}-channel; NCX, Na^{+}/Ca^{2+} exchanger; Oral1, calcium-release–activated calcium channel protein 1; P, phosphorylation; PKA, protein kinase A; PLB, cardiac phospholamban; PP1, protein phosphatase 1; RyR2, ryanodine receptor 2; SERCA2a, sarcoplasmic/endoplasmic reticulum calcium ATPase 2a; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1; TrnC, tropinin C.

ly secondary to mutations in RyR2 channels. Some of these mutations manifest as triggers of fatal ventricular arrhythmias during exercise due to the increased SR Ca\(^{2+}\) uptake, which increases the Ca\(^{2+}\) gradient and increases the diastolic SR Ca\(^{2+}\) leak. This activates inward depolarizing currents via the Na\(^+\)/Ca\(^{2+}\) exchanger that cause delayed afterdepolarizations (DADs) and trigger fatal cardiac arrhythmias.\(^{11,12}\) Figure 2 depicts a schematic of the predicted structure of the RyR2 receptor.\(^{13}\)

Small molecules to improve cardiac diseases by preventing Ca\(^{2+}\) leak

K201, also known as JTV-519, was shown to inhibit muscle Ca\(^{2+}\) overload and to reduce Ca\(^{2+}\) leak through the RyR2 channel along with its consequences in HF models.\(^{7,14}\) However, as this compound can inhibit several ion channels and transporters,\(^{15}\) it is unclear whether these beneficial effects are RyR2 dependent or involve other types of action.

In 2004, derivatives of this compound (among them, S107 and S 44121/ARM036) were identified by Dr A. Marks and his colleagues. These small molecules, known as Rycals\(^{8}\), prevent pathologic Ca\(^{2+}\) leak from RyR2 without blocking the channel's pore. They also improved cardiac function in mice with dysfunctional RyR2 (Figure 3, page 352).\(^{16-18}\) ARMGO Pharma Inc, a privately held biopharmaceutical company founded in 2006 to develop a Rycal for treatment of chronic HF, established in 2006 to develop a Rycal for treatment of chronic HF.

**ARMGO-Servier collaborative partnership for the selection of compounds targeting HF and CPVT**

Evidence of mortality reduction with ivabradine in HF patients (as seen in SHIFT [Systolic Heart failure treatment with \(\mathrm{I}\) \textsubscript{3} inhibitor ivabradine Trial])\(^{19}\) rewards Servier’s research efforts in postischemic cardiopathies. Indeed, in recent decades, Servier has been particularly committed to the fight against these pathologies. As a growing body of preclinical evidence has specified a relationship between dysfunctional RyR2 in cardiomyocytes and the development of HF, strategies described by Marks and colleagues were obviously of interest for Servier. Consequently, a collaborative partnership with ARMGO was established in 2006 to develop a Rycal for treatment of chronic HF.

In permeabilized cardiomyocytes isolated from a mouse model in which RyR2 has a mutation at residue 4497 where arginine is replaced by cysteine (R4497C-RyR2), compounds that stabilize the RyR channel decreased the occurrence of Ca\(^{2+}\) sparks, suggesting that, as expected, they target RyR2 to limit Ca\(^{2+}\) leak. Furthermore, at Servier, we demonstrated in a rat model of post–myocardial infarction (MI) chronic HF (1-hour occlusion, 3 months of reperfusion) that RyR-stabilizing compounds induced limitation of cardiac dysfunction, both systolic and diastolic. The effect was not restricted to preservation of myocardial contractility, as cardiac structural changes (reduction in cardiomyocyte volume and in cardiac collagen content) were also observed. In addition, in a mouse model of chronic HF post-MI (permanent left anterior descending coronary artery [LAD] ligation), an antiarrhythmic effect with
a decrease in ventricular extrasystoles was demonstrated. This antiarrhythmic effect was directly related to a decrease in number of Ca²⁺ sparks and triggered activity occurrence measured in isolated permeabilized post-MI cardiomyocytes.

Moreover, in healthy conscious pigs studied in Servier labs, at a plasma concentration above those able to prevent in vitro and in vivo RyR2 dysfunction, no pharmacological effects, eg, on hemodynamic parameters or on global cardiac function, at rest or during catecholaminergic stress have been observed.

In view of these considerations, and the standard preclinical development program demonstrating appropriate safety, one compound, S 44121/ARM036, was proposed for clinical development for the prevention of cardiovascular morbidity and mortality in patients with chronic HF and left ventricular dysfunction and for cardiac arrhythmia in patients with CPVT1. S 44121/ARM036 has now completed phase 1 and phase 2a studies, with results validating the approach of targeting RyR2 in HF patients.

**ARMGO-Servier research collaboration: moving forward**

The observed activity of S 44121/ARM036 in the completed phase 2a trials supports the likelihood that clinically meaningful effects can be achieved by effectively targeting leaky RyR2 channels in patients with chronic HF and cardiac arrhythmias. Meanwhile, the ongoing goal of the ARMGO-Servier chemistry program is to select follow-on compounds for further clinical development that improve upon the profile of the first-generation Rycal candidates. Current screening tools available to guide these efforts are in vitro pharmacological as-

**Figure 3. S107 improves cardiac function in RyR2-S2808 D+/+ mice.**

(A) Echocardiographic measurements during a 10-week treatment period, showing that the S107-treated (20 mg/kg/d via osmotic pump) group exhibited preserved cardiac function compared with the vehicle-treated group (P<0.01 versus vehicle-treated group). (B and C) Cardiac catheterization was performed at the end of study, and both dP/dt max and (dP/dt max)/Pid, where Pid indicates the instantaneous developed pressure in mm Hg and s⁻¹ is the unit of measure for (dP/dt max)/Pid, showed a significant improvement in the S107-treated group (*P<0.05 versus vehicle-treated group).

Abbreviations: dP/dt max, maximum rate of pressure change in the ventricle; S107, a Ca²⁺-channel stabilizer (a Rycal).


**Figure 4. Cameleon is expressed in cells by viral transduction.**

(A) This sensor is targeted to the endoplasmic reticulum. (B) As Ca²⁺ binds to calmodulin (CaM), the Ca²⁺-binding domain undergoes a conformational change, interacting with its binding peptide. This brings YFP closer to CFP, increasing the efficiency of FRET. (C) Representative traces of endoplasmic reticulum Ca²⁺ measurements into HEK293 cells stably expressing cameleon and RyR2. SOICR (store-overload–induced Ca²⁺ release) was induced by adding 2mM Ca²⁺ and results in Ca²⁺ oscillations. Decrease in Ca²⁺ during the first phase of the oscillations can be explained by a massive opening of RyR2 channels. Increase of Ca²⁺ in the second phase is mainly due to SERCA activity. As expected, 1µM ryanodine completely blocks the oscillations.

Abbreviations: Ca²⁺, calcium ion; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; GECl, genetically encoded calcium indicators; HEK293, human embryonic kidney 293 cells; M13, calmodulin peptide-binding protein; RyR2, ryanodine receptor 2; YFP, yellow fluorescent protein.
says, along with absorption, distribution, metabolism, and excretion (ADME) tests. A dynamic high-throughput screening (HTS) assay first developed at Servier uses fluorometric analysis of the Ca\(^{2+}\) signal from stimulated RyR2 activity in human embryonic kidney 293 cells (HEK293) overexpressing RyR2 (HEK-RyR2).

Two robust functional assays using Ca\(^{2+}\) indicator dye–loaded cardiomyocytes have been developed (1 at ARMGO and 1 at Servier) to assess RyR2-associated Ca\(^{2+}\) abnormalities.

Using this strategy, several new-generation Rycals that significantly inhibit Ca\(^{2+}\) release via a leaky RyR2 channel have been identified. Similarly to what has been done previously, subsequent in vivo efficacy studies will need to confirm the efficacy of these new-generation Rycals in vivo models of HF.

In parallel, Servier has developed new assays to confirm the mechanism of action of selected compounds. Firstly, measurement of SR Ca\(^{2+}\) in HEK-RyR2 cells using fluorescence resonance energy transfer (FRET)-based Ca\(^{2+}\) sensors and epifluorescence microscopy allows the direct evaluation of the consequences of the Ca\(^{2+}\) leak (Figure 4). Secondly, confocal microscopy–facilitated measurement of Ca\(^{2+}\) sparks in cardiomyocytes from pathological models provides insight into RyR2 dysfunction. These Ca\(^{2+}\) sparks are intracellular Ca\(^{2+}\) release events arising from the activation of a cluster of RyRs.

At the same time, the exciting and significant progress achieved in induced pluripotent stem cell (iPS) technology gives us an invaluable opportunity to evaluate the effects of new Rycals on iPS-derived cardiomyocytes obtained from skin fibroblasts or keratinocytes from patients with RyR2 mutations.

**Summary**

Fundamental research has highlighted that abnormal diastolic Ca\(^{2+}\) leak from cardiac SR RyRs is one of the pathophysiological mechanisms leading to cardiac contractile dysfunction and arrhythmias. First evidence of therapeutic potential in patients with HF has been obtained with a compound—S 44121/ARM036—targeting this pathological process. Continued collaborative efforts are under way between Servier and ARMGO Pharma to select follow-on compounds for further clinical development that improve upon the profile of first-generation Rycal candidates.

Nicole Villeneuve was trained in Animal Biology (PhD) at the University of Poitiers (France) and specialized in Electrophysiology. She joined Servier Research Institute in 1983 where she participated in the setup of a Cardiovascular Research Division, especially the in vitro group. She now runs a basic science research group within the Cardiovascular Research Discovery unit, directed by Jean-Paul Vilaine, that comprises postdoctoral scientists, technicians, and students. This group is leading research programs focusing on myocardial ischemia, heart failure, and hypertension.

Jean-Paul Vilaine was trained in Medicine (MD) at the Faculté de Médecine Cochin Port-Royal (Paris, France) and specialized in Cardiology at the Assistance Publique, Hôpitaux de Paris (France). He was also trained in Experimental Cardiovascular Pharmacology at the Faculté de Médecine Paris Sud (France) and at the Heymans Institute (Gent, Belgium). Jean-Paul Vilaine joined Servier Research Institute in 1983 where he participated in the setup of a Cardiovascular Research Division. He is Director of Servier Cardiovascular Research Discovery. His research programs are in the fields of myocardial ischemia, heart failure, hypertension, thrombosis, and venous diseases. He initiated and developed a research program that permitted the discovery and selection of ivabradine for clinical development. Ivabradine is an inhibitor of the cardiac pacemaker current and selectively reduces heart rate. This compound (Procoralan\(^{\circledR}\)) is the first with this mechanism of action to have obtained marketing authorization and is presently used for the treatment of stable angina and heart failure.

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L’insuffisance cardiaque (IC) est la première cause de morbi-mortalité dans les pays développés, la mortalité étant principalement attribuée à l’arrêt cardiaque subit, souvent en raison d’arythmies ventriculaires. Les jeunes ne sont pas épargnés comme le montrent les tachycardies ventriculaires catécholaminergiques polymorphiques de type 1 (CPVT1) typiquement associées aux morts subites de l’enfance ou de l’adolescence. Les résultats des traitements actuels, bien qu’améliorés, restent décevants. Le calcium joue un rôle clé dans la fonction du cardiomyocyte, un comportement aberrant du calcium intracellulaire entraînant détérioration de la contractilité myocardique et arythmies dans l’IC. En termes de cible thérapeutique, l’intérêt s’est porté sur la fuite pathologique de calcium à partir du canaux RYR2 cardiaques de type 1 (CPVT1) typiquement associées aux morts subites de l’enfance ou de l’adolescence. Les résultats des traitements actuels, bien qu’améliorés, restent décevants. Le calcium joue un rôle clé dans la fonction du cardiomyocyte, un comportement aberrant du calcium intracellulaire entraînant détérioration de la contractilité myocardique et arythmies dans l’IC. En termes de cible thérapeutique, l’intérêt s’est porté sur la fuite pathologique de calcium à partir du Retículo sarcoplasmático de los canales de ryanodina en fase diastólica. A la suite de la découverte de los Rycal®, pequeñas moléculas oponentes a la fuga calcica al nivel de canales de receptores 2 a la ryanodina, ARMGO-Pharma y Servier han unificado un partenariato en 2006 en el but de desarrollar un candidato Rycal en el tratamiento de la IC. A l’issue de l’études de fase 1 et 2a de l’campaign Rycal S 44121/ARM036, le potentielle thérapeutique des canaux Ryr2 perméables chez les patients souffrant d’IC a été confirmé. Le partenariato actuel vise a sélectionner des successeurs améliorant le profil des candidats Rycal de première génération, en vue de les développer.
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- The winners of the Servier Research Grant in Hypertension were Konstantin Kotliar (Munich, Germany) in 2011 and Stefano Masi (London, United Kingdom) in 2013.
- The Servier Research Grant in Hypertension is limited to PhDs or MDs under 45 years of age on July 1 in the year of the award.

Next deadline for applications: January 31, 2015
Applications should be sent to: Prof Giuseppe Mancia, Clinica Medica, Ospedale San Gerardo, Via Pergolesi 33, 20052 Monza (MI), Italy
E-mail: giuseppe.mancia@unimib.it
More information is available at www.eshonline.org and on the Servier Web site: www.servier.com

- The G. B. Morgagni Prizes were instituted in 1984 by a group of postdoctoral researchers working at the Medical School of the University of Padua (Italy). Named in honor of Giovanni Battista Morgagni, the Italian anatomist called the founder of pathologic anatomy (1682-1771), the prizes were set up to promote research in the field of metabolism. Servier has been the sole sponsor of the Awards Program since 1997.
- The prizes, consisting of one Morgagni Medal awarded to a senior scientist (Gold Medal and €20 000) and two Young Investigator Awards (€8000), are given every 2 years for outstanding scientific achievements. Every April in the year of the prize, an international panel of prominent experts evaluates the candidates’ qualifications, curriculum vitae, and scientific publications. The Awards Ceremony takes place in October and is held at an international symposium.
- Previous winners were Markus Stoffel (Zurich, Switzerland), Timothy Mark Frayling (Exeter, United Kingdom), and Roberto Malone (Paris, France).
- In 2014, the Gold Medal was awarded to Hans-Henrik Parving (Copenhagen, Denmark), while the Young Investigator Awards were presented to Patrick Collombat (Nice, France) and to Anna Glyn (Oxford, United Kingdom) during the Morgagni Prizes Symposium, held in Padua on October 31, 2014, organized by G. Crepaldi.
- The Morgagni Prizes will next be awarded in 2016 during an annual congress on metabolism in Padua.

Next deadline for applications: February 1, 2016
Applications should be sent to Prof Gaetano Crepaldi, G. B. Morgagni Prizes Committee, Centro Studi per l’Invecchiamento-C.N.R., Via Giustiniani 2, 35128 Padova, Italy
E-mail: crepaldi.metabolism@unipd.it
More information is available on the Servier website: www.servier.com

- Servier is a partner of the Union Internationale de Phlébologie (UIP). Every 2 years, the UIP/Servier Research Fellowship provides a €25 000 grant for a 2-year research project consisting of original clinical or basic research in the areas of phlebology and lymphology, including the following topics: anatomy, physiology, pathophysiology, diagnostic methods, and clinical research.
- Review of the proposals submitted and selection of the best candidate are carried out by a committee of internationally recognized specialists in the field of phlebology and lymphology, including the President of the UIP.
- The 2013 Servier/UIP fellowship was awarded to Markus Fokou (Cameroon) at the World Congress of the UIP in 2013 (Boston, Massachusetts, USA).
- The next grant will be awarded at the World Congress of the UIP in 2015 (Seoul, Korea).
- Candidates less than 45 years old and belonging to a national society affiliated with the UIP may apply.

Next deadline for applications: March 31, 2015
More information is available at www.servier.com, together with the electronic application file.

For further information and deadline applications please visit our Web site: www.servier.com
Inflammation plays a major role at all stages of the atherosclerotic process, from the early events, whereby leukocytes are recruited at sites of subendothelial LDL cholesterol accumulation, to the late events, when plaque rupture occurs and leads to thrombus formation. Clinical complications of atherosclerosis, such as myocardial infarction or critical limb ischemia, are also featured by the immunoinflammatory reaction within the affected territory that plays an active role in posts ischemic vascular and tissue remodeling. A complex continuum of molecular, cellular, and extracellular responses is controlled by the different actors of the inflammatory reaction and determines the extent of atherosclerosis development, as well as the homeostasis of the ischemic tissue. Although therapeutic targeting of inflammatory cells and/or cytokines may be envisioned for a short period of time following acute coronary events, such as a therapeutic approach should target a specific type (or subtype) of inflammatory cells and should deeply evaluate the long-term effects of inflammatory mediators on atherosclerosis and ischemic tissue regeneration. This review provides an overview of our current knowledge regarding the role of innate and adaptive immunity in atherosclerosis and related ischemic diseases.

Medicographia. 2014;36:355-361 (see French abstract on page 361)
Innate immunity

The innate response is instigated by the activation of vascular cells and monocytes/macrophages. Subsequently, an adaptive immune response develops against an array of potential antigens presented to effector T lymphocytes by antigen-presenting cells. Experimental studies in murine models of atherosclerosis have shown that proinflammatory and T helper 1 (Th1)–related cytokines promote the development and progression of the disease, whereas anti-inflammatory and regulatory T cell–related cytokines exert clear antiatherogenic activities. As an example, signaling through the interleukin-1 receptor (IL1R) severely aggravates vascular inflammation and atherosclerosis. IL1R-deficient (IL1RN−/−) mice exhibit less atherosclerosis, and overexpression of the IL1R antagonist (IL1RA) ameliorates the disease. Conversely, mice deficient in IL1RA (IL1RN−/−) display exacerbated atherosclerosis, and spontaneous and fatal vascular inflammation. In the early stages of atherosclerosis, proinflammatory cytokines can alter endothelial functions. Tumour necrosis factor α (TNFα), for example, increases cytosolic Ca2+ and activates myosin light chain kinase and ras homolog gene family, member A (RhoA), which disrupts endothelial junctions, leading to loss of barrier function and facilitation of leukocyte transmigration. Cytokines also induce the expression of chemokines and adhesion molecules in endothelial cells, favoring the recruitment, adhesion, and migration of lymphocytes and monocytes into the inflamed vessel wall. Once in the intima, leukocytes can be permanently activated by locally generated cytokines, which can accelerate the transformation of macrophages into foam cells by stimulating the expression of scavenger receptors and enhancing cell-mediated oxidation. Cholesterol crystals act as metabolic triggers of the NLRP3 inflammasome, which promotes the maturation of IL-1β and IL-18. At an advanced stage of the disease, proinflammatory cytokines destabilize atherosclerotic plaques by promoting cell apoptosis and matrix degradation. A number of proinflammatory cytokines can induce SMC and macrophage apoptosis, particularly the association of IL-1, TNFα, and interferon γ (IFN-γ). Macrophage apoptosis results in the formation of cell debris, which contributes to the enlargement of the lipid core. Plaque SMC apoptosis leads to thinning in the fibrous cap, favoring its rupture. Proinflammatory cytokines significantly affect the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), inducing substantial remodeling of many components of the extracellular matrix. For example, IFN-γ inhibits collagen synthesis, whereas IL-1 and TNFα induce a broad range of MMPs in vascular cells, including MMP-1, 3, 8, and 9. Finally, the antithrombotic properties of endothelial cells are deeply altered by cytokines. Downregulation of anticoagulant mediators may in turn affect inflammation. Proinflammatory cytokines also modify the fibrinolytic properties of endothelial cells, decreasing the production of tissue plasminogen activator and increasing the production of type I plasminogen activator inhibitor. As a result, proinflammatory cytokines might precipitate thrombus formation and promote the development of acute coronary syndrome (ACS).

A variety of plasma inflammatory markers have been shown to predict future cardiovascular risk well. They can be useful for risk stratification and also to identify those patients who might benefit from targeted interventional therapy. Of these markers, C-reactive protein (CRP), an acute-phase protein, has been the most extensively studied. There is robust evidence from primary prevention cohorts and amongst patients presenting with ACS that elevated CRP levels predict future cardiovascular events. Also, IL-6 levels appear to be predictive of cardiovascular events and are elevated in patients with unstable angina compared with those with stable angina. In a recent meta-analysis including up to 133,449 individuals, an IL-6 receptor (IL6R) single nucleotide polymorphism (rs7529229) was associated with increased circulating IL-6 concentration, consistent with IL6R blockade, and decreased coronary heart disease events, suggesting that IL6R signaling has a causal role in the development of coronary artery disease.
Hitherto, the strongest evidence that inflammation plays a major role in atherosclerosis in humans stems from studies in patients with autoimmune disease who are at very high cardiovascular risk, and who benefit from anti-inflammatory treatments. Patients with autoimmune diseases, such as systemic lupus erythematosus or rheumatoid arthritis, are at particularly high risk of cardiovascular disease. In patients with rheumatoid arthritis, anti-TNF therapy reduced inflammation, thrombotic risk, and the incidence of cardiovascular events.9

◆ Adaptive immunity

T-cell responses are initiated when specific molecular epitopes on antigens, including oxLDL and heat shock proteins, are presented by antigen-presenting cells and recognized by T cell antigen receptors. Although macrophages can also present antigens to T cells, dendritic cells are the main cell type responsible for the activation of naïve T cells and therefore play a crucial role in triggering adaptive immunity. In atherosclerotic plaques, dendritic cells localize with T cells, suggesting that they are involved in T-cell activation within the plaque. However, sensitization of naïve T cells most likely occurs in the regional lymph nodes. A number of experimental studies have clearly shown a critical pathogenic role for the T_{H1} response, associated with the production of IFN-γ.5

Atherosclerosis is also associated with B-cell activation. The main function of B cells is to secrete antibodies of various isotypes. Immunoglobulin G (IgG) antibody production by B2 B cells, the most common type of B cells, requires T cell co-stimulation, whereas innate production of natural immunoglobulin M (IgM) antibodies by B1 B cells does not require the help of T cells. Both IgG and IgM antibodies against oxLDL have been described. In mouse models, recent studies have provided evidence for a proatherogenic role of B2 B cells.10

Natural regulatory T lymphocyte (Treg) cells develop in the thymus and recognize a specific self-antigen. They are characterized by the expression of CD4, high levels of CD25, and the transcription factor Foxp3. They home to peripheral tissues to maintain self-tolerance and prevent autoimmunity by inhibiting pathogenic lymphocytes. These cells mediate suppressor function through the production of IL-10 and transforming growth factor β (TGFβ). Treg cells are detected in much lower amounts in atherosclerotic plaques than in other chronically inflamed tissues, such as in the skin of patients with eczema or psoriasis, where Treg cells can represent up to 25% of all T cells, suggesting an impairment of local tolerance against potential antigens in atherosclerotic lesions.11 Using mice with genetically altered Treg cells, or by application of CD25-neutralizing antibodies, it has been shown that Treg cells exert a protective role in atherosclerosis (Figure 1).12 The effects of Treg cells are dependent on TGFβ and IL-10. In humans, a decrease in the percentage of these cells has been observed in patients with ACS.13

◆ Therapeutic perspectives

Inflammation plays a major role at all stages of the atherosclerotic process, from the early events, whereby leukocytes are recruited at sites of sub-endothelial LDL cholesterol accumulation, to the late events, when plaque rupture occurs, leading to thrombus formation and adverse clinical outcomes. The chronic inflammatory disease of the arterial wall is promoted by both innate and adaptive T_{H1}-driven immunity, and is orchestrated by a complex network of proinflammatory cytokines. Murine experimental models of atherosclerosis provide clear evidence that blockade of proinflammatory cytokines results in limitation of plaque development and progression. In humans, anticytokine therapies have proven very successful against autoimmune disease. However, most of the proinflammatory cytokines are central to a successful host defense against microbial pathogens. Therefore,
Innate immunity

Monocytes/macrophages

In an experimental model of CLI, the presence of monocytes is strongly associated with the local proliferation of endothelial cells and SMCs and the extent of the postischemic neovascularization process closely depends on the number of circulating monocytes. Monocytes can be mobilized from the bone marrow or the spleen. In mice with MI, 40% of the monocytes infiltrating the infarcted myocardium 24 hours after the ligation originate from the spleen. Monocytes promote angiogenesis and collateral growth in a paracrine manner, by secreting diverse growth factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). They are also a major source of MIP-9, which is involved in the emergence and branching of the newly formed vascular network. Monocytes constitute a heterogeneous population with two major subtypes in mice: Ly6C<sup>hi</sup>-CCR2<sup>-</sup>CX3CR1<sup>hi</sup> monocytes and Ly6C<sup>lo</sup>-CCR2<sup>-</sup>CX3CR1<sup>lo</sup> monocytes, corresponding in humans to the CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup> subpopulations, respectively. These two subtypes of monocytes are recruited sequentially to the ischemic tissue and display different functions. They have similar capacities for the phagocytosis of dead cell debris, but Ly6C<sup>hi</sup> monocytes secrete large amounts of diverse proteases (MMPs, cathepsins), whereas Ly6C<sup>lo</sup> monocytes express VEGF strongly. The chemokine (C-C motif) ligand 2/chemokine (C-C motif) receptor 2 (CCL2/CCR2) pathway seems to act specifically on the Ly6C<sup>hi</sup> subpopulation of monocytes (Figure 2). Similarly, two subtypes of macrophage can be distinguished on the basis of various markers and functional criteria: M1 macrophages express the inducible nitric oxide synthase and proinflammatory cytokines, whereas M2 macrophages produce large amounts of arginase 1, anti-inflammatory cytokines IL-10, and VEGF. It is widely agreed that this M2 population favors tissue regeneration.

Neutrophils

The role of neutrophils in the revascularization of ischemic tissues is less well defined. The elastase secreted by neutrophils has a strong antiangiogenic effect and inhibits the proangiogenic effect of other cell types. The serine proteases produced.
by neutrophils (elastase and proteinase 3) induce the apoptosis of endothelial cells. Neutrophils are a major source of reactive oxygen species, an overabundance of which can damage endothelial cells, blocking the angiogenesis process. Conversely, neutrophils stimulated with granulocyte colony-stimulating factor secrete VEGF and may favor angiogenesis. Neutrophils also participate in the proangiogenic effect obtained after inhibition of plasminogen activator inhibitor-1 in a mouse model of CLI.14

A crucial interaction between mature B lymphocytes and monocytes has recently been unravelled. After acute MI in mice, mature B lymphocytes selectively produce chemokine (C-C motif) ligand 7 (CCL7) and induce Ly6C<sup>high</sup> monocyte mobilization and recruitment to the heart, leading to enhanced tissue injury and deterioration of myocardial function. Of interest, high circulating concentrations of CCL7 and B cell-activating factor (BAFF) in patients with acute MI predict increased risk of death or recurrent MI (Figure 4, page 360).23,24

**Therapeutic perspectives**

The overall effect of the various actors of the inflammatory component depends on the local environment. The activation and differentiation states of each cell type of inflammatory cells are also important. Macrophages and differentiated dendritic cells are, for example, less able to promote vascular neogenesis than monocytes.15,25 In addition, proinflammatory cytokines, growth factors, and chemokines may also affect the production of the chemokines CCL2 and CXCL8 in endothelial cells. Mast cell granules might be protective in MI; the injection of these granules into the ischemic heart of rats decreases cardiomyocyte apoptosis and promotes neovascularization by activating the Akt/protein kinase B–dependent pathway. Nevertheless, mast cells may also have deleterious effects on cardiac remodeling. In ischemia/reperfusion, the degranulation of resident mast cells containing active TNFα and proinflammatory cytokines can participate in the deleterious remodeling of the ischemic myocardium. The inhibition of mast cell degranulation decreases myocardial ischemia/reperfusion lesions. Combined inhibition of the angiotensin I-converting enzyme and the mast cell chymase, resulting in the inhibition of the two principal pathways responsible for angiotensin II production, has been found to have a beneficial effect on postinfarction cardiac remodeling.14
Inflammation and cardiovascular disease

Figure 4. Role of B lymphocytes in postischemic cardiac remodeling.

After acute myocardial infarction in mice, the release of necrotic debris by the infarcted heart activates circulating mature B lymphocytes. Activated B lymphocytes selectively produce chemokine (C-C motif) ligand 7 (CCL7) and induce Ly6C<sup>high</sup> monocyte mobilization from the bone marrow and recruitment to the heart through activation of chemokine (C-C motif) receptor 2 (CCR2), leading to enhanced tissue injury and deterioration of myocardial function. B lymphocytes are also recruited in the cardiac tissue where they may also coordinate cardiac remodeling. Genetic (B cell activating factor [BAFF]-receptor deficiency) or antibody-mediated (CD20- or BAFF-specific antibody) depletion of mature B lymphocytes impedes CCL7 production and monocyte mobilization, limits myocardial injury, and improves heart function. (1, 2)

Abbreviations: Myd88, myeloid differentiation primary response gene 88; Trif, TIR domain-containing adapter-inducing interferon β.


such as IL-18 and IL-12, have antiangiogenic activities. Antiangiogenic molecules are produced during inflammatory reactions and participate in the tissue response to ischemia. The effect of inflammation on tissue remodeling may depend on the homeostasis of ischemic tissues. Hence, results obtained in models of moderate tissue ischemia, such as experimentally induced hindlimb ischemia, may differ from those obtained from ischemic mouse models, which are associated with severe tissue ischemia and massive cellular death. Moreover, the type of immunoinflammatory response may trigger adverse tissue remodeling despite its neovascularization-activating effect. Lymphocytes, like myeloid cells, display activities potentially deleterious in tissue remodeling, particularly after an MI. Excessive inflammation is associated with a decrease in left ventricle ejection fraction in mice with MI. T lymphocytes of patients with MI are hyperactivated and produce the proapoptotic protein FasL (Fas/CD95/Apo-1 receptor ligand). A protective role of Treg cells has been shown in models of brain ischemia and MI. Similarly, despite their angiogenic activity, certain proinflammatory interleukins favor an excessive immune reaction in infarcted myocardium and are involved in deleterious remodeling (fibrosis) of the left ventricle. For example, the inhibition of IL-6 by neutralizing antibodies decreases inflammation and attenuates adverse cardiac remodeling after MI in mice. The overexpression of the natural antagonist of the IL-1β, IL1RA, or the administration of a recombinant form of human IL1RA, anakinra, decreases postinfarction cardiac remodeling. On the other hand, antiangiogenic interleukins may exert beneficial effects on postischemic cardiac remodeling. IL-10 inhibits postischemic revascularization, but exerts a protective effect in postischemic cardiac remodeling. These results indicate that therapeutic strategies aimed at regulating inflammation should target a specific type (or subtype) of inflammatory cells, and evaluate not only short-term, but also long-term effects of inflammatory mediators on both vascular and tissue regeneration. Interestingly, nanoparticle-facilitated silencing of CCR2, the chemokine receptor that governs inflammatory Ly6C<sup>high</sup> monocyte subset traffic, reduce infarct inflammation and improve ejection fraction in apoE<sup>−/−</sup> mice after MI.

Ailain Tedgui (born 4 June 1953) obtained his PhD thesis in “Fluid Mechanics”. After his postdoctoral fellowship at Imperial College, London, he joined the Institut Nationale de la Santé et de la Recherche Médicale (Inserm) in 1983. He was head of an Inserm laboratory at Hospital Lariboisière, Paris, from 2000 to 2004. He is currently Director of the Paris Cardiovascular Research Center at the Hôpital Européen Georges Pompidou (Paris, France). He coordinated the European Vascular Genomics Network from 2004 to 2008. His primary research is aimed at elucidating the role of apoptosis and inflammation in atherosclerosis. More recently, he bridged the interface between vascular biology and immunology in showing that a subset of immune cells—regulatory T cells—limit the development of atherosclerosis and can be used as a promising antiatherosclerotic strategy to curtail inflammation. He acted as the European editor of Arteriosclerosis Thrombosis and Vascular Biology (2007-2012).

Jean-Sébastien Silvestre (born 27 November 1971) is a French biologist and physiologist. From 1999 to 2007, he was assistant professor at Paris-Diderot University and an honorary member of the Institut Universitaire de France. Since 2008, he is research director at Institut Nationale de la Santé et de la Recherche Médicale (Inserm) and head of team 6, “regenerative therapies for cardiac and vascular diseases” at the Paris Cardiovascular Research Center, Inserm UMR 970 (Paris, France). His central interest lies in cardiovascular physiology, and the role of vascular growth and remodeling in ischemic diseases. He has coauthored more than 100 published articles deciphering the molecular and cellular mechanisms involved in postischemic revascularization and tissue regeneration.
ATHÉROSCLÈROSE ET MALADIES ISCHÉMIQUES ASSOCIÉES : L’INFLAMMATION, UN BIENFAIT CONTRADICTOIRE


Les traitements ciblés des cellules inflammatoires et/ou des cytokines peuvent être imaginés pour de courtes périodes après des événements coronaires aigus mais devraient s’adresser à un type spécifique (ou sous-type) de cellules inflammatoires et évaluer en profondeur les effets à long terme des médiateurs inflammatoires sur l’athérosclérose et la régénération du tissu ischémique. Cet article passe en revue nos connaissances actuelles sur le rôle de l’immunité innée et acquise dans l’athérosclérose et les maladies ischémiques associées.

Keywords: atherosclerosis; B lymphocyte; inflammation; interleukin; ischemia; T lymphocyte

References
Cardiovascular diseases are the leading cause of death worldwide, and atherosclerosis is the main underlying etiology. Atherosclerosis is an inflammatory, dynamic, and complex disease involving multiple cell types, and many anti-inflammatory strategies have recently emerged as potential therapeutic approaches for atherosclerotic diseases. In this review, we focus on the role of the proinflammatory cytokine interleukin 1 (IL-1) in atherosclerosis and the potential benefits of IL-1 inhibition. A number of ongoing clinical studies of IL-1 inhibition will be introduced, with an in-depth look at gevokizumab. Gevokizumab is an antibody directed against IL-1 that is being tested in clinical trials of patients with coronary artery disease. The first study is evaluating the effects of gevokizumab on arterial inflammation measured by positron emission tomography.

Medicographia. 2014;36:362-370 (see French abstract on page 370)

Cardiovascular diseases remain the leading cause of mortality in the world, and atherosclerosis is the main underlying etiology. The long-standing view supporting that development of the atherosclerotic lesion solely depends on lipid deposition has been replaced by the current concept that activation of immune and inflammatory responses has a central role in plaque initiation and progression. Subsequently, different anti-inflammatory strategies have emerged as potential treatments of atherosclerotic disease, in addition to the existing lipid-lowering therapies.

Atherosclerosis is an inflammatory disease that consists of the formation of an atherothrombotic plaque in the arterial wall, causing stenotic and thrombotic complications. The atherosclerotic plaque formation begins early in childhood by the accumulation of lipids and inflammatory cells in the arterial wall (known as the fatty streak). Further lipid deposition and oxidation triggers the phagocytosis of the accumulated lipids by macrophages, stimulating the inflammatory reaction that contributes to the plaque’s growth. Recruitment of inflammatory cells and increased secretion of proinflammatory cytokines, such as interleukin (IL) 1, enhance the inflammatory reaction. Atherosclerosis is therefore a complex process involving diverse cell types (including monocytes and lymphocytes) and molecules (including selectins, other adhesion molecules, chemokines, and growth factors). Numerous studies dissecting the interplay between lipid deposition and oxidation, as well as inflammatory cell recruitment and cytokine secretion, have revealed multiple potential targets that could interfere with and potentially inhibit the development and progression of the atherosclerotic plaque.
Patients with chronic extracardiac inflammatory diseases such as rheumatoid arthritis or extensive psoriasis have a higher risk of cardiovascular diseases and related mortality compared with the general population. Systemic inflammation appears to be independently involved in this increased cardiovascular risk and leads to coronary artery disease, myocardial infarction, cerebrovascular disease, and heart failure. Indeed, the level of inflammation seems to be an independent risk marker and possibly a risk factor for cardiovascular diseases in patients with extracardiac inflammatory diseases, especially in patients with rheumatoid arthritis. Taken together, these studies indicate that treatment of the underlying inflammatory process could contribute to improved cardiovascular outcomes.

Despite treatment with the most powerful statins, patients remain exposed to a high risk of complications (including death and reinforcement) after an acute coronary syndrome or a stroke. Based on the model that inflammation is a central process in

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**Figure 1.** Cholesterol crystals can promote inflammation by activating the NLRP3 inflammasome. NLRP3 inflammasomes can activate the inflammatory cascade by binding to and cleaving pro-caspase-1, forming caspase-1, important for the cleavage and secretion of various interleukins, including IL-1β (detected by ELISA and immunoblot analysis) is released into the supernatants of PBMNC cultures treated with cholesterol crystals. Resting or LPS-primed human PBMNC were treated with cholesterol crystals as indicated, MSU crystals (250 μg/mL), or ATP in the presence or absence of the caspase-1 inhibitor zYVAD-fmk (10μM). Results are represented as means and SEM of 4 donors. (C) Cleaved caspase-1 was assayed by immunoblot in the supernatants and cell lysates and (D) IL-1β was measured by ELISA in supernatants from LPS-primed wild-type, NLRP3- or ASC-deficient macrophages stimulated with cholesterol crystals, dAdT, nigericin, or ATP. One out of 3 independent experiments are shown.

**Abbreviations:** ASC, an adaptor protein involved in inflammasome binding to pro-caspase-1; ATP, adenosine triphosphate; dAdT, transfected double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; IB, immunoblot; IL, interleukin; KO, knockout; LPS, lipopolysaccharide; MSU, monosodium urate; NLRP3, nucleotide-binding domain, leucine-rich-repeat–containing family, pyrin domain–containing 3; PBMNC, peripheral blood mononuclear cell; SEM, standard error of the mean; zYVAD-fmk, a caspase-1 inhibitor.

Inflammation and cardiovascular disease

Involvement of IL-1β in cardiovascular diseases

IL-1β is a potent proinflammatory cytokine secreted by different cell types, including macrophages and endothelial cells, and is involved in the differentiation of lymphoid cells. IL-1β seems to be a key regulator of several inflammatory and autoimmune diseases. The important role of IL-1 in atherosclerosis has been highlighted by experimental studies showing reduced atherosclerosis in IL-1 knockout or IL-1–type I receptor knockout mice. The role of IL-1 is largely documented in the pathophysiology of atherosclerosis, where it specifically targets endothelial cells and promotes vascular smooth muscle cell proliferation leading to intima thickening in mice. IL-1β has been shown to upregulate adhesion molecules in endothelial cells, thereby increasing recruitment of inflammatory cells, such as macrophages, in response to atherogenic stimuli such as cholesterol deposits. IL-1β also promotes inflammatory-cell transmigration to the atherosclerotic site. In addition, IL-1β has been shown to increase the levels of matrix metalloproteinases, thereby promoting extracellular matrix degradation. Also, IL-1β has been shown to enhance the reactivity of endothelial cells by inducing the expression of inducible nitric oxide synthase and vascular endothelial growth factor.

In atherothrombotic coronary disease, IL-1 has been shown to promote atheromatous lesions, modulate cholesterol metabolism, enhance vascular inflammation, and contribute to plaque rupture. Indeed, IL-1 showed proadhesive activity by increasing the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) in mice.

The increased expression of VCAM-1 and monocyte chemotactic protein-1 (MCP-1) results in increased accumulation of atherosclerotic disease development and subsequent complications and on the hypothesis that targeting specific inflammatory proteins or pathways can be effective in reducing the risk of cardiovascular events, many anti-inflammatory drugs have been developed and some have shown promising results when administered on a background of statin therapy.

In this article, we focus on the potential role of IL-1β inhibition in coronary and ischemic diseases.

**Selected abbreviations and acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
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<tr>
<td>CANTOS</td>
<td>Canakinumab Anti-inflammatory Thrombosis Outcomes Study</td>
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<tr>
<td>hs-CRP</td>
<td>high-sensitivity C-reactive protein</td>
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<td>PET</td>
<td>positron emission tomography</td>
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<td>FDG</td>
<td>fluorodeoxyglucose</td>
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<td>TBR</td>
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**Figure 2.** Microscopic and macroscopic views of atherosclerotic lesions in apoE-deficient mice having either the IL-1β+/- or IL-1β-/- genotype. (A) Microscopic appearance around the aortic valves of 12- and 24-week-old male apoE-deficient mice (an animal model of atherosclerosis with hypercholesterolemia when fed on a normal diet) having either the IL-1β+/- or IL-1β-/- genotype. At both 12 and 24 weeks of age, the atherosclerotic lesion sizes of IL-1β-/- mice were smaller than those of IL-1β+/- mice. For each mouse, 3 sections at 100-μm intervals around the aortic valve were observed. The section where all valves were visible was regarded as the O-μm section. The left (+100 μm) and right (-100 μm) photomicrographs are sections 100 μm above and below the O-μm section, respectively. All sections were stained with Oil red O (magnification, ×40). Bar in lower right panel equals 200 μm. (B,C) Macroscopic findings of total aortas of apoE+/- (IL-1β+/-) (n=14) and apoE-/-IL-1β-/- male mice (C, n=13) at 24 weeks of age. These aortas were stained with Oil red O. The percentage of the atherosclerotic lesion to total aorta area was significantly lower in apoE-/-IL-1β-/- mice than in apoE+/-IL-1β+/- mice.
monocytes in the arterial intima which will differentiate into macrophages and foam cells, leading to atherogenesis. In humans, IL-1 has been shown to result in upregulation of matrix metalloproteinases, which induce collagen breakdown in atheromatous plaques. Increased expression of matrix metalloproteinases by IL-1 might therefore induce atheromatous plaque rupture and lead to superimposed thrombosis. IL-1 may also modulate cholesterol plasma levels by serum amyloid A induction and stimulate angiogenesis and vessel wall inflammation via vascular endothelial growth factor and other inflammatory pathways. Of note, IL-1 levels were found to be elevated in atherosclerotic human coronary arteries.

**IL-1 antagonism**

Pharmacological inhibition of IL-1 or IL-1 receptor has been shown to decrease experimental atherosclerotic plaque formation. The selective loss of IL-1 signaling in the vessel wall by bone marrow transplantation reduced plaque burden in a mouse atherosclerosis model. This reduction was associated with restored endothelium-dependent vasodilation and decreased levels of arterial oxidative stress. These findings were further confirmed by studies targeting the IL-1 receptor. Subsequently, therapies including soluble truncated IL-1 receptor, IL-1–receptor antagonist, IL-1 trap fusion protein, or anti–IL-1 antibodies have been developed and tested in animal models and in clinical settings.

The soluble IL-1–receptor antagonist molecule is an IL-1 antagonist, which binds to the IL-1 receptor, but does not activate the IL-1–receptor signaling pathway. IL-1–receptor antagonist has a key role in the development of atherogenesis; indeed, mice lacking the IL-1Ra gene develop lethal arterial inflammation. IL-1–receptor antagonist has also been shown to inhibit neointima formation after coronary artery injury. This impact on vascular inflammation and atherosclerosis has been proposed to be dependent on the ratio of IL-1 to IL-1–receptor antagonist. Finally, genetic associations between IL-1Ra and coronary artery disease as well as the development of restenosis after stenting have been reported.

**Ongoing clinical trials evaluating the effects of anti–IL-1 therapies**

There are a number of clinical trials studying the effects of anti–IL-1 therapy currently under way. Briefly introduced here are studies evaluating the effects of IL-1 inhibition with anakinra, and more selectively, IL-1β inhibition with canakinumab. We will then take a more in-depth look at the IL-1β inhibitor gevokizumab and the ongoing initial clinical assessment of its effects on plaque inflammation.

**The IL-1 inhibitor anakinra**

Anakinra is a recombinant form of human IL-1–receptor antagonist that competitively inhibits IL-1 by binding the IL-1 type I receptor, acting as an antagonist to the IL-1 receptor. A clinical trial (NCT01566201) is ongoing to evaluate the effects of anakinra on vascular processes (ie, coronary flow reserve, aortic deformation) and ventricular function (ie, systolic and diastolic function on echocardiography) as well as apoptotic and inflammatory biomarkers in 80 patients with both rheumatoid arthritis and coronary artery disease.

**The IL-1β inhibitor canakinumab**

A human monoclonal antibody against IL-1β is currently indicated for the treatment of autoinflammatory diseases such as cryopyrin-associated periodic syndromes (CAPS). Canakinumab selectively neutralizes IL-1β, resulting in a rapid and sustained inhibition of the inflammatory acute phase response. CANTOS (Canakinumab ANti-inflammatory Thrombosis Outcomes Study) is a large randomized, double-blinded, placebo-controlled, event-driven trial (NCT01327846) of 17 200 stable post–myocardial infarction patients with persistent elevation of high-sensitivity C-reactive protein (hs-CRP) that is evaluating the effects of canakinumab, at doses of 50, 150, or 300 mg administered subcutaneously every 3 months, as an add-on to optimized treatment. All participants are followed up over an estimated period of up to 4 years for the trial’s primary end point (ie, cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke) as well as for other end points including vascular events, total mortality, adverse events, and specific end points associated with inflammation, including new-onset diabetes, venous thrombosis, and atrial fibrillation. CANTOS is the first large study evaluating the effect of anti–IL-1 therapies on atherosclerosis-related end points. If such therapy has beneficial effects, it would open new avenues for direct targeting of inflammation to reduce atherosclerosis and its complications.

**Focus on the IL-1β inhibitor gevokizumab**

Gevokizumab is a potent anti–IL-1β neutralizing antibody, which acts as a regulator of the IL-1 pathway (Figure 3, page 366). It has been shown to reduce pathologically high IL-1β activity, while allowing homeostatic signaling in biologically important processes. Gevokizumab is a recombinant Human Engineered monoclonal antibody that binds human IL-1β with 0.3pM affinity and regulates the activation of IL-1 receptors. The antibody was humanized using proprietary technology with the goal of reducing the probability of eliciting anti-drug immunogenic responses. Gevokizumab is produced in Chinese hamster ovary (CHO) cells. It is an immunoglobulin G subclass 2 (IgG2) isotype, thereby reducing the probability of antibody-dependent cell-mediated cytotoxicity should binding to the cell surface occur. The terminal half-life of the antibody is estimated to be between 22 to 28 days. The high affinity and potency of gevokizumab is expected to allow for lower doses and more convenient dosing regimens than currently available with other IL-1β inhibitors.

In vitro binding studies show that gevokizumab binds with similar affinity to human, rat, rabbit, and cynomolgus and rhesus monkey IL-1β and with over 1000-fold less affinity to mouse IL-1β. Gevokizumab shows selective binding to recombi-
niant human IL-1β, but not to recombinant human IL-1α (another inactive subtype of IL-1). In vitro cell culture studies show the ability of gevokizumab to inhibit IL-1β-mediated IL-6 expression: the inhibition induced by gevokizumab was greater than that induced by the recombinant IL-1–receptor antagonist anakinra (data on file). Evaluation of Toll-like–receptor agonist stimulation in human whole blood cultures also indicate that 0.1pM gevokizumab inhibited 50% of the production and release of IL-1β, IL-1α, and interferon gamma, and to a lesser degree tumor necrosis factor α and IL-6, but not IL-1–receptor antagonist and IL-8. These results demonstrate that gevokizumab was able to reduce, but not fully suppress, the cytokine induction under physiological conditions of this assay.

In vivo studies confirmed in mice the ability of gevokizumab to inhibit, in a dose-dependent manner, recombinant human IL-1β induction of IL-6. Approximately 70% inhibition of activity was obtained with the highest dose tested (3 mg/mouse, data on file). The in vivo pharmacological evaluation of gevokizumab has included studies that show that it is safe in a rat model of myocardial infarction and has beneficial effects in a mouse model of atherosclerosis. When administered at the time of reperfusion following 30 minutes of complete occlusion of a coronary artery, gevokizumab (10 mg/kg) did not exert a deleterious effect on left ventricular scar formation or on scar size (J. C. Tardif et al, unpublished observations, 2012). Gevokizumab was recently shown to have antiatherosclerotic effects in apolipoprotein E (apoE)-knockout mice: indeed,
Figure 4. Effect of a chimeric murine version of gevokizumab (XMA052 MG1K) on atherosclerotic lesions in the aortas and brachiocephalic arteries of apoE-deficient mice.

ApoE-deficient mice were fed an atherogenic diet for 16 weeks and dosed as indicated. Aortic lesion area was measured by en face analysis and expressed as percent Sudan IV–positive pixels. (A) En face images from representative individuals with lesion size approximating the mean are shown. (B) Lesion area was reduced by 37%, 22%, and 29% at the 0.1, 1.0, and 10 mg/kg doses, respectively. Results are represented as mean ± SEM (n=10). *P<0.05 versus IgG and vehicle. (C) Representative images of brachiocephalic arteries for IgG and XMA052 MG1K (1.0 mg/kg).

Abbreviations: mlgG, mouse immunoglobulin G; SEM, standard error of the mean; XMA052 MG1K, chimeric murine version of gevokizumab.

lesion area in the aorta was reduced by 37%, 22%, and 29% at doses of 0.1, 1, and 10 mg/kg administered twice weekly for 16 weeks, respectively, with no significant differences between doses (Figure 4, page 367). 40

The initial clinical cardiovascular assessment of gevokizumab will target arterial inflammation evaluated by positron emission tomography (PET) in patients at high cardiovascular risk. Symptomatic unstable arteriosclerotic plaques accumulate more 18F-fluorodeoxyglucose (18FDG) than asymptomatic lesions, and 18FDG uptake in the ascending aorta and the left main coronary artery is higher in patients with a recent acute coronary syndrome as compared with patients with stable angina. 41, 42 In addition, recent studies demonstrated that 18FDG uptake is frequently elevated in high-risk patients with type 2 diabetes, coronary artery disease, or obesity. 43 In order to maximize the possibility of observing a difference between placebo and gevokizumab, the study population includes patients with active plaques: high-risk patients with a high 18FDG uptake in at least 1 main arterial region (thoracic aorta or carotid) following a recent acute coronary syndrome.

This first study is a prospective, international, multicenter, randomized, double-blinded, parallel-group trial. As a pilot exploratory study, the multiple administration of gevokizumab is compared with placebo, and the randomization is unbalanced (2:1 ratio). The duration of treatment (12 weeks between first and last monthly injection) was chosen in order to allow sufficient exposure to therapy allowing the assessment of drug activity on plaque inflammation. The assessment of plaque inflammation with PET will be performed both before study drug initiation and approximately 2 weeks after the last administration of study drug, at the theoretical peak of plasma concentration. There is a 3-month observation period following the last drug intake; its duration is considered to be sufficient given the expected gevokizumab pharmacokinetic profile (half-life of 22 to 26 days, residual concentration of gevokizumab 3 months after last injection <15%).

The standardized uptake value is the unit used to quantify 18FDG uptake in body tissue and is determined as the decay-corrected tissue concentration of 18FDG (in kBq/mL) divided by the injected dose per body weight. The target-to-background ratio (TBR) was developed in order to reduce the interpatient variability due to injected dose and body weight. It is calculated by dividing the arterial wall standardized uptake value by the venous blood value measured in the corresponding venous area (vena cava or jugular vein). A significant correlation has been demonstrated to exist between the TBR measured in the arterial wall and macrophage staining from the corresponding histological sections (r=0.85; P<0.0001). 44 An elevated TBR has been shown to be a strong predictor of subsequent cardiovascular events 45 and was associated with several cardiovascular risk factors.

The goal of this pilot study is to evaluate the effect of 4 successive monthly subcutaneous administrations of gevokizumab versus placebo on the reduction of arterial wall inflammation in patients with marked arterial wall inflammation following a recent acute coronary syndrome. The primary objective is to evaluate the effect of gevokizumab compared with placebo on arterial wall inflammation assessed by 18FDG PET in the most diseased region of interest of both carotid and thoracic aortic walls. The secondary objectives are to evaluate the effects of gevokizumab compared with placebo on cardiac and vascular biological blood biomarkers, including hs-CRP and IL-6, on the safety profile of gevokizumab, and on its pharmacokinetics in this specific population.

All 18FDG PET measurements will be performed in 3 regions of interest: left carotid, right carotid, and thoracic aorta. All comparisons will be performed within the most diseased region of interest (region with the highest maximum mean TBR at baseline). The study end points include the changes of the maximum TBR, mean TBR, and most diseased segment TBR assessed by 18FDG PET.

Conclusion
IL-1 appears to play a significant role in the pathophysiology of coronary artery disease. Thus, IL-1 inhibition is being investigated in patients with cardiovascular diseases. Clinical trials, such as those touched on in this review, are required to test in a definitive way the hypothesis that anti-inflammatory approaches like IL-1 inhibition will improve cardiovascular outcomes in patients with coronary disease treated with optimal standard of care including intensive statin use.
Jean-Claude Tardif is Professor of Medicine and holder of the Endowed Research Chair in Atherosclerosis at the University of Montreal and of the Canada Research Chair (tier 1) in Translational and Personalized Medicine, and Cardiologist and Director of the Research Center at the Montreal Heart Institute. He is also the Scientific Director of the Montreal Heart Institute Coordinating Center (MHICC), the Canadian Centre of Excellence in Personalized Medicine (Cepmed), and the Canadian Atherosclerosis Imaging Network (CAIN). Professor Tardif is the principal investigator of several large international clinical trials including SPIRE (the Study of PCSK9 Inhibition and Reduction of Vascular Events), INITIATIVE (iNTerventional Trial on the Treatment of angina with IVabradine versus atenolol), and ASSOCIATE (evaluation of the Anti-anginal efficacy and Safety of the aSociation Of the IL-1 Current Inhibitor IVabradine with a beTa-blockEr). He is Chairman of the Executive Committee for the MODIFY trial (Modification of COronary atherosclerosis by ivabradine in patients with CAD who have a clinical Indication For coronaryY angiogram) and is also a member of the Executive Committee of SIGNIFY (Study assessInG the morbidity-mortality benefits of the IL-1 inhibitor ivabradine in patients with coronary artery disease) and CLARIFY (prospective observational LongitudInAl Registry of patients with stable coronary artery disease). He has authored and coauthored more than 400 articles in peer-reviewed journals. In addition, he has written 30 book chapters and has edited several books.

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Étude de la modulation de l’interleukine 1-β dans la maladie coronaire et ischémique

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IMPROVING COGNITION AND NEUROPLASTICITY

AMPA receptor modulation for enhancing plasticity and treating neuropathology

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AMPAR receptor modulation for enhancing plasticity and treating neuropathology

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Positve allosteric modulators of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPAR)–type glutamate receptors (“ampakines” and functionally related compounds) constitute a relatively new class of psychoactive drugs that enhance fast, excitatory transmission in the brain. Because of this effect, ampakines reduce the threshold for inducing memory-related changes to synapses and improve learning in animals across species and paradigms. While most CNS drugs target neurons that project in a one-step, parallel fashion to a multitude of sites, ampakine-type agents act on the multiple connections found in serial brain networks. This results in a multiplier effect for the drug, likely to be most pronounced in the elaborate circuits found in the cortex, thereby intensifying cortical regulation of lower brain areas (“pharmacological encephalization”). Evidence that the compounds are effective in animal models of psychiatric disorders associated with abnormal brainstem activity is in agreement with this hypothesis. The possibility that expanding cortical networks will lead to cognitive, as opposed to memory, enhancement in normal brains is largely unexplored. Finally, positive modulators increase the production of brain growth factors that promote plasticity and neuronal viability; upregulation is associated with neuroprotection, growth, and improved functional outcomes in different disease models.

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Twenty years have passed since the introduction of peripherally administered compounds that positively modulate α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPAR)–type glutamate receptors (ie, ampakines) and thereby rapidly enhance fast, excitatory transmissions in the brain. The motivation for developing such drugs was relatively straightforward: increasing the size of postsynaptic responses should facilitate the opening of voltage sensitive N-methyl-D-aspartic acid (NMDA) receptors colocalized with AMPAR receptors. Given that NMDA receptors trigger the learning-related long-term potentiation (LTP) effect, ampakines were expected to potentially facilitate memory encoding. These predictions have been confirmed by several groups. However, it quickly became evident that the drugs might have broader uses due to two effects, one obvious and the other not, that follow from augmented transmission at excitatory contacts. First, glutamatergic synapses mediate communication within the myriad networks used by the cortical telencephalon to regulate lower brain regions and to execute the dense computations underlying cognition. Positive modulators therefore have potential therapeutic applications in psychiatric disorders involving brainstem biogenic amine...
systems\(^2\) and, more speculatively, for enhancing cognition. Second, greater than normal excitatory postsynaptic currents (EPSCs) will increase the likelihood of cell discharges, an effect known to upregulate transcription of brain-derived neurotrophic factor (BDNF).\(^4\) Such an effect raised the possibility of using ampakine-type drugs to treat brain damage.

These ideas received considerable preclinical support and, as might be expected, the positive modifier strategy evolved significantly over the subsequent two decades. This is an appropriate point to survey the status of the field and to consider possible future directions. This review begins with a brief description of the mode of action for ampakine-like drugs and then turns to specific applications. The issue of why, given their substantial success in animal studies, the compounds have not progressed further in clinical development will also be discussed.

**Mode of action**
The first, centrally active, positive AMPA receptor modulators were small benzamide structures (\\(\sim 300\) Da)\(^7\) that, with further development, resulted in two large subtypes. Later studies by other investigators, primarily at Servier, Eli Lilly, and Glaxo, generated pyridothiadiazine,\(^6\) biarylpseudothiazone,\(^7\) and 5'-alkylbenzothiadiazide\(^9\) families that similarly enhance AMPA receptor currents.\(^5\) Compounds from each of these groups proved effective in various animal tests.

AMPA receptors are tetramers composed of several possible combinations of four homologous subunits (GluA1, GluA2, GluA3, and GluA4) each with RNA splicing variants (“flip” and “flop”).\(^10\) X-ray crystallography\(^11\) and site-directed mutagenesis\(^12\) work found that extracellular domains for individual GluA subunits create a V structure that contains the glutamate binding pocket. Notably, adjacent subunits form dimers (ie, two dimers per one tetrameric receptor). Ligand binding causes the extracellular V domains to converge and thereby trap the ligand; this generates tension on the transmembrane segment of the receptor and a shift to the channel open state. The V then reopens, releases the transmitter, and terminates ion flux, events referred to as “deactivation”. However, binding can disrupt dimerization, leaving the V structures and the channel closed. This paradoxical (bound, but closed receptor) state is likely responsible for the pronounced desensitization of AMPA receptors following extended agonist treatment or repetitive high frequency transmitter release.\(^13\) While it was commonly thought that desensitization terminates synaptic EPSCs, the decay phase of AMPA receptor–mediated synaptic currents appears to be governed by the rate of deactivation.\(^14\) Notably, the rate constants for deactivation and desensitization are not greatly dissimilar, and routinely used experimental methods (eg, whole cell recording) can shift the balance between them in favor of the latter.\(^15\) This is a point of some significance for the design of modulator experiments.

The binding pocket for ampakines was identified by x-ray crystallography of the receptor–drug complex. The pertinent site is found at the hinge of the extracellular V near the dimer interface,\(^16\) a position that: (i) controls the kinetics for reopening of the V and transmitter release (deactivation); and (ii) maintains dimerization, thus preventing the receptor from becoming desensitized. These crystallography findings accord with physiological results showing that ampakines can slow in both deactivation and desensitization.\(^13\) However, the relative effect on the two processes is variant-dependent; some modulators act predominantly on deactivation, others on desensitization, and still others on both. The first group increases the amplitude of synaptic responses while having minor effects on their duration (referred to as type A ampakines), while the second group (type B) causes a significant prolongation of the excitatory postsynaptic potential (EPSP) (Figure 1, page 374).\(^17\) As will be described, these two categories have substantially different functional effects.

**Enhancement of memory and cognition**

- **Synaptic plasticity and memory**

Brain waves during learning in mammals, including humans, contain high levels of 4-8 Hz \(\theta\) activity; recordings from individual neurons routinely detect multiple discharges (bursts) during the peaks of the rhythm. Remarkably, activation of inputs to the cells in a pattern that mimics \(\theta\) bursts causes a marked and extremely persistent increase in the amplitude of EPSCs elicited by subsequent single stimulation pulses.\(^18,19\) This long-term potentiation (LTP) effect is broadly accepted to be the substrate for many types of memory. If so, then there is a good possibility that enhancing LTP will produce corresponding effects on long-term memory. As mentioned, positive modulation of AMPA receptors potently facilitates the induction of LTP by reducing the voltage block on NMDA receptors, and there is now a large amount of literature showing that this is accompanied by the expected improvement in memory scores.

An overview of this material, the entirety of which cannot be adequately reviewed here, points to several general conclusions. A diverse array of positive modulators are effective across many different learning tests in animals and, where tested, without significant disturbances to behavior (Figure 2, page 375).\(^20\) Perhaps the most extensively tested single drug is Servier’s S18986, which has positive effects on many hu-
man-relevant memory types (including episodic, declarative, and relational)\(^\text{23}\) and is effective after peripheral or central administration.\(^\text{22}\) Work from other laboratories, using structurally different compounds, extends this list even further.\(^\text{28,24}\) That positive modulators work in so many circumstances is perhaps surprising given that some forms of learning are not likely to be dependent on LTP.\(^\text{25}\) This could indicate that drug effects on network processing, in these presumably non-LTP cases, facilitate attention and other processes required for acquisition, as opposed to acting directly on encoding mechanisms. Distinguishing between actions on processing vs encoding is thus a major issue with regard to interpreting memory enhancement effects.

Another noteworthy feature of memory enhancement with positive modulators is that it occurs in multiple species, including mice, rats, rabbits, and monkeys. This is encouraging with regard to human outcomes. The literature for humans is sparse, but at least one study, using an early, relatively weak ampakine, obtained evidence for enhancement in young, adult subjects.\(^\text{26}\)

Most experiments searching for memory enhancement use a fixed amount of training followed by retention tests at some later time. These paradigms do not discriminate between accelerated learning vs strengthening of the memory trace. Acceleration has been established in a few cases, such as fear learning\(^\text{27}\) and odor discrimination; in the latter, an ampakine acceleration has been established in a few cases, such as fear conditioning experiment in which ampakine-treated rats showed faster acquisition, but learned extinction proceeded at a normal rate, suggesting that the stability of the memory trace was not increased.\(^\text{28}\) Additional work is needed on this topic, particularly with regard to type A vs type B drug variants. In all, an impressive body of work from many laboratories has confirmed the prediction that positive modulation will enhance memory in well-established learning paradigms. It will be of great interest to test for such effects in more cognitively demanding circumstances.\(^\text{29}\)

**Network throughput and cognition**

Positive AMPA receptor modulators are thus far the only agents known to enhance communication within cortical networks. Predictions about outcomes need to consider the high likelihood that the drugs act at many stages in serial circuits, something that is not the case for the great majority of psychoactive compounds. Serotonergic neurons, for instance, do not string together to form long serial networks, but instead release more or less simultaneously at a very large number of scattered locations. The “serial sites of action” effect strongly suggests that the ultimate influence of an ampakine will depend upon the length and complexity of the network under study. Facilitation of transmission at one connection will lead to a greater number of cells that discharge at the next; repeated across many stages, each responding to the modulator, this would result in a multiplier effect for drug action. Confirmation was obtained in an experiment comparing the magnitude of EPSP increases at the first and third steps in a trisynaptic circuit that runs through the hippocampus: an ampakine concentration that produced a minor increase in synaptic responses at the initial step caused marked facilitation at the third.\(^\text{31}\) Note that these arguments suggest that positive AMPA receptor modulation will have much greater effects in long networks, such as those found within the cortex, than in shorter ones.

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**Figure 1. Mode of action for positive modulators of AMPA receptors.**

(A) Chemical structures for two functionally distinct, early stage ampakines (type A: CX516; type B: CX614). (B) The middle schematic illustrates the binding pocket for the drugs as deduced from site-directed mutagenesis and x-ray crystallography. Shown are two subunits of the tetrameric receptor. Note that each subunit has two extracellular domains (“1” and “2” in the schematic); the binding site for glutamate is found within the “V” formed by the two domains (not shown). The subunits form a dimer whose interface contains the ampakine pocket (orange circle). (C) Inward currents elicited by one-millisecond (ms) pulses of glutamate delivered to a patch excised from a hippocampal pyramidal cell. Note that application of an ampakine (green asterisk) greatly slows the recovery of the AMPA receptor–mediated current. This is the standard effect for positive modulators. Not shown here are effects in the presence of one-second glutamate pulses, during which the response quickly decays to 10% of its maximum value. Agents that block the decay are referred to as type B drugs; those that do not block the receptor desensitization, and yet produce the illustrated effect, are classified as type A. The bottom two traces show synaptic responses elicited by single stimulation pulses in a hippocampal slice. Type A ampakines increase the amplitude of field excitatory postsynaptic potential (fEPSP), while type B ampakines produce this effect, but also prolong the response.

**Abbreviations:** H, hydrogen; N, nitrogen; O, oxygen.

The question then arises as to why the multiplier effect doesn’t produce grossly enlarged EPSPs and seizures (notably, seizures are the primary unacceptable side effect of positive modulators). The likely reason that moderate concentrations do not cause epileptiform activity involves interneurons: enhancement of glutamatergic synapses on these inhibitory cells results in a slightly delayed suppression of further firing by projection neurons. In all, ampakines produce a potent, but brief, facilitation of rapid communication along a sequence of excitatory connections, thereby allowing a modest input to generate a sizable circuit output. Computational studies using integrated, multi-scale networks have provided a formal description of how enhanced excitatory transmission can lead to new and functionally useful outcomes from complex cortical systems. 32

Could such effects enhance cognition in the sense of adding capabilities in normal humans? The only work addressing the issue used nonhuman primates and a very difficult problem. Peripheral injection of an ampakine allowed monkeys to perform well beyond the level that could be achieved with weeks of training. Subsequent brain imaging studies showed that the drug produced a striking network effect: the monkeys engaged additional cortical areas while dealing with multiple, complex

**Figure 2.** Memory enhancement in two very different learning paradigms.

(A) Well-trained rats were placed in an arena in which two novel odors were ejected simultaneously from any of six locations randomized from trial to trial. (B) Vehicle injected animals require 10 trials to form stable memory, as assessed on tests given 1 to 3 days later (red line). Five training trials produce chance scores on long-term retention (gray bars). However, the same animals showed robust memory when given a pretraining injection of an ampakine (green bars). (C) Enhancement in a complex, delayed nonmatch to sample task. Rats were trained for weeks to master a problem in which they were required to learn the following sequence: (i) press a bar to receive a small reward; (ii) move to the opposite side of test arena and perform a nose poke that activated a light; (iii) remain at that position until the light extinguished; (iv) return to the original location where two bars were now presented; and (v) press the bar that had not been originally selected. (D) As expected from the overtraining, control rats (open circles) did not improve over three additional weeks of testing. Rats given injections of an ampakine every other day (green circles) showed a steady increase in correct responses over days and this persisted after the end of drug treatment. These results suggest that the ampakine enabled the animals to acquire novel information about the testing paradigm.

Future directions

An intriguing complexity to the ampakine/LTP interaction was introduced by the discovery that drug variants causing increases in EPSC amplitude only (type A) simply lower the threshold for potentiation, whereas those that increase both amplitude and duration (type B) produce this effect, but also elevate the LTP ceiling. Both classes improve memory scores, but tests for behavioral differences are lacking. However, recent work describing new LTP timing rules suggests interesting possibilities. Those experiments showed that hippocampal LTP exhibits an analogue of the “spaced trials effect” (wherein retention is much better after multiple, temporally separated training sessions than after a single massed trial) that is a fundamental feature of learning. Specifically, a second episode of θ burst stimulation doubled the magnitude of LTP, but only when delivered with a one hour delay after a first train. This phenomenon appears to depend on the presence of a large population of synapses with high plasticity thresholds. Pertinent to the present discussion, a type B ampakine caused a twofold increase in LTP after the first θ burst stimulation, but a subsequent train delayed by one hour produced no further enhancement. In essence, the drug removed the need for spaced trials to produce maximum potentiation. These results make clear predictions regarding behavior: (i) type A ampakines (which do not affect the LTP ceiling) will work within the distributed practice rules to reduce the amount of training needed to achieve stable memory; and (ii) type B compounds will eliminate the spaced trials effect by producing maximal encoding in a single training session.

AMPA receptor modulators and neuropsychiatric disorders

The interaction between the magnitude of modulator effects and network size is expected to produce a greater influence on the cortex than the lower brain. This would result in a kind of “pharmacological encephalization” in which the ampakine-influenced cortex exerts greater than normal regulation of: (i) brainstem and hypothalamic operations; and (ii) ascending projections to the forebrain. Such an outcome has considerable therapeutic potential for the many psychiatric conditions in which aberrant activity in subcortical systems is a contributing factor. Experimental testing of the idea produced encouraging results. Early work showed that acute ampakine treatment suppresses stereotypic activity caused by methamphetamine. Follow-on experiments found that the ampakine increased neuronal activity in the cortex while depressing striatal fields, thereby shifting cortico-subcortical balance in favor of the higher brain region. Excessive dopaminergic activity is widely thought to be a causal factor in schizophrenia, and antagonists of dopamine receptors are the most common treatment for the disease. It is of interest, then, that such compounds act synergistically with ampakines in animal tests. Unusual activity in ascending dopamine projections has also been implicated in attention deficit hyperactivity disorder (ADHD) and here again there is evidence that ampakines have positive effects. The compounds were found to produce dose-dependent suppression of hyperactivity in mice genetically modified to exhibit an ADHD-like syndrome. Those studies were followed by successful phase 2 human trials using the type A ampakine CX717, developed in collaboration with Servier. Notably, a second group has recently reported positive results for attenuating locomotor activity in rats and reducing ADHD symptoms in a human trial.

The pharmacological encephalization hypothesis suggests that enhanced cortical activity produced by ampakines will normalize activity in multiple ascending biogenic amine systems. Perhaps the most interesting test case involves serotonin, a transmitter intimately related to depression. Structurally diverse AMPA receptor modulators are multiply reported to be effective in different rodent models of the disorder. Moreover, synergies with serotonin-based antidepressant medicines are evident: researchers at Eli Lilly found ampakine-like drugs cause a remarkable five- to tenfold leftward shift in dose-response curves for typical and atypical antidepressants in animal models. Significant improvements have been described in at least one (modulator only) clinical study.

Positive modulators and upregulation of brain growth factors

BDNF, a neurotrophin that is anterogradely transported and released from axon terminals, promotes neuronal viability and facilitates learning-related synaptic modifications. Heightened levels of neuronal discharges cause a rapid increase in BDNF transcription, leading to prolonged elevations in pro-
tein levels. These findings suggested that ampakine-mediated increases in excitatory drive would increase BDNF protein levels, an idea confirmed using peripheral injections. Importantly, upregulation was induced by type B variants that have very short half-lives; this is in line with evidence that BDNF transcription responds quickly to increased firing and produces relatively stable products. The following sections consider two classes of potential clinical applications for compounds that increase brain levels of BDNF.

**Intellectual disability and memory loss**

Defects in LTP are found in animal models for each of nine, quite different, cases of intellectual disability or memory impairment so far tested. Although these conditions have different etiologies (e.g., stress, inflammation, aging, gene mutation, etc.), the plasticity loss in each was traced to a shared end point failure in the cytoskeletal machinery responsible for LTP consolidation. There are reasons to suspect that increasing brain BDNF levels will circumvent this failure.

Induction of LTP by 8–100-MHz pattern stimulation activates multiple, small GTPase-initiated signaling pathways that trigger the assembly and later stabilization/elongation of actin filament networks in the zone underlying excitatory synapses. Manipulations that disrupt these events do not affect the initial expression of potentiation, but prevent its stabilization (consolidation) over the 10 to 15 minutes following induction. The actin signaling sequences involved in rapid LTP consolidation are regulated by multiple modulatory receptors found at synapses, prominent among which are tyrosine receptor kinase B (TrKB) receptors for BDNF. As anticipated from this, brief infusions of the neurotrophin potently facilitate the production of LTP in normal animals. They also rescue actin polymerization and LTP in models of late onset Huntington’s disease (HD), Fragile X syndrome, and aging. These encouraging findings set the stage for tests of whether chronic BDNF upregulation can be used as a general therapy for intellectual disability and memory impairment.

Several studies of this type have been conducted using 4 to 5 daily peripheral injections of an ampakine, which, despite a half-life of only several minutes, reliably increases BDNF levels in the hippocampus by 40% to 60%. Restoration of actin signaling and LTP was found in long-term ovarectomized rats and in mouse models of both HD and Angelman syndrome (Figure 3, page 378); LTP recovery has also been described for middle-aged rats. Where tested, learning defects were reduced or eliminated in conjunction with normalization of 8–100-MHz-driven actin filament assembly and LTP consolidation.

Notably, newly published work using small molecule BDNF receptor (TrKB) agonists obtained positive results in rat aging. Collectively, the above results suggest the possibility, using daily treatments of very short duration, of a therapeutic strategy with a broad spectrum of potential neuropsychiatric applications.

**Degenerative diseases and brain damage**

While BDNF potently enhances activity-driven mechanisms underlying memory-related plasticity, it is more widely discussed with regard to positive actions on neuronal viability. The question that naturally arises in the latter context is whether the increased production elicited by positive modulation minimizes neuropathology. Several groups have reported positive tests of this idea. Experiments from Eli Lilly, using chronic peripheral administration, obtained a profound reduction in the degree of dopamine loss within the striatum in a rodent model of Parkinson’s disease, a result that is most readily explained by extensive sprouting of remaining, intact axons. Effects on the integrity of dopamine projections were accompanied by a pronounced improvement in locomotor scores. Of great clinical interest, these normalizing actions were also obtained when drug injections began two weeks after the destruction of dopaminergic neurons in brainstem.

A similar reduction in pathology was obtained in the R6/2 knock-in mouse model of early onset HD. These animals begin to exhibit motor symptoms at 3 to 4 weeks postnatal and then show progressively more intense signs of HD pathology in the striatum. Daily ampakine injections for seven weeks, starting at week three postnatal, substantially reduced multiple markers of the disease, including shrinkage of the striatum. This was accompanied by a striking improvement to near wild-type levels in motor functioning (Figure 4, page 379). Similar reductions in HD pathology in mouse models were recently described using peripheral administrations of a BDNF receptor agonist. Impressive evidence of neuroprotection associated with modulator-induced BDNF elevation was obtained by Servier researchers in a model of excitotoxic brain injury. A parallel study using Servier compounds provided the first evidence that AMPA receptor modulators upregulate BDNF in the neonatal brain, and then showed that this effect is associated with a marked reduction of excitotoxic and inflammatory damage to the cortex. Perinatal brain injury leads to profound clinical problems, and the results just described point to a novel, mechanism-based strategy for treating it.

Stroke is a leading cause of disability in adults, with treatment largely restricted to rehabilitative training. The positive effects of BDNF on learning-related plasticity suggested the possibility of using upregulation to enhance the beneficial effects of postinfarct practice. Positive results with a well-characterized animal model were recently described: daily peripheral injections with an ampakine, at dosages shown to substantially increase EPSPs, elevated cortical BDNF concentrations and markedly improved functional outcomes (motor performance) after six weeks of testing. Critically, the ampakine-induced improvement was dependent upon BDNF: prolonged infusion into the peri-infarct zone of an extracellular BDNF scavenger eliminated the positive effects of drug treatment. This conclu-
sion was reinforced by a demonstration that a type A ampa-
kine did not elevate neurotrophin levels or improve stroke re-
covery. It seems clear from the selection of papers considered
here that upregulating BDNF holds considerable promise for
ameliorating the debilitating effects of brain injury.

Future directions
Despite the relatively short time since the discovery that am-
pakines and allied compounds elevate brain BDNF levels,
there is a now a surprisingly large amount of preclinical liter-
ature concerning the potential utility of the effect in treating
intellectual disability. Missing from this is a description of how
the treatments affect other, seemingly noncognitive compo-
nents of syndromes associated with intellectual disability. How-
ever, a recent study in a mouse strain that exhibits many of the
diagnostic features of autism, showed that daily injections with
a type B compound reduced disturbances in social interaction.
More work of this type is needed to arrive at a reason-
able appraisal of the broad utility of the ampakine/BDNF strat-
egy for treating concomitants of intellectual disability.

This section of the review, dealing as it does with the most severe
of conditions (degenerative diseases and brain injury),
brings translational issues to the forefront. It does not appear
from broadly available reports that any positive modulator has
progressed to phase 3 trials, despite encouraging results in
earlier stages of testing. There are likely to be several reasons
for this. First, type B compounds (ie, those that increase both

Figure 3. Positive modulation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)–receptors rescues actin signaling, learning-related synaptic plasticity, and learning in a mouse model of Angelman syndrome.63
(A) TBS stimulation (TBS), which mimics neuronal activity occurring during learning, is known to cause actin polymerization in dendritic spines. (i) Low background labeling for spines with dense concentrations of polymerized action in hippocampus given low-frequency stimulation (LFS) of inputs. (ii) The number of labeled spines is markedly increased by TBS in wild type (WT) mice. (iii) However, TBS has little, if any, effect on spine actin polymerization in theUBE3A mutant mouse model of Angelman syndrome. (iv) Pretreating the mice with an ampakine (CX929) for 4 days completely restored TBS-driven actin polymerization in the mutants. (B) The positive modulator produced a full rescue of an essential step in producing learning-related synaptic plasticity. (C) As predicted from the actin studies, TBS does not cause stable increases in synaptic strength in vehicle-treated UBE3A–knock-out (KO) mice; four days of ampakine pretreatment restored long-term potentiation (LTP) in the mutants. (D) Angelman syndrome mice are defective in encoding long-term memory in a conventional behavioral task (context conditioning). This impairment is eliminated by the same four-day ampakine treatment that rescued actin signaling and LTP.

Abbreviation: fEPSP, field excitatory postsynaptic potential.
the amplitude and duration of EPSPs are generally more effective across indications and for elevating BDNF than type A variants (ie, those that affect amplitude only), but are more likely to cause seizures. Developing versions of these type B compounds with an acceptable safety margin may have been a major barrier to translation. Second, as is not uncommon, promising drugs can have side effects unrelated to their primary mode of action; this seems to be the case for an ampakine that proved effective in phase 2 ADHD work. Third, in view of the many potential indications being discussed, it is difficult to arrive at general conclusions regarding tolerable side effects, required efficacy levels, and safety margins; results for one situation may not apply to others. Additional points relevant to translation are noted below.

Final comments
The large body of work on positive AMPA receptor modulators establishes the perhaps surprising point that increasing the strength of fast excitatory transmission does not disturb a broad range of brain operations. It does, however, produce a number of beneficial effects either directly, via improved neuronal communication, or indirectly through increased growth factor production. Despite the many studies documenting these points, the field cannot be viewed as being well structured; put simply, it lacks broadly accepted guidelines and fundamental principles. A prominent example of this can be seen in the absence of data on how large an increase in EPSPs (amplitude and/or duration) is needed to produce a targeted functional outcome. Currently, efficacy is considered

Figure 4. Chronic treatment with an ampakine reduces pathology and restores motor functioning in a mouse model of early onset Huntington’s disease (HD).

R6/2 transgenic mice, overexpressing a fragment of mutant huntingtin protein, were given 7 weeks of daily vehicle (veh) or ampakine injections beginning at 3 weeks postnatal, when HD symptoms first appear. (A) The ampakine (CX929) treatments substantially elevated concentrations of mature brain-derived neurotrophic factor (mBDNF) in the striatum, the primary region showing HD-related pathology in the mutants. (B) A key feature of HD is the accumulation of huntingtin protein aggregates (Htt) in striatal neuronal nuclei (arrows). This effect is substantially reduced by chronic administration of the drug. (C) Another characteristic of HD is a sizable loss of the kinase, dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32), from striatal neurons. As shown, the 7-week treatment with the positive modulator restored striatal DARPP-32 in R6/2 mice to normal levels. (D) An image of a coronal section through mouse forebrain shows the striatum densely labeled with acetylcholinesterase histochemistry. HD reduces striatal volume, an effect that is also evident in the R6/2 mouse by postnatal week 10. The ampakine (weeks 3 to 10) blocked shrinkage, yielding near wild type (WT) mean area measures. (E) R6/2 mice have a severe motor deficit in a “pole descent” test. The upper panel shows mean results for a set of descent attempts; note that chronic drug treatment caused a dramatic improvement in the mutants. Trial-by-trial analysis showed that vehicle-injected R6/2 mice (open circles) do not improve with practice; mutants that received the drug (green filled circles) were indistinguishable from WTs (boxes) at the end of testing.

only in terms of conventional dose-response curves with no discussion of neurobiological mechanisms, despite there being a clear primary mode of action. Discrepancies between effective doses for learning vs EPSP enhancement would have a major impact on further development of a candidate modulator, because it would point to the presence of unknown factors that regulate outcome potency.

There are also multiple reasons (eg, variations in AMPA receptor composition), and some evidence, to assume that ampakine variants are differentially effective across brain systems. This constitutes a major barrier to the rational design of compounds intended for particular indications, since a drug could be exerting its greatest effects outside areas responsible for the desired effect. What seems called for is a type of regional “mapping” enterprise, perhaps using activity-dependent gene expression, to identify brain regions most affected by a candidate agent. Routine construction of such a map, during drug screening, was not a realistic possibility in the recent past, but advances in computer technology and the advent of automated microscopes suggest that it is now entirely feasible. Note that maps would not only help explain differential behavioral effects, but could also point to unsuspected applications. Importantly, emerging methods not only allow for the analysis of regional actions, but also effects on interneuron-projection cell connections that constitute local circuits. Such information will likely prove critical for identifying agents that increase network throughput without greatly increasing the likelihood of seizures.

There is also considerable confusion about the duration of drug action needed to initiate BDNF production. Much of the work with animal models used an ampakine with a half-life of less than 15 minutes, and yet caused substantial increases in BDNF and positive effects in several animal models of intellectual disability. This raises the possibility that minimizing drug half-life might be a useful step towards reducing side effects of type B compounds. To conclude, while much has been accomplished, further progress will likely benefit from recognition of the unique complexities created by increasing excitatory transmission and the implementation of new analytical tools.

G ary Lynch received his doctorate from Princeton University and then moved to the University of California, Irvine, where he is currently a Professor (Above-Scale). His early work produced foundational discoveries concerning two aspects of brain plasticity: growth after damage (axon sprouting) and learning-related synaptic modifications (long-term potentiation [LTP]). A search for drugs to enhance LTP and memory led to the invention of the first peripherally administered compounds (ampakines) to selectively enhance communication in cortical networks. Subsequent collaboration with Professor Gall revealed that ampakines increase transcription of a potent brain growth factor. Studies from their laboratories, and elsewhere, have shown that ampakines and closely related drugs have positive effects in animal models of psychiatric disorders and neuropathological conditions. Professor Lynch has recently described the synaptic chemistry responsible for LTP, and has shown that it is engaged by learning and is defective in many animal models of intellectual disability.

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Keywords: ADHD; ampakines; brain-derived neurotrophic factor; depression; glutamate receptors; hippocampus; intellectual disability; learning; long-term potentiation; synaptic plasticity

AMPAR receptor modulators: promises and problems – Lynch and Call

Improving cognition and neuroplasticity

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MODULATION DU RÉCEPTEUR AMPA POUR STIMULER LA PLASTICITÉ ET TRAITER LES PROCESSUS NEUROPATHOLOGIQUES

Les modulateurs positifs allostériques des récepteurs au glutamate AMPA (∅-amino-3-hydroxy-5-méthyl-4-isoxazole-propionate), les « ampakines » et composés fonctionnellement reliés, représentent une classe relativement nouvelle de médicaments psychoactifs qui favorisent une transmission cérébrale rapide des signaux synaptiques excitatoires. En raison de cet effet, les ampakines diminuent le seuil d'induction des modifications synaptiques liées à la mémoire et améliorent l’apprentissage parmi les espèces et les modèles animaux. Alors que la plupart des médicaments du système nerveux central agissent sur les neurones qui se projettent avec une seule connexion, de façon parallèle vers une multitude de sites, ceux du type ampakine agissent sur les nombreuses connexions des réseaux cérébraux en série. Ceci a pour conséquence de multiplier les effets du médicament, qui tendront de ce fait à plus prononcés dans les circuits corticaux complexes, intensifiant donc la régulation corticale induite par les aires cérébrales d’aval (« encéphalisation pharmacologique »). L’efficacité avérée de ces agents dans les modèles animaux de troubles psychiatriques associés à une activité anormale du tronc cérébral conforte cette hypothèse. Des réseaux corticaux étendus pourraient stimuler la fonction cognitive, et non mnésique, des cerveaux normaux mais cette éventualité est peu étudiée. Enfin, des modulateurs positifs augmentent la production cérébrale de facteurs de croissance favorisant la plasticité et la viabilité neuronales ; une régulation positive s’associe à la croissance, à la neuroprotection, et à des résultats fonctionnels améliorés dans différents modèles lésionnels.
Diabetes
Innovation-driven partnerships

IMIDIA: a precompetitive consortium for the β cell
B. Thorens, Switzerland
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The role of TXNIP in the pathophysiology of diabetes and its vascular complications: a concise review
G. Leibowitz, A. Ktorza, and E. Cerasi, Israel and France
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In an absolute or relative deficit in insulin secretion underlies the pathogenesis of type 1 and type 2 diabetes, respectively. Restoring normal pancreatic \( \beta \)-cell insulin secretion capacity and preventing the demise of \( \beta \) cells are, therefore, major goals to improve treatment or prevent development of diabetes. Although \( \beta \)-cell physiology has been investigated for decades, there is still insufficient knowledge of the metabolic, signaling, and differentiation pathways that control \( \beta \)-cell plasticity and failure in adult life. This stems largely from the relative scarcity of \( \beta \) cells, which limits classic cell biological and biochemical investigations, and from the lack of satisfactory imaging techniques to assess their mass and function in living individuals. There is thus an urgent need to develop new tools and experimental approaches to investigate \( \beta \)-cell function and mass. These include fundamental investigations on molecular mechanisms controlling \( \beta \)-cell plasticity, the development of human \( \beta \)-cell lines, the identification of plasma biomarkers of \( \beta \)-cell function and response to drug treatments, and new imaging techniques to monitor \( \beta \)-cell mass and function in vivo. Because of the formidable complexity of the task, no single laboratory or industry can hope to independently achieve these goals. The Innovative Medicine Initiative for DIAbetes (IMIDIA) project is a precompetitive consortium of 15 academic laboratories, 1 small and medium enterprise (SME) and 8 major pharmaceutical companies, which has been set up to work in a highly integrated and coordinated manner to address these key challenges.


da) Within IMIDIA, innovative imaging markers are being developed with the help of chemists. For example, chemists are developing new derivatives of sulfonylureas and the gluco-incretin hormone glucagon-like peptide 1 (GLP-1), as well as molecules sensitive to Zn\(^{2+}\), an ion secreted by \( \beta \) cells at the time of insulin granule exocytosis. These probes are used for optical and magnetic resonance imaging (MRI), and positron emission tomography (PET).

b) Insulin action on liver, muscle, and fat controls glucose absorption and utilization to generate metabolic energy and precursors for biosynthetic reactions, such as nucleotides synthesis, or for conversion of glucose into glycogen or fat. Insulin is also required to block hepatic glucose production. Insulin resistance, ie, a progressive decline in the efficacy of insulin to control these essential actions, occurs in conditions such as obesity, pregnancy, or aging. Unless pancreatic \( \beta \) cells compensate for insulin resistance, by increasing their insulin secretion capacity, hyperglycemia will develop. This compensation process involves not only an increase in insulin secretion capacity by individual \( \beta \) cells, but also an augmentation of their total number, which can arise from replication of mature \( \beta \) cells, differentiation from progenitors, or transdifferentiation of \( \alpha \) or even exocrine cells into \( \beta \) cells. This adaptation capacity is, however, limited, and if insulin resistance continues to develop, insulin secretion becomes insufficient, leading to the onset of diabetic hyperglycemia.

IMIDIA: a precompetitive consortium for the \( \beta \) cell

by B. Thorens, Switzerland
What are the intracellular signaling, metabolic, and differentiation pathways that control β-cell plasticity and failure in type 2 diabetes? The β-cell mass present in the pancreas of healthy individuals can increase to compensate for the development of insulin resistance and to maintain normoglycemia. As insulin resistance continues to develop, β cells reach a stage where they can no longer increase their total insulin secretion capacity. This leads to the development of hyperglycemia. The combination of hyperglycemia (and higher plasma free fatty acids released from insulin-resistant adipocytes) may precipitate a decrease in the secretion capacity of the β cells and their progressive death by apoptosis. Unpublished data.

Objectives of the IMIDIA precompetitive network
Recognizing the importance of a β-cell–centered investigation program, the EU and the European Federation of Pharmaceutical Industries and Associations (EFPIA) created a public–private partnership (PPP) project to address key bottlenecks in β-cell research. This PPP would bring together academic laboratories and small and medium enterprises (SMEs), in collaboration with the pharmaceutical industry. A call for application was launched in 2009, asking academic networks to propose a specific action plan to address a set of key β-cell–related questions. The IMIDIA academic network was selected to complete a full proposal in close interaction with representatives from eight major pharmaceutical companies. The IMIDIA precompetitive network was officially launched on February 1st, 2010. This 5-year project integrates the research activities of more than 100 collaborators in academia, one SME, and the participating pharmaceutical companies with a global budget of ≈25 million euros (Figure 2, page 386; refer to footnote for a full list of participants and general goals).

Specific issues related to the study of adult β-cell plasticity
Progress towards elucidating the molecular basis of adult β-cell plasticity and failure in type 2 diabetes is faced with several difficulties, due to unique β-cell specificities.

- First, their relative scarcity. Indeed, pancreatic islets represent ≈1% of the total mass of the pancreas and β cells only ≈70% of the islet cells. This represents an important limitation when working with rodent islets and an even greater one when working with human islets, which can only be obtained from autopic pancreas or infrequently from biopsy material obtained after elective pancreas surgery, usually for tumor removal. Thus, identification of key intracellular signaling, metabolic, and differentiation pathways using classic cell biological and biochemical techniques is very limited, and must be combined with alternative approaches, such as genetic, genomic, and metabolomic techniques.

- Second, identification of plasma biomarkers, for monitoring β-cell mass and function in diabetes and during therapeutic treatment, requires the availability of cohorts of individuals who...
have progressed from a normal state to type 2 diabetes, and who have been well phenotyped during the pathogenic progression.

Third, the distribution of the endocrine pancreas in small islets, consisting of 1-2000 cells, scattered in the exocrine pancreas, imposes particular difficulties in developing contrasting agents and imaging modalities that can specifically and precisely identify these cells and assess their functions.

These intricate questions need to be approached in a global, coordinated manner, requiring diverse experimental approaches and technical developments. The IMIDIA network has selected to work along the following lines:

- Develop new knowledge about the mechanisms controlling adult β-cell function, expansion, and death.
- Establish new tools for β-cell research, in particular new, functionally validated, human β-cell lines.
- Identify plasma biomarkers diagnostic of β-cell function and deregulation in the pathogenesis of type 2 diabetes and in response to treatment.
- Develop new imaging techniques for in vivo monitoring of β-cell mass and function in diabetes and upon therapeutic treatments.

Specific research projects

Undoubtedly, the major benefit of the IMIDIA consortium was the possibility to address multiple questions and to develop new investigative tools related to β-cell biology, using broad-based technological approaches contributed to by both academic and pharmaceutical partners in a highly synergistic way. Some of the ongoing projects have been presented at a meeting preceding the European Association for the Study of Diabetes (EASD) annual meeting in Barcelona (September 2013), where the three Innovative Medicines Initiative (IMI) diabetes networks (IMIDIA, SUMMIT [SUrrogate markers for Micro- and Macrovascular hard end points for Innovative diabetes Tools], and DIRECT [DIabetes REsearch on patient stratifiCaTion]) highlighted their key progress.

- A systems biology approach to discover novel genes, gene regulatory pathways, and biomarkers of functional β-cell mass

In order to obtain a new view of the functional architecture of β cells in health and in response to metabolic stress, a large Systems Biology approach was undertaken. This consisted of two investigative arms: one focused on mouse islets and the other on human islets. Both arms proceeded independently until the stage of bioinformatics analysis of the collected data, where comparative analysis of mouse and human data reinforced each other in the quest for new essential information about β-cell biology.

In the animal study, mice from different genetic backgrounds were fed a regular or high fat diet for different periods of time (2, 10, 30, and 90 days; six different inbred strains of mice were chosen because they differentially adapt to metabolic stress, allowing information on the interaction between genetics and nutrition to be obtained). Each mouse was then fully genotyped for basic parameters such as glycemia and insulinemia, but also for glucose and insulin tolerance, and their β-cell mass was assessed. In parallel, lipidomic analysis was performed to determine the plasma concentration of a few hundreds lipid species and the lipid composition of isolated islets; islet gene expression was determined by RNA sequencing analysis, a technique providing highly accurate and very extensive information about which genes are expressed in islets and at which level.

A critical aspect of these studies was that each piece of data generated for each mouse was deposited, with a unique identification tag, in a database organized and maintained by partners of the Swiss Institute of Bioinformatics. These data could be interrogated in a global manner to find correlations between the measured phenotypes and the gene expression profile of islets, or the particular lipid composition of the plasma. Two types of information have been extracted: (i) the composition of new modules (groups) of β-cell genes whose expression correlates with any of the measured phenotypes;
and (ii) identification of modules of plasma lipids, which correlate with a particular β-cell phenotype. Modules of gene expression have been identified, which highlight the involvement of so far unsuspected signaling or metabolic pathways with, for instance, insulin secretion capacity or glucose intolerance (Figure 3). These data are now being validated using basic cell biological studies on insulin cell lines or primary islets; these studies confirm that our Systems Biology approach is extremely powerful in generating new discoveries that can hopefully lead to new molecular targets to improve β-cell plasticity.

On the other hand, the plasma lipidomic analysis reveals tight correlation between the concentration of a small group of lipids, with insulin secretion and glycemic control. These studies may lead to identification of novel biomarkers diagnostic of β-cell function. Confirmation of these initial experiments in human plasma samples is now required, a task that is again facilitated by the availability, within the IMIDIA consortium, of longitudinal cohorts of individuals who have developed type 2 diabetes, and for whom plasma samples have been collected before and after the development of diabetes.

In the second arm of this Systems Biology approach, a unique European network has been established to isolate and characterize human islets using highly standardized methods. These islet preparations, which come from healthy individuals and type 2 diabetic patients, have been analyzed for gene expression profiling, lipidomic analysis, and the presence of diabetes susceptibility genes. Global analysis of gene expression modules in islets from humans and mice is now providing a unique view on the functional organization of β cells in health and disease, and provides an extraordinary new base for the discovery of new targets for β-cell–centered therapy for diabetes and for the identification of biomarkers for monitoring β-cell mass and function.

**Human β-cell lines**

Rodent insulin-secreting cell lines have been available for several decades. Even though they differ in many respects from primary β cells, they have been invaluable for the study of various aspects of β-cell biology, including insulin biosynthesis and the glucose signaling pathway that controls insulin secretion. In contrast, human insulin cell lines have been much more difficult to obtain, since the transgenic approach used to express proliferation-inducing genes in mouse β cells could not be used in human. An alternative strategy used by IMIDIA researchers led to the first successful development of human cell lines (Figure 4, page 388). This approach was based on the transplantation, under the kidney capsules of immunoincompetent mice, of human fetal islet cells transduced with a transforming oncogene. This success was acclaimed as a major breakthrough in human β-cell research, which could have major implications in the development of new drugs controlling insulin secretion and β-cell proliferation. The effort to establish and validate this first cell line as well as the ongoing development of second-generation lines has benefited from a unique combination of academic and private research performed in a SME. IMIDIA is now providing further support for validation of these cell lines by several pharmaceutical partners, to establish them as industry-relevant research tools.

**Centralized database**

From the planning stage of IMIDIA’s activities, it was recognized that for a successful integration of all network activities, strong database management and bioinformatics supports were essential. Indeed, to identify novel genes and gene networks controlling β-cell plasticity, to find novel biomarkers of β-cell function, and to be able to compare data obtained in animal models and in human tissues, it is essential that all data generated throughout the network are stored in a centralized database in a format that allows their retrieval for global bioinformatics analysis. This was achieved by creating unique identifiers for each data (individual mice, islet preparation, RNASeq data, phenotype of donors for human islets, plasma lipidomic, glucose tolerance tests, etc).

This initial effort to uniquely label each piece of data has been critical for the bioinformatics analysis. As mentioned above, the first goal was to generate a new view of β-cell gene net-
works that are involved in the control of their function, or failure to adapt to metabolic stress. Gene modules have now been discovered that are associated with phenotypes such as insulin secretion, oral glucose tolerance, insulin resistance, body weight, etc. In each of these modules, genes with highest impact on the module classification have been identified, and in many cases these can be placed in a gene interaction network, allowing identification of genes with important hub positions, and thus probably critical function in β-cell adaptation to metabolic stress.

Importantly, crossing mouse and human islets expression data allows the finding of genes with similar hub positions in both species, further supporting their functional importance. These genes are now actively being investigated in a coordinated manner by several IMIDIA laboratories.

Figure 4. Characterization of the human β-cell line EndoC-βH1.
Left: Immunofluorescence microscopy analysis insulin (Ins) and C-peptide (C-PEPT), the β-cell–specific transcription factors PDX1 and NKX6-1. Right: Insulin secretion by EndoC-βH1 cells exposed to indicated glucose concentrations and to GLP-1 (glucagon-like peptide 1), glibenclamide (Gli), the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), diazoxide (DZ), or leucine (Leu).

Figure 5. Different mouse islet imaging modalities.
(A) Immunofluorescence microscopy detection of insulin (Ins; green) and glucagon (Glu; red) in sections of pancreas from a control mouse or from a mouse treated with diphtheria toxin to ablate β cells (these mice express a transgenic diphtheria toxin receptor in β cells). (B) Blood glucose levels from control- and diphtheria toxin–treated mice. (C) Luminescence emission from β cells expressing a transgenic luciferase gene, without (control) or after (DT-treated) diphtheria toxin treatment.
Imaging the β cells

One critical shortcoming in the assessment of β-cell plasticity is the lack of suitable in vivo imaging techniques to accurately determine β-cell mass and secretion activity. This precludes the real appreciation of the relative contribution of β-cell number and function in the progression towards type 2 diabetes and the impact of pharmacological or lifestyle interventions on β-cell function.13,15

Development of imaging modalities is therefore an area of intense research across the world. Because of the complexity in developing appropriate β-cell imaging markers that can be used with various imaging modalities, and because these techniques need to be applied to humans, the cost of development and the expertise required need a highly integrated approach, which can be best obtained by combining academic and pharmacological partners.

Within IMIDIA, innovative imaging markers are currently being developed. For example, chemists are developing new derivatives of sulfonylureas and the gluco-incretin hormone glucagon-like peptide 1 (GLP-1), as well as molecules sensitive to Zn2+, an ion secreted by β cells at the time of insulin granule exocytosis.14,16-18 These probes are used for optical and magnetic resonance imaging (MRI), and positron emission tomography (PET); see Figures 5 and 6 for examples.16,18

They are tested in cellular and animal models available in different laboratories, and the successful ones will be tested in humans, as already occurs as part of some of IMIDIA’s partner activities.

General considerations

There is a challenge in establishing a large-scale academic-pharmaceutical partnership such as IMIDIA. This requires both parties to be willing to work beyond their usual comfort zones. IMIDIA has demonstrated that this is, however, not only possible, but also highly productive. The examples mentioned above illustrate that completely new avenues of research and tool development are being successfully pursued. The mass of data generated, and the new tools developed will feed years of productive research. The challenge, which is now addressed, is to fully integrate this new knowledge into translational research for improved diabetes treatment. With the planning of a new wave of Innovative Medicine Initiative projects by the EU, we trust that sufficient resources will be made available to fruitfully conduct this translational effort.

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Footnote: The IMIDIA consortium is coordinated by Sanofi-Aventis (Werner Kramer), Servier (Alain Ktorza), and the University of Lausanne (Bernard Thorens). The other pharmaceutical partners are: AstraZeneca, Boehringer Ingelheim, F. Hoffmann-La Roche, Lilly Deutschland, Novo Nordisk A/S, and Novartis Institute. The academic partners are: CEA/Institut d’Imagerie Biomédicale, CNRPS UMR 7001, CNRS-University Paris Diderot, Dresden University of Technology, Hannover Medical School, Imperial College London, INSERM U845, Swiss Institute of Bioinformatics, University of Geneva, University of Pisa, Vrije Universiteit Brussel, and the small and medium enterprise (SME) ENDOCELLS, Paris.

Bernard Thorens is a professor at the Center for Integrative Genomics of the University of Lausanne. He studied biochemistry at the University of Geneva where he received his PhD. He did a postdoctoral fellowship at the Whitehead Institute for Biomedical Research at the Massachusetts Institute of Technology, Massachusetts, USA. He then returned to Switzerland to join the University of Lausanne. His laboratory focuses on the molecular control of glucose homeostasis with, in particular, the study of gluco-incretin hormone action, and the role of glucose sensing in the central nervous system in the control of pancreatic islet function. He is part of the coordination team of the Innovative Medicine Initiative for DIAbetes (IMIDIA).

References

Innovating in type 2 diabetes

Keywords: \( \beta \) cells; biomarkers; imaging; lipidomic; signaling pathways; systems biology; transcriptomic; type 2 diabetes


**IMIDIA : UN CONSORTIUM PRÉCONCURRENTIEL POUR LA CELLULE \( \beta \)**

Un déficit absolu ou relatif de sécrétion d’insuline préside à la pathogenèse respectivement des diabètes de type 1 et 2. La restauration d’une capacité de sécrétion d’insuline pancréatique \( \beta \)-cellulaire normale et la prévention de la mort des cellules \( \beta \) sont donc des objectifs majeurs pour améliorer le traitement ou éviter le développement d’un diabète. La physiologie \( \beta \)-cellulaire est étudiée depuis des décennies, mais la connaissance des voies métaboliques, des voies de signalisation et des voies de différenciation qui contrôlent leur plasticité et leur insuffisance chez les adultes est toujours incomplète. Cette situation résulte principalement de la relative rareté des cellules \( \beta \), ce qui limite les recherches classiques de biologie et de biochimie cellulaires, ainsi que du manque de techniques d’imagerie satisfaisantes pour évaluer leur masse et leur fonction chez l’être vivant. Il devient donc urgent de développer des outils et approches expérimentales nouveaux pour étudier la fonction et la masse des cellules \( \beta \). Ceci passe par la recherche fondamentale sur les mécanismes moléculaires contrôlant la plasticité \( \beta \)-cellulaire, le développement de lignées humaines \( \beta \)-cellulaires, l’identification des biomarqueurs plasmatiques de la fonction \( \beta \)-cellulaire et de la réponse aux traitements médicamenteux et l’instauration de nouvelles techniques d’imagerie pour contrôler la fonction et la masse des cellules \( \beta \) in vivo. En raison de l’énorme complexité de la tâche, aucune industrie ou laboratoire ne peut espérer réaliser ces objectifs isolément. Le projet IMIDIA (Innovative Medecine Initiative for DIAbetes) est un consortium « préconcurrentiel » de 15 laboratoires universitaires, d’une petite et moyenne entreprise (PME) et de 8 grands laboratoires pharmaceutiques, réunis pour travailler ensemble de façon complémentaire et coordonnée pour relever ces défis.
The role of TXNIP in the pathophysiology of diabetes and its vascular complications: a concise review

by G. Leibowitz, A. Ktorza, and E. Cerasi, Israel and France

Type 2 diabetes (T2D) results from failure of the pancreatic β cell to cope with the increased insulin demand of nutrient overload and obesity-associated insulin resistance. Although many studies in the 1960s demonstrated clearly diminished insulin secretion in T2D patients, for a long period thereafter the role of the β cell in the pathogenesis of diabetes was regarded as controversial at best, more often as nonexistent. However, numerous studies in more recent decades have convincingly shown that β-cell dysfunction is the driving force of the metabolic disturbance both in the initiation and the progression of T2D. β-Cell dysfunction in diabetes is multifactorial, involving both genetic and environmental factors. The latter include hyperglycemia and elevated levels of free fatty acids (FFA) and inflammatory cytokines, all factors that impair mitochondrial and endoplasmic reticulum (ER) function, leading to mitochondrial and ER stress. Accumulation of reactive oxygen species (ROS), ie, oxidative stress, can cause ER stress, and vice versa, thus generating a vicious feedforward cycle which impinges on β-cell function, survival, and differentiation. Figure 1 (page 392) presents a schematic illustration of these events.

The β cell is particularly prone to develop oxidative stress, due to low activities of catalase, selenium-dependent glutathione peroxidase 1 and Cu/Zn-superoxide dismutase 1. By contrast, the NADPH-dependent oxidoreductase thioredoxin is abundant, which suggests that it has an important role in β-cell defense against cellular stress. Thioredoxin partners with thioredoxin reductase and thioredoxin peroxidase to reduce oxidized proteins and scavenge ROS.
Thioredoxin-interacting protein (TXNIP), a member of the arrestin family, binds to the redox-active cysteine (Cys) residues of thioredoxin and inhibits its oxidoreductase activity, thus functioning as an endogenous inhibitor of thioredoxin. Importantly, in β cells, as well as in other tissues, TXNIP expression is robustly induced by glucose; hence, it may play a central role in the pathophysiology of T2D and its vascular complications. Indeed, TXNIP has been implicated in β-cell apoptosis in T2D, diabetic microvascular complications, and atherosclerosis, which is the leading cause of death among diabetic patients. In this concise review, we discuss the regulation of TXNIP in diabetes and its impact on β-cell function, glucose homeostasis, and vascular complications. We also evaluate the implications of these findings for envisaging future diabetes therapies.

TXNIP structure and function

TXNIP, also known as vitamin D3–upregulated protein-1 (VDUP-1) and thioredoxin-binding protein-2 (TBP-2), is a 50-kDa protein that was originally discovered by yeast 2-hybrid screen for thioredoxin-interacting protein. An intramolecular disulfide bond between Cys63 and Cys247 in TXNIP is susceptible to disulfide exchange with reduced thioredoxin at Cys247, leading to generation of stable mixed disulfide bonds; this inhibits the ability of thioredoxin to scavenge ROS and to interact with other signaling molecules. The crystal structure of the thioredoxin-TXNIP complex shows that upon binding to thioredoxin, TXNIP undergoes a structural rearrangement that involves intermolecular switching of disulfide domains between different TXNIP molecules that eventually leads to de novo intermolecular interactions between TXNIP Cys247 and the active site of thioredoxin at Cys32.15

**Figure 1.** Mechanisms of β-cell stress in type 2 diabetes.

In type 2 diabetes, the β cells are exposed to a noxious environment including hyperglycemia, elevated free fatty acids, and pro-inflammatory cytokines, all leading to cellular stress. This is, in part, the consequence of excessive reactive oxygen species (ROS) production, in turn leading to mitochondrial fragmentation and impairment of adenosine triphosphate (ATP) production. Alterations in the redox state, as well as other poorly defined mechanisms, also lead to morphological changes in the endoplasmic reticulum (ER) that are associated with impaired protein folding, leading to ER stress. Impaired mitochondrial and ER functions culminate in β-cell dysfunction and apoptosis, with worsening of diabetes as a consequence. Thioredoxin (TXN) is the main antioxidant of β cells, regulating its adaptation to stress. TRX is inhibited by TRX-interacting protein (TXNIP), whose expression is markedly stimulated by high glucose concentrations.

**Figure 2.** Transcriptional regulation of the Txnip gene.

Glucose stimulates the translocation of carbohydrate-response-element-binding protein (CHREBP) or Mondo from the cytosol to the nucleus and its binding to the carbohydrate response element (ChoRE) of the Txnip promoter, in complex with Max-like protein X (MLX). Several other factors participate in the activation of transcription (see text for details). Insulin, cyclic adenosine monophosphate (cAMP), as well as AMP-activated protein kinase (AMPK) signaling pathways exert negative actions on Txnip transcription (blue arrows). The inhibition of Txnip involves also the activation of FOXO.

**Abbreviations:** AMPK, AMP-activated protein kinase; cAMP, cyclic adenosine monophosphate; ChoRE, carbohydrate response element; CHREBP, carbohydrate-response-element-binding protein; CREBP, cAMP-response-element-binding protein; FOXO, forkhead transcription factor; GLP-1, glucagon-like peptide-1; GTF, general transcription factors; HAT, histone acetyltransferase; HDAC, histone deacetylase; MLX, Max-like protein X; NF-Y, nuclear factor Y; P3 kinase, phosphatidylinositol 3-kinase; PKB/Akt, protein kinase B (also known as Akt); Pol II, RNA polymerase II; TBP, TATA-binding protein.

**Selected Abbreviations and Acronyms**

- cAMP: cyclic adenosine monophosphate
- Cys: cysteine
- ER: endoplasmic reticulum
- FFA: free fatty acids
- Glut-1: glucose transporter 1
- ROS: reactive oxygen species
- T2D: type 2 diabetes
- TXNIP: thioredoxin-interacting protein
TXNIP is a member of the α-arrestin–protein superfamily that is involved in receptor recycling and endocytosis. The molecular function of the α-arrestin domain of TXNIP, as well as of other proteins belonging to the family, is currently unknown. TXNIP has important effects on glucose homeostasis that are independent of its binding to thioredoxin.11 TXNIP inhibits glucose uptake, including in cells unresponsive to insulin.12 This effect is mediated through enhanced endocytosis of glucose transporter 1 (GLUT1) by plasma membrane–localized TXNIP and by inhibition of GLUT1 transcription.13 In addition to inhibiting glucose uptake, TXNIP may also modify intracellular glucose metabolism; indeed, TXNIP deficiency reduces mitochondrial oxidation while promoting glycolysis,14 thus potentially protecting cells against oxidative stress.

Regulation of TXNIP expression in diabetes
Glucose is the most potent physiological regulator of TXNIP expression. Glucose regulates production of TXNIP by increasing the binding of the transcription factor carbohydrate-response-element–binding protein (ChREBP) to the Txnip promoter, with recruitment of histone acetyltransferase p300 and histone H4 acetylation, thereby stimulating Txnip transcription (Figures 2 and 3).15 Consistently, TXNIP expression was markedly increased in islets of different animal models of T2D. To give an example, in Psammomys obesus, an animal model of nutrition-induced diabetes, TXNIP was undetectable in the islets of normoglycemic animals. When the animals were fed a high-energy diet, they rapidly developed hyperglycemia, which was then accompanied by a rapid and strong induction of TXNIP; this persisted throughout the course of the disease (Figure 3).16 Furthermore, when islets of normoglycemic animals were incubated in vitro, increasing glucose concentrations markedly augmented the expression of TXNIP; this was associated with a strong stimulation of Txnip transcriptional activity (Figure 4).16 In human islets, the expression of TXNIP was robustly increased when incubated in the presence of a high glucose concentration.17 Similarly, TXNIP expression was elevated in muscle tissue of human subjects with impaired glucose tolerance or diabetes.18 No association was found between common genetic variations in the TXNIP gene and T2D, and its expression was not increased in normoglycemic subjects with a family history of diabetes, suggesting that TXNIP is increased in response to glucose dysregulation, rather than being a genetic trait of diabetes.

In contrast to glucose, insulin suppresses TXNIP expression in peripheral tissues (muscle and fat) and in β cells.16,18 In T2D, hyperglycemia along with insulin deficiency and insulin resistance may thus be expected to further increase TXNIP expression, and hence markedly exacerbate the oxidative stress of the diabetic state.

Role of TXNIP in the β-cell dysfunction of T2D
Recent studies have shown that TXNIP is a crucial factor in β-cell biology, and its upregulation is one of the key events leading to β-cell dysfunction and apoptosis in diabetes. β-Cell mass in TXNIP loss–of–function mutant mice was increased, and consequently the mice were resistant to the diabetes–inducing effect of streptozotocin (a drug which destroys β cells).19 Moreover, intercrossing TXNIP-deficient mice with an animal model of diabetes (ob/ob mice) prevented the appearance of hyperglycemia and β-cell apoptosis, producing instead a 3-fold increase in β-cell mass.19 TXNIP-deficient islets, and β cells in which TXNIP was knocked down, were completely protected from the apoptosis–inducing effect of high glucose.
concentrations. These findings indicate that TXNIP is critical for diabetes-induced β-cell apoptosis. The mechanism of action of TXNIP includes shuttling of the molecule within the β cell between the nucleus and the mitochondria, where it promotes apoptosis by activating the mitochondrial apoptotic pathway.

In addition to the proapoptotic effects of TXNIP, it also impairs β-cell function, and might therefore be an important mediator of the deleterious effects of hyperglycemia (glucotoxicity) on insulin production and secretion. Of note, overexpression of TXNIP in β cells repressed the expression of genes regulating insulin secretion. Recently, TXNIP has been reported to induce the expression of microRNA 204 (miR-204), which inhibits insulin production by directly targeting and downregulating MAFA, a well-established transcription factor involved in proinsulin gene transcription. Previous reports showed that decreased MAFA expression is responsible for inhibited insulin production in states of hyperglycemia and high FFA (glucolipotoxicity). Thus, the TXNIP–miR-204–MAFA sequence constitutes a novel pathway in the pathogenesis of diabetic β-cell dysfunction.

Finally, in diabetic β cells, ER stress generates a proinflammatory response mediated by the nucleotide-binding domain, leucine-rich-repeat–containing family, pyrin domain–containing 3 (NLRP3) inflammasome, which contributes to β-cell dysfunction and apoptosis in diabetes, both type 1 and type 2. Importantly, TXNIP has been reported to serve as a critical link between ER stress and inflammation, further supporting its prime role in mediating the deleterious effects of the diabetic environment on the β cell. Figure 1 summarizes this section.

TXNIP regulation of insulin sensitivity and hepatic glucose production

In healthy individuals, TXNIP expression is inversely correlated with total body glucose uptake, suggesting that it may have a role in regulating insulin sensitivity. Indeed, forced expression of TXNIP in cultured adipocytes significantly reduced glucose uptake, while silencing it with RNA interference in adipocytes and in skeletal muscle enhanced glucose uptake, confirming that TXNIP exerts a detrimental effect on glucose transport. Interestingly, TXNIP expression was elevated in ovarian granulosa cells and in the serum of insulin-resistant women with polycystic ovary syndrome, further supporting its role in regulating insulin sensitivity in human disease.

Noteworthy, TXNIP also regulates hepatic glucose production: HcB-19 mice, which harbor a missense mutation in the TXNIP gene, exhibit fasting hypoglycemia. Similarly, conditional knockout of TXNIP in the liver led to lower fasting blood glucose concentrations and impairment of hepatic glucose production. Furthermore, forced expression of TXNIP in the liver of normal mice resulted in enhanced glucoseogenesis, along with insulin resistance and impaired glucose tolerance.

Figure 5. Expected effects of reduction in thioredoxin-interacting protein (TXNIP) expression on several vascular cell functions. See text for details.

This was probably mediated by increased expression of the key gluconeogenic enzyme glucose-6-phosphatase and decreased expression of the glycolytic regulator glucokinase. These observations have important implications for the pathophysiology of T2D, as augmented hepatic glucose production is among its fundamental defects, and is considered a determining factor for fasting hyperglycemia in this disease.

Altogether, it is now established that TXNIP is increased in diabetes and has multiple detrimental metabolic effects, including impairment of insulin production and secretion and increased insulin resistance in peripheral tissues and the liver, thus leading to exacerbation of the metabolic disorder in T2D.

The role of TXNIP in vascular complications of diabetes

TXNIP has been implicated in various processes that increase the risk for vascular disease, including metabolic dysregulation, most notably hyperglycemia, oxidative and ER stress, and inflammation. TXNIP has been shown recently to mediate endothelial cell inflammation in response to disturbed blood flow by increasing monocyte adhesion. Lipid peroxidation products also inhibit thioredoxin activity by inducing a conformational change; this has been associated with increased ROS production and enhanced monocyte adhesion to vascular endothelial cells. Taken together, these studies suggest that the thioredoxin system is important for vascular endothelial cell function and protects against atherosclerosis. We found that TXNIP is an important mediator of oxidative stress–induced cellular dysfunction and senescence in endothelial cells exposed to oxidized low-density lipoprotein (unpublished data, manuscript in preparation). This further supports the hypothesis that increased TXNIP expression results in endothelial cell dysfunction, thereby promoting atherosclerosis. The fact that TXNIP is increased in response to alterations of blood flow may suggest that it plays an important role in the pathophysiology of atherosclerosis, independently of mediating the deleterious effects of hyperglycemia, thus also in the context of diabetes-unrelated atherosclerosis.
The driving force behind T2D disease progression

Hyperglycemia and insulin resistance increase TXNIP expression in vascular tissues of diabetic patients, which may contribute to the accelerated atherosclerotic process of diabetes. Dysregulation of TXNIP expression by hyperglycemia has also been implicated in diabetes-induced impairment of angiogenesis, probably due to inhibition of vascular endothelial growth factor (VEGF) production and action. In addition, TXNIP has been shown to enhance ischemia-reperfusion injury in response to acute hyperglycemia. Indeed, inhibition of TXNIP in the myocardium alleviated oxidative stress and decreased myocardial infarct size.

Finally, TXNIP may also play a role in the microvascular complications of diabetes, such as retinopathy and nephropathy. This is suggested from work in models of diabetic retinopathy and retinal degeneration, where high glucose concentrations increased the expression of TXNIP, leading to oxidative stress, cellular dysfunction, and death. TXNIP deletion protected against retinal vascular degeneration and preserved retinal function. The beneficial effect of reducing TXNIP expression in vascular cells is schematically demonstrated in Figure 5.

**TXNIP: a therapeutic target in diabetes**

The accumulating data that have been briefly summarized above clearly suggest that TXNIP plays a central role in the pathophysiology of T2D as well as in mediating glucotoxicity, the driving force behind T2D disease progression (Figure 6). Hence, downregulation of TXNIP has potential to become a powerful therapeutic approach in diabetes, capable of improving hyperglycemia and preventing oxidative stress–induced tissue damage, including β-cell apoptosis and diabetic vascular complications.

The regulation of TXNIP is multifactorial, and many agents, including some pharmaceuticals, modify its expression. For example, insulin suppresses TXNIP expression by stimulating the canonical phosphatidylinositol 3-kinase signaling pathway. Glucagon-like peptide-1 (GLP-1) analogs inhibit TXNIP in β cells by increasing cyclic adenosine monophosphate (cAMP) levels. The inhibition of TXNIP by insulin and cAMP is mediated by repression of Tnpi transcription and destabilization of the protein. We and others have shown that AMP-activated protein kinase (AMPK) activation also inhibits TXNIP. However, the efficacy by which such medications inhibit TXNIP in vivo is not clear. Therefore, novel TXNIP inhibitors that can specifically and effectively prevent the glucose stimulation of TXNIP expression would be highly desirable; their discovery could pave the way for the development of a new class of antidiabetic medications that target key pathways involved in the pathophysiology of diabetes and its vascular complications. If successful, such an approach may fully satisfy the present requirement for antidiabetic drugs: action not only on hyperglycemia, but on the global pathology of diabetes, including, most importantly, cardiovascular and microvascular morbidity.
Innovating in type 2 diabetes

Born in 1935 in Istanbul, Prof Errol Cerasi received his MD at the University of Istanbul in 1960 and another at the Karolinska Institute (Stockholm) in 1964, where he also received his PhD in 1967. Trained at the Karolinska Institute hospitals, he was a senior physician and Associate Professor in the Department of Endocrinology there until 1977. During 1973 to 1975, he was also an Invited Professor at the University of Geneva Medical School. In 1977, he was recruited to the Hebrew University Hadassah Medical Centre in Jerusalem as Professor of Endocrinology & Metabolism. He created a clinical and research department at the Hadassah Hospital, which he directed during the years 1977 to 2001. Since 2003, he has been an active Professor Emeritus. Among his major awards and honors are the Minkowski Prize of the European Society for the Study of Diabetes (EASD) (1974); Presidency of the Scandinavian Society for Diabetes Research (1975-1977); Honorary Membership, Turkish Diabetes Association (1995); Maurice Dérot Award of the French Diabetes Association, Journées de Diabétologie (1997); Presidency of the Israel Endocrine Society (1996-2000); Samuel and Paula Elkeles Prize for Outstanding Scientist in Medicine, Israel (2000); Dr honoris causa, Université Catholique de Louvain (2002); Life Achievement Award, Scientific & Technological Research Council, Turkey (2006); Sam and Sadie Roth Visiting Professorship in Medicine, McGill University (2008); Dr honoris causa, Erciyes University, Turkey (2014). He has been Chairman of the International Group on Insulin Secretion (IGIS) since 1999. His research interests include regulation of insulin production in normal and diabetic β cells in man and animal models of type 2 diabetes; the molecular control of glucose transport; the molecular control of insulin gene expression; cell biology of the β cell; and generation of antidiabetic agents. He has published around 300 research papers.

Born in 1962 in Haifa, Israel, Prof Gil Leibowitz received his MD at the Hebrew University in Jerusalem in 1989. Between 1989 and 1995, he trained in Internal Medicine and Endocrinology and then spent 2 years as a postdoctoral fellow at the Center for Molecular Genetics at the University of California, San Diego (UCSD). He serves as a senior physician and Associate Professor in the Endocrine Service and the Division of Medicine at the Hebrew University - Hadassah Medical Center. He is Head of the Hebrew University Diabetes Research Center. He was awarded the Wolfson Prize for diabetes research from the Israel Diabetes Society (2001) and the Hans Lindner Prize of the Israel Endocrine Society (2010). His research interests include different aspects of β-cell biology, focusing on cellular stress and autophagy, and the regulation of insulin production and secretion in normal and diabetic β cells in animal models and man. He has published around 70 papers.

A lain Ktorza was trained in Life Sciences (receiving his PhD) with a specialization in metabolic diseases and nutrition. He was Professor of Endocrinology and Metabolism at the Université Denis Diderot – Paris 7 and Director of the CNRS UMR 7059 (Centre national de la recherche scientifique – unités mixtes de recherche) until 2004, when he joined Servier as Director of Discovery Research on Metabolism Therapeutic Innovation Poles. This division is in charge of research programs for drug discovery in type 2 diabetes with a special focus on the improvement of function and survival of the pancreatic β cells, which produce insulin, as well as on the metabolic environment.

References
Innovating in type 2 diabetes

Keywords: glucose; insulin; thioredoxin; thioredoxin-interacting protein; type 2 diabetes; vascular complications

Le rôle de la TXNIP dans la physiopathologie du diabète et ses complications vasculaires : brève mise au point


Special focus
Innovation-driven partnerships

SPECIAL FOCUS ON CHINA

The SIMM-Servier partnership for drug discovery:
looking back, looking forward
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The SIMM-Servier partnership for drug discovery: looking back, looking forward

by J. Ding and Y. Ye, China

The Shanghai Institute of Materia Medica (SIMM) of the Chinese Academy of Sciences (CAS), evolved from the Institute of Materia Medica of Peking Academy of Sciences, founded in 1932 by Professor Zhao Chenggu (T. Q. Chou). SIMM has developed and commercialized over 100 new drugs in the past 60 years. With the implementation of a high-end talent program in 1998, SIMM has successfully recruited and trained a group of domestic and overseas leading talents, forming a structured, professional, open-minded, and innovative research team. Collaboration between SIMM and Servier in 1996 has resulted in various projects in the fields of oncology, neurodegenerative disease, and diabetes. In the last three years, SIMM has set up new strategic world-class centers; the research capabilities of such new centers will soon be involved in the ongoing SIMM-Servier collaborative research programs. Both parties trust that new technologies in each other’s laboratories, a joint laboratory, and talented people from both sides will help identify either novel lead compounds targeting currently untreated diseases, or new, previously unobserved mechanisms of drug actions.

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The Institute

The Shanghai Institute of Materia Medica (SIMM) of the Chinese Academy of Sciences (CAS), has the longest history as a comprehensive research institute for drug discovery in China. SIMM has evolved from the Peking Institute of Materia Medica, Academia Sinica, founded in 1932 by Professor Zhao Chenggu (T. Q. Chou; Figure 1) in Beijing.

Professor Zhao was the first Chinese person in history to receive a PhD degree in chemistry. He received his PhD degree from the University of Geneva in 1914, under the supervision of Professor Amé Pictet, a famous Swiss chemist studying organic natural products. In 1916, Professor Zhao became a researcher in the French company ROC. He became fluent in French and married a Frenchwoman, with whom he had one daughter.

In 1933, the institute was relocated to 395 Wu Kang Road, Shanghai, which was a French concession at that time. In 1953, the institute moved to 319 Yue Yang Road, the residence of the former Consulate General of France in Shanghai. Since 2003, the institute has been based at 555 Zu Chong Zhi Road, Zhang Jiang Hi-Tech Park, Pudong New Area, Shanghai (Figure 2).
In line with the mission of “discovering new drugs to relieve patients’ suffering from various diseases,” SIMM has developed and commercialized over 100 new drugs in the past 60 years. Among them, a number of innovative drugs such as artemether (antimalarial), dimercaptosuccinic acid (antidote for heavy metal poisoning), and hyperzine A (Alzheimer’s disease) gained recognition both nationally and abroad. SIMM has also developed some novel drugs in recent years, including depside salts (angina), a modern Traditional Chinese Medicine (TCM), and antofloxacin hydrochloride, a novel fluoroquinolone antibacterial agent. SIMM currently has a series of drug candidates in clinical stages.

Since the implementation of the Knowledge Innovation Program of CAS, developing novel drugs has become a paramount research focus of SIMM. In line with frontiers in life sciences, with the aim of solving key scientific problems in drug discovery, SIMM carries out both basic and applied studies, and develops new theories, methods, and technologies. Research priorities are given to treat major diseases, such as oncology, cardiovascular, neuropsychiatric, metabolic, autoimmune, and infectious diseases. SIMM also pays high attention to the development of modern TCM.

There are 4 national research centers in SIMM (State Key Laboratory of Drug Research, National Center for Drug Screening, National Engineering Laboratory for TCM Standardization Technology, and Chinese National Compound Library); 5 research departments (Medicinal Chemistry, Natural Products Chemistry, Pharmacology I, II, and III, and CAS Key Laboratory of Receptor Research); 11 technological platforms and research centers; and 4 institutional service and logistic departments, including a United Editorial Officer responsible for the publication of two academic journals (Acta Pharmacologica Sinica and Asian Journal of Andrology).
With the implementation of a high-end talent program in 1998, SIMM has successfully recruited and trained a group of domestic and overseas leading talents, forming a structured, professional, open-minded, and innovative research team. SIMM currently has 850 employees; among them 92 are professors and 50 are associate professors, including 6 academicians. The graduate education department has more than 600 masters and PhD students, as well as 40 postdoctoral fellows.

SIMM enjoys a wide range of international partnerships around the world. The SIMM-AstraZeneca alliance project, focused on the joint establishment of a Center for Drug Safety Evaluation and Research, has become the first organization in China to be accredited by in terms of compliance with the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (GLP). Based on the transfer of Novo Nordisk 0.5 million small molecule compounds, the number of compounds collected in the Chinese National Compound Library has now exceeded 1.3 million.

SIMM has established an innovative partnership with the World Health Organization (WHO) for drugs against poverty-related diseases. SIMM has established dynamic scientific collaborations with universities and institutions in North America, Europe, the Middle East, and Asia.

Through several generations’ efforts, SIMM has become one of the leading interdisciplinary centers of excellence in drug discovery in China. It is recognized worldwide for its outstanding achievements and distinguished research team.

**The SIMM-Servier joint laboratory**

The initial contact between Servier and SIMM started in 1996 when Professor Paul Vanhoutte and Dr Pierre Renard made the groundbreaking visit to SIMM in the campus of Yue Yang Road, thus starting the long-lasting partnership of 18 years (Figures 3 and 4).

**Trust each other: develop together**

Over these 18 years, scientists working at SIMM and Servier have established various collaborative projects in the areas of oncology, central nervous system disease, and diabetes, sparing no effort to identify drug leads, drug candidates, or new mechanisms of drug action and drug targets. Along with the trust the collaborative teams have built up through the years, the management teams from both sides have established an increasingly constructive, productive structure of the collaboration. Collaboration goals between SIMM and Servier are gradually shifting from focusing on the identification of single projects limited by one compound series or one target, to multilevel cooperation programs involving, for example, medicinal chemistry, chemical proteomics, pharmacology, and structural biology. Both parties hope that such multilevel partnerships will facilitate innovative discovery thanks to optimized interactions among the team, thus saving time and accelerating each step involved.

In 2013, the collaboration of SIMM and Servier entered into a new era by the fact that the rights of the first SIMM clinical candidate lucitanib were licensed to Servier. Lucitanib is a kinase inhibitor that targets fibroblast growth factor receptors 1 and 2 (FGFR1-2) and vascular endothelial growth factor receptors 1-3 (VEGFR-1-3). It has specific antitumor activity in FGFR1-2–dependent tumors and a strong antiangiogenic effect. SIMM and Servier are co-owners of the marketing authorizations for China. In addition, SIMM will conduct specific research in biomarkers and support Servier regarding the participation of China in international clinical studies.
Trust innovation: building new drug discovery technology platforms together

In the last four years, SIMM has set up new strategic world class centers, such as the Center for Structure and Function of Drug Targets, where scientists are focusing on the elucidation of G protein–coupled receptor structures, and the Center for Chemical Proteomics, where scientists are focusing on the identification of new posttranslational modifications. The research capabilities of such new centers will soon be involved in the ongoing research collaborative programs between SIMM and Servier.

Moreover, in 2012, SIMM and Servier agreed to enter into a new agreement in order to establish a joint discovery and biopharmaceutical research laboratory. Advanced technology platforms in pharmacokinetics, metabolism, and in vitro toxicology have been successfully transferred and have become operational at SIMM. The joint laboratory serves not only the SIMM-Servier joint research programs, but also SIMM’s own discovery programs (Figure 5).

Trust natural products: exploiting treasures from folk medicine together

The first collaborative project between Servier and SIMM in 1997 was already focusing on a famous cluster of natural products represented by artemisinine. Throughout the years, the enthusiasm of scientists from both sides remains, with current programs in the areas of oncology, neurodegenerative disease, and diabetes. Both parties trust that new technologies in each other’s laboratories, a joint laboratory, and talented people from both sides could help us to identify either novel lead compounds targeting currently untreated diseases, or new, previously unobserved mechanisms of drug actions (Figures 6 and 7).

Conclusion

Drug discovery research is a long and complex journey where we always need state-of-the-art skills as well as talented people. The long-lasting partnership between Servier and SIMM reflects the inspiration of recognizing, trusting, and sharing each other’s knowledge, skills, and expertise, according to the needs of drug discovery programs. SIMM has full confidence and expectations, through joint efforts with Servier’s expertise, to continue delivering successful results in our long-lasting and ongoing collaborative projects.

Keywords: diabetes; drug discovery; neurodegenerative diseases; oncology; SIMM (Shanghai Institute of Materia Medica)
**Special Focus**

**Special Focus on China**

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**Professor Yang Ye,** BS in Chemistry, Chemistry Department, East China Normal University in 1987; PhD in Medicinal Chemistry, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (SIMM-CAS) in 1992. Alexander von Humboldt postdoctoral fellow at the Institute of Organic Chemistry, University of Munich; currently the Deputy Director General of SIMM and full professor in the Department of Natural Products Chemistry, SIMM, and adjunct professor at the ShanghaiTech University. Research interests focus mainly on the secondary metabolites in traditional herbal medicinal plants and their bioactivities. Professor Ye is on the editorial/advisory board of seven international journals and book series.

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**PARTENARIAT SIMM-SERVIER POUR LA DÉCOUVERTE DU MÉDICAMENT : HISTORIQUE ET PERSPECTIVES**

L’Institut de Matière Médicale de Shanghai (SIMM) de l’Académie Chinoise des Sciences est l’émancipation de l’Institut de Matière Médicale de l’Académie des Sciences de Pékin fondé en 1932 par le Professeur Zhao Chenggu (T. Q. Chou). Le SIMM a développé et commercialisé plus de 100 nouveaux médicaments au cours des 60 dernières années. La mise en place d’un programme d’excellence en 1998 a permis au SIMM de recruter et former un groupe de collaborateurs talentueux venant de Chine et de l’international, constituant une équipe de recherche structurée, professionnelle, innovante et de grande ouverture d’esprit. La collaboration entre le SIMM et Servier, qui remonte à 1996, a été à l’origine de plusieurs projets dans les domaines de l’oncologie, des maladies neurodégénératives et du diabète. Au cours des trois dernières années, le SIMM a essaimé de nouveaux centres de recherche de niveau mondial, dont les capacités de recherche profiteront aux programmes de recherche collaborative entre le SIMM et Servier. Forts des technologies issues de leurs laboratoires respectifs, d’un laboratoire exploité en commun, et de leurs excellentes équipes de recherche, le SIMM et Servier espèrent identifier de nouvelles molécules chefs de file, efficaces dans des pathologies pour lesquelles il n’existe jusqu’à présent aucun traitement, ainsi que mettre en évidence de nouveaux mécanismes d’action thérapeutique.
Design is the buzzword of the 21st century, and this is particularly true of today’s cutting edge drug discovery and design scene, as this issue of Medicographia has purported to show by evoking several ground-breaking biotech endeavors. Our Touch of France section now looks at a different, but no less cutting edge kind of design, and documents a distinctive “French Touch” whether in the design of objects of daily life and medicine, by leading and visionary designer Mathieu Lehanneur, or of buildings, with such internationally hailed architects as Paul Andreu, Christian de Portzamparc, Jean Nouvel, Dominique Perrault, Jean-Michel Wilmotte, and Odile Decq.

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A revolution in design: reinventing our relationships with objects and interiors

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Display stands created by Mathieu Lehanneur for Japanese designer Yohji Yamamoto and Italian specialist in leather goods, Mandarina Duck: the material chosen was an extruded laser-cut lightweight, resistant milky-white ABS tube structure resembling a honeycomb. © M. Lehanneur.

Jean Nouvel’s Doha Tower (Qatar) surfaced with four layers of aluminum “butterfly” elements of different scales to create different densities according to orientation with respect to the sun, reminiscent of Islamic screens, which contribute to shielding the building from high temperatures. © J. Nouvel.
Transgression and innovation: French contemporary architecture outside of France

by P. Jodidio, Switzerland

Philip Jodidio was born in 1954 in New Jersey. He studied art history and economics at Harvard University, graduating in 1976. He was the Editor in Chief of the widest circulation French art monthly Connaissance des Arts from 1980 to 2002. Jodidio is the author of more than 100 books on contemporary architecture and art, notably the Architecture Now! series (Taschen). He is the author of monographs on Paul Andreu (Birkhäuser Publishers, Berlin, 2004), and Jean Nouvel (Jean Nouvel by Jean Nouvel, Taschen, Cologne, 2008), and is presently working on a monograph about Christian de Portzamparc. He has also published a survey entitled Architecture in France (Taschen, Cologne, 2006).

In the works of Paul Andreu—as in those of the other architects presented here—the French sense of organization comes forward. It is this sense that allowed Aéroports de Paris under his direction to be the leading world-wide designer of airports. It was Andreu’s audacity that made Terminal 1 at Roissy–Charles-de-Gaulle one of the most noted airport buildings of its time, an audacity that surprised and delighted the Chinese in the heart of their capital with the National Center for the Performing Arts, which they call ‘a Pearl on the Water.’

The local, and sometimes foreign, press puts a good deal of energy into detailing evidence of the “decline” of France. It is true that a century or so ago, the diplomacy and language of France may have had broader influence than they do today. Art too is often referred to, with the present unfavorably compared to the glories of the School of Paris that counted amongst its members Picasso, Matisse, Modigliani, Mondrian, Chagall or Mondrian. But what of architecture? Might it be that the importance and presence of contemporary French architecture has rarely if ever been as significant as it is today? Some call the Pritzker Prize the “Nobel” of architecture. France counts two active winners of that award, Christian de Portzamparc (1994) and Jean Nouvel (2008). Germany, for example has just one (Gottfried Böhm, 1988) as does Spain (Rafael Moneo, 1996). Few living architects have had as broad a presence outside their own country as Paul Andreu, who for thirty years (1967-97) was the Chief Architect of Aéroports de Paris, developing no less than fifty airports around the world as well as the Paris–Charles-de-Gaulle hub at Roissy. Though conceivably slightly less well-known to the general public, other figures such as Dominique Perrault, author of the new French National Library (Paris,1989-95); Jean-Michel Wilmotte, award winning architect and interior designer responsible for the galleries in a large part of the Louvre, but also for the Rijksmuseum in Amsterdam (2013); or Odile Decq, the colorful architect of the MACRO Museum of Contemporary Art of the City of Rome (2004-10), count amongst the significant international figures of contemporary architecture. Each of these architects has completed major projects outside of France in the past ten years that have left a considerable trace in the cities where they were built.
In the heart of Beijing

Paul Andreu was born in 1938 in Caudéran in the Gironde region of France. He obtained an unusual and prestigious combination of diplomas from the École Polytechnique (1961), the École Nationale des Ponts et Chaussées (1963, as an engineer) and as an architect from the École Nationale Supérieure des Beaux-Arts in Paris (1968). As Chief Architect of the Aéroports de Paris he was responsible not only for the architecture of Paris–Charles-de-Gaulle (Roissy) Airport (Paris, 1967–97), but also for the development of approximately 50 airports around the world such as those of Jakarta (1986); Tehran, Iran (1996); Harare, Zimbabwe (1996) or Shanghai-Pudong, China (1999). Andreu has also worked on other large-scale projects such as the French terminal for the Eurotunnel project (1987) and the National Center for the Performing Arts 国家大剧院 (Beijing, China, 2007). Other work outside of France carried out after he began to leave Aéroports de Paris to create his own practice in Paris, includes the Museum of Maritime History of Osaka, Japan (2000), a sports complex in Guangzhou, China (2001), the Center for Oriental Arts (Shanghai, China, 2004), and the Archaeological Museum in Taiyuan, Shanxi, China, nearly completed.

The National Center for the Performing Arts was awarded to Paul Andreu in August 1999 as a result of an international competition that he won in the last phase against Carlos Ott, author of the Paris Bastille Opera and the English architect Terry Farrell, as well as a Chinese group from Xinghua University. This large structure was erected just behind the Great Hall of the People, near Tienanmen Square, and thus very close to the entrance to the Forbidden City. Its 212-meter long ellipsoidal titanium shell houses three halls of 2416 seats (opera), 2017 (concerts) and 1040 seats (theater). The shorter axis of the structure is 143 meters and the height of the shell is 46 meters. In order to leave the external shell intact, the architect decided to provide public access through a 60-meter long tunnel passing beneath the basin that surrounds the building.

Andreu emphasizes that the tunnel is an essential design element since it represents a transition space between the busy outside world and the world of culture within. Andreu faced resistance to his project, both within China, and curiously in France as well, officially because of the building’s apparent lack of reference to its Chinese context. In response to critics at the time, Andreu stated, “What I look for in every project, is its internal coherence and its intelligibility, but at the same time, its relation to what surrounds it. Each project seems to me to be a closed and complete world, at the same time as it is a part of a larger whole, which is to say its physical location, its site, and in a more general way the environment. The whole that I imagine is very often an entity that exists only in my mind… a mental reconstruction of a disparate group of elements. That is why I often think of my projects as elements that are detached from the broken up body of the city.”

National Center for the Performing Arts (the “Giant Egg”), Beijing, China (Paul Andreu with ADP and BIAD). Photo © Paul Maurer.
The Taiyuan Archeological Museum (under construction), Shanxi, China (Paul Andreu architecte paris with Richez Associés and BIAD). Photo © PAAP.
One57, tallest residential building in New York, NY, USA (Christian de Portzamparc Architect). © WADE ZIMZEMAN.
From 57th street to Rio de Janeiro

Christian de Portzamparc was the first French winner of the Pritzker Prize in 1994. He was born in Casablanca, Morocco in 1944. He studied at the École Nationale Supérieure des Beaux-Arts, Paris (1962-69) and created his own firm in 1980. His work includes the Crédit Lyonnais Tower (Euralille, Lille, 1992-95); Nexus World Housing (Fukuoka, Japan, 1989-92); the widely praised LVMH Tower on 57th Street in New York (1996-99); and an addition to the Palais des Congrès in Paris (1996-99). He also designed the French Embassy in Berlin (1997-2003); La Philharmonie Concert Hall in Luxembourg (1997-2005); and the headquarters of Le Monde in Paris (2001-05). More recently he has completed the Ciudad de la Musica (Rio de Janeiro, Brazil, 2002-07); and One57 (New York, NY, USA, 2010-14).

One57 is quite simply one of the most visible new buildings to emerge on the Manhattan skyline in many years. This 75-story, 306-meter high tower dominates its neighborhood on West 57th Street, just across the street from Carnegie Hall. The project was based on a 2005 direct commission from Gary Barnett, President of the Extell Development Company. Heights up to 400 meters were envisaged during the development process, but the completed tower remains the tallest residential building in Manhattan. Aside from luxurious apartments on the upper floors, One57 includes the Park Hyatt Hotel. Residential interior design was by Thomas Juul-Hansen while the hotel interior is by Yabu Pushelberg. The site has an unusual L-shaped configuration. As he did for the earlier LVMH Tower also located on 57th Street, the architect carefully studied the city’s alignment regulations and the air rights specific to this site. The architects state, “The building’s volumes are linked by an ascending and descending cascading movement that flows over curved transitional surfaces containing inhabited terraces. A vertical pattern of contrasting stripes comprised of two different glass types (with uniform visibility from the interior) distinguishes the north façades and recall the vertical energy of New York’s cascading skyline, in contrast with the east and west façades that resemble the aesthetic of the Le Monde and Nantes projects.”

Another of Christian de Portzamparc’s recent and very visible projects is the Cidade das Artes, located at the crossing of Americas and Ayrton Senna Avenues, an intersection originally designed by Lucio Costa in the Barra da Tijuca area of Rio. It forms a single large structure with a vast terrace set ten meters above ground level. Intended for chamber music (500 seats) as well as popular music, three movie theaters, dance studios, ten rehearsal rooms, exhibition spaces, restaurants, a media library, the design is characterized by the two horizontal plates that form the roof and the main terrace. Between these two horizontal limits, curved concrete walls contain the halls and establish an interplay of solids and voids. The 1800-seat Philharmonic Hall can be converted into a 1300-seat Opera. The architect describes this work as a “public symbol” and a landmark for a new area of Rio. Echoing his own Cité de la Musique in Paris, Portzamparc undoubtedly also intends this Cidade das Artes as a kind of homage to the great Brazilian architect Oscar Niemeyer (1907-2012) who mastered the art of concrete forms that appear to float above their site.

Provocative, forbidden, visible

Jean Nouvel is certainly one of the most visible French architects, both within the country and abroad. Born in 1945 in Fumel, he studied in Bordeaux and then at the École Nationale Supérieure des Beaux-Arts (Paris, 1964-72). From 1967 to 1970, he was an assistant of the noted architects Claude Parent and Paul Virilio. He created his first office with François Seigneur in Paris in 1970. Jean Nouvel received the RIBA Gold Medal in 2001 and the Pritzker Prize in 2008. His first widely noted projects were the Institut du Monde Arabe (Paris, 1981-87, with Architecture Studio) and the Fondation Cartier (Paris, 1991-94). He has worked frequently outside of France, completing the Agbar Tower (Barcelona, Spain, 2001-03); an extension of the Reina Sofia Museum (Madrid, Spain, 1999-2005) and the Danish Radio Concert House (Copenhagen, Den-
In France he also designed the Quai Branly Museum, dedicated to the arts and civilizations of Africa, Asia, Oceania, and the Americas (Paris, 2001–6). Current work includes the somewhat controversial new Philharmonic Hall in Paris (2015); the Louvre Abu Dhabi (UAE, 2009–15); and the National Museum of Qatar (Doha, Qatar, 2015).

Jean Nouvel’s Summer Pavilion for the Serpentine Gallery in London’s Kensington Gardens (2010) was a 500–square meter ode to the color red. “Red,” he says, “is the heat of summer. It is the complementary color of green. Red is alive, piercing. Red is provocative, forbidden, visible. Red is English like a red rose, like objects in London that one has to see: a double-decker bus, an old telephone booth, transitional places where one has to go.” Nouvel’s pavilion in Hyde Park had retractable awnings but most visibly, a freestanding 12-meter high sloping wall. Intended as a public space, the Pavilion was the venue for the Serpentine’s Gallery’s program of public talks and events, Park Nights. Red outdoor ping-pong tables emphasized Nouvel’s interest in play in this outdoor context. Edwin Heathcote wrote in the Financial Times (July 9, 2010), “His pavilion is another step into something new. A series of theatrical red planes, bars and canopies, it stands somewhere between a hip Ibiza nightclub and Soviet constructivist agit-prop.”

A six-hour flight from London, Nouvel recently completed his Doha Tower (Doha, Qatar, 2007–11) for Sheikh Saud Al Thani. This 238-meter structure has a cylindrical plan measuring 45 meters in diameter. It is located in the center of the downtown area of Doha that is called West Bay. There are 41 stories of rental office space. Each floor provides panoramic views towards the Gulf, the Bay, the dense urban perspective of West Bay or even the desert according to orientation. The client has reserved the uppermost three stories of the building for his own use. The building has an unusual entrance—with a sloping, landscaped ramp leading down to the main entrances that are below grade and are covered by a circular canopy. Landscape design is by Jean-Claude Hardy and takes into account the desert climate. As seen from street level the building in fact appears to have no entrance—it becomes a purely sculptural object on the skyline, showing no visible surfaces in glass. The building has an unusual skin, formed with four “butterfly” aluminum elements of different scales, which are superimposed to create different densities according to orientation vis-à-vis the sun: 25% toward the north, 40% toward the south, and 60% on the east and west. Inside, a slightly reflective glass provides further protection, complemented by roller blinds where necessary. This combined system substantially reduces solar gain. The nighttime presence of the Doha Tower is enhanced by a computer controlled LED system.
designed for the façade by Yann Kersalé. During the design process, the Doha Tower has a formal relation to two earlier projects by Jean Nouvel, the unbuilt Tour Sans Fins (Paris, 1989) and the Torre Agbar on the Avenida Diagonal in Barcelona (2000).

Contradicting modernist tenets

Dominique Perrault, eight years younger than Jean Nouvel, was born in Clermont-Ferrand. He studied in Paris and received his diploma as an architect from the École Nationale Supérieure des Beaux-Arts in 1978. He received a further degree in urbanism at the École Nationale des Ponts et Chaussées in 1979, as well as a Masters in History at the Écoles des Hautes Études en Sciences Sociales (EHESS) in 1980. He created his own firm in 1981 in Paris. His most significant projects include the French National Library in Paris (1989-95), and the Olympic Velodrome, Swimming, and Diving Pool (Berlin, Germany, 1992-99). Recent projects include an extension of the Court of Justice of the European Community (Luxembourg, 2004-08); the Ewha Women's University in Seoul (2004-07); the Olympic Tennis Center in Madrid (2002-07); and the redevelopment of the banks of the Manzanares in Madrid (2005-08). Perrault’s Olympic Swimming Pool (Berlin, Germany, 1998-99) was originally intended as an element in the application of Berlin to host the 2000 Olympic Games. This swimming pool is situated next to the Velodrome also designed by Perrault for the same event. Echoing the low-lying geometric circle traced in the ground for the Velodrome, Perrault here uses a strict rectangle, also set deeply into the surrounding orchard of 450 apple trees. Obviously intending to avoid the heavy-handed symbolism that accompanied the 1936 Games, Perrault opts for a minimalist discretion, a digging into the earth that is atypical of modernist designs. Although the idea of situating a good part of the structure below ground level may initially entail somewhat higher construction costs, it also ensures the thermal stability of the complex, and reduces the energy consumption of the buildings.

More recently Perrault worked on the large (70 000 square meter) and rather unusual Ewha Woman’s University (Seoul, South Korea, 2004-08). Founded in 1886, Ewha has 22 000 female students. Dominique Perrault won the international competition to design these new facilities in 2003, inaugurating the building on April 29, 2008. The program includes spaces for study, sports, including outdoor areas, offices, a cinema and parking. A great emphasis was put on the energy efficiency of the structure, with its green roof, water-use efficiency, and renewable energy sources. In winter fully 80% and in summer 70% of the power demands are provided by natural resources such as geothermal energy or natural ven-
Velodrome and Olympic Swimming Pool, Berlin, Germany (Dominique Perrault). © Georges Fessy/DPA/ADAGP.

Ewha Woman’s University, Seoul, South Korea (Dominique Perrault). © André Morin/DPA/ADAGP.
The project resembles a work of landscape architecture as much as it does more traditional structures—with its long avenue slicing through the middle of the site and revealing the academic spaces below a green roof. The architect calls the main spaces the Sports Strip and the Campus Valley—emphasizing the landscape elements of the design. As he wrote at the beginning of the project, “A new seam slices through the topography revealing the interior of the Ewha campus center. A void is formed, a hybrid place, in which a variety of activities can unfold. It is an avenue, gently descending, controlling the flow of traffic, leading to a monumental stair carrying visitors upwards, recalling the Champs-Elysées or the Campidoglio in Rome.”

Perrault’s relation with digging into the earth has followed his career and his attitude, expressed about the earlier French National Library, makes his gestures vis-à-vis the earth in Berlin and at Ewha clearer. “The modern movement has always had a very Puritan relationship with the earth. When Le Corbusier imagined setting buildings up on pilotis so that would not touch the earth, one must admit that his attitude was very peculiar. In my project, the idea of the natural level of the earth disappears, and the building blends with nature. In Paris, one has the impression that the garden of the Library is at the level of the Seine, but in fact, it is ten meters lower. One almost has the feels that the garden was there before the building and that the Library somehow protects it. This relationship with the earth is complex and contradictory, and it contradicts the usual Modernist tenets.”

Jean-Michel Wilmotte, born in 1948, is a graduate of the Camondo School in Paris. He created his own firm in 1975. Although he is best known for his work in interior design, including numerous galleries of the Louvre (in collaboration with I. M. Pei), Wilmotte joined the Order of Architects in France in 1993. With approximately 200 employees, his office works on industrial and furniture design, such as the lighting fixtures and benches installed on the Champs-Elysées. As an architect, Wilmotte has completed the Gana Art Center, Seoul (South Korea, 1996-98) and a museum for objects given to French President Jacques Chirac in Saran, France. Wilmotte also completed the interior design of the Museum of Islamic Arts (I. M. Pei architect, Doha, Qatar, 2003-08), the Ullens Center for Contemporary Art (UCCA, Beijing, China, 2006-07) and...
the Mandarin Oriental Hotel in Paris (2011). He recently completed the interior refurbishment of the Rijksmuseum (Amsterdam, The Netherlands 2013); the new Nice football stadium (France, 2010-13); and the Arsenal Museum in Kiev (Ukraine, 2010-ongoing).

Built in 1885 by the architect Pieter Cuypers (1827-1921), the Rijksmuseum in Amsterdam, home to such masterpieces as Rembrandt’s Night Watch, was refurbished by Jean-Michel Wilmotte (with the Spanish architects Cruz y Ortiz) subsequent to a 2004 competition. The 12 000–square meter museum design project was completed in 2013. A very particular constraint of the project was that the new work should not alter the work of Pieter Cuypers. Wilmotte and his team completed the mezzanine level which is dedicated to special collections and works from the Middle Ages, level 1 for objects from the 18th and 19th centuries, level 2 with the works of the 17th century including the Night Watch, and level 3, which is for 20th-century art. The minimalist display cases designed for the Rijksmuseum by Wilmotte are very much in the spirit of the rest of the architect’s work where modernity and a sensitivity to existing buildings are brought together in an effortless way.

**An abstract art garden**

Odile Decq was born in 1955 in Laval, and obtained her degree in Architecture (DPLG) at UP6 in Paris in 1978. She studied Urbanism at the Institut d’Études Politiques (IEP, “Sciences Po” in Paris (1979) and founded her office in 1980. Her former partner Benoît Cornette died in 1998. She has designed a number of apartment buildings in Paris; three buildings for Nantes University (France, 1993-99);
MACRO Museum of Contemporary Art of the City of Rome, entrance to Didactic Room (Odile Decq). © Odile Decq / Photo: Luigi Filetici.
a refurbishment of the Conference Hall of UNESCO in Paris (France, 2001); renovation of the Cureghem Veterinary School in Brussels (Belgium, 2001); and the Liaunig Museum (Neuhaus, Austria, 2004). Decq was amply praised in the press for her MACRO Museum of Contemporary Art in Rome (Italy, 2004-10). Set into an area of historic industrial buildings, Odile Decq’s new MACRO Museum of Contemporary Art of the City of Rome MACRO has been hailed as a worthy competitor for Zaha Hadid’s MAXXI. The architect explains that the architectural section of the building is expressed through “the translation from horizontal to vertical, from inside to outside, from the foyer to the roof-landscape-garden.” Because of the complex location, she has developed the project as a series of transitions, or as an “abstract art garden” at least where the roof is concerned, and a “landscape”. Although they are “non-regular” her interior spaces are nonetheless “simple spaces given to the artists,” offering multiple exhibition possibilities. She also recently completed the Opéra Restaurant (Paris, France, 2008-11); and the FRAC Contemporary Art Center in Rennes (France, 2009-12). She was the winner of the Golden Lion at the Venice Architecture Biennale (1996) and the 1999 Benedictus Award for the Faculty of Economics and the Law Library at the University of Nantes. She is currently working on the Great Site of Homo Erectus Fossils Museum (Nanjing, China, 2012-ongoing).

**Breaking the molds**

The projects presented here and surely a good number of others, such as Jean Nouvel’s emerging museums in Doha and Abu Dhabi confirm not only the geographic spread of French contemporary architecture, but also its interest and originality. Designers like Wilmotte can take on the architecture of the past easily because they were born with it all around them. The spirit of transgression and innovation that animates the work of Nouvel, Perrault and Decq makes them ideally suited to breaking the molds of modernism, reaching out and creating the forms that already have begun to define the 21st century. The lyrical forms of Christian de Portzamparc add another feature to what might amount to a description of the character of the French, an appreciation of beauty and a capacity for reasoning that some link back to René Descartes (1596-1650). In each of these projects, but perhaps particularly in the work of Paul Andreu, the French sense of organization comes forward. It is this sense that allowed Aéroports de Paris under his direction to be the leading worldwide designer of airports. It was his audacity that made Terminal 1 at Roissy–Charles-de-Gaulle one of the most noted airport buildings of its time, an audacity that also surprised and finally delighted the Chinese in the heart of their capital with the National Center for the Performing Arts, that they call “a Pearl on the Water.”

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**Transgression et innovation : l’architecture française contemporaine hors de France**

A revolution in design: reinventing our relationships with objects and interiors

by M. Lehanneur, France

Mathieu Lehanneur, 39, is a French designer at the forefront of the international design scene. He is one of the very few of his generation to apply his talents to a wide variety of areas, with an urgent sense of mission: to combine design, art, science, and technology into innovative poetic-communicative components with which he creates humanistic projects. Ultimately, he seeks to achieve maximum welfare for human beings, considered by him as complex structures who over and above aesthetically designed objects or settings need pure air to breathe, sustainable food to eat, good health to enjoy, in short, who need a better life. In 2001, his graduation project at the renowned French design school ENSCI–Les Ateliers (École Nationale Supérieure de Création Industrielle), entitled “Therapeutic Objects,” reflected his early fascination with the relationship between patients, their medicines and their welfare, and is now part of MoMA’s permanent collection in New York, together with his iconic (Popular Science magazine’s Best Invention Award 2008) indoor plant-based air purifier “Andrea.” His works feature in the permanent collections of Centre Pompidou (Paris) and SFMoMA (San Francisco). Mathieu Lehanneur was invited to lecture at a TED Conference in 2009; a monograph about his work entitled “Mathieu Lehanneur” was published by Gestalten in 2012.

Described by Paola Antonelli, Senior Curator in the Department of Architecture and Design at New York’s Museum of Modern Art (MoMA), as “a champion of the intellectual agility of today’s design,” Mathieu Lehanneur is intent on showing how design should invest every moment of our lives with meaning, intelligence, emotion, and well-being. After graduating in 2001 from the famous Paris school of design ENSCI–Les Ateliers (École Nationale Supérieure de Création Industrielle), Lehanneur gained international fame in 2007 by inventing, in collaboration with NASA, an air purifier—Andrea—that unites technology, well-being, and a natural air filter, a plant. He works in a variety of fields, but has a particular interest in health care. His Drugs of Tomorrow project is now part of the MoMA permanent collection. For the Hôtel du Marc de Veuve Clicquot Ponsardin, in Reims (France), he improved the quality of the guests’ sleep by means of light, sound, and temperature. Even more striking is his work in the palliative care department at the Hôpital des Diaconesses in Paris, where he has eased the last moments of patients’ lives by installing in each room a digital porthole that shows a skyscape that changes round the clock, like a window onto a possible “tomorrow.” An international speaker, Lehanneur is much sought after for his views as a designer concerned for our planet’s future, particularly following his talk at the TED Global 2009 Conference. The leitmotif of his work is that design is not just shape, style, or color, but above all a tool intended to create a veritable relationship between us and “the object,” to lighten, to enhance, to improve our lives.

Medicographia. 2014;36:419-429 (see French abstract on page 429)
Three figures huddle in a shadeless wasteland. Sprawled on the ground, his back propped against the leg of a standing friend, the arrowed cowboy is grimacing, perspiring, groaning. Windborne dust and grit coat the beads of sweat on his brow. Crimson blood flows around the arrow buried in his thigh, darkening as it congeals on its course. The standing man braces his knee against the cowboy’s back and slips a stick between his teeth. The third figure straddles the wounded leg, grips the arrow shaft and yanks. Blood spurts, clenched teeth snap the wooden gag, a muffled howl wells up and dies in some corner of that godforsaken plain, and the stand-in sawbones tosses aside the arrow.

The Western I watched that day in 2001 was formulaic, hackneyed even, its scene of makeshift surgery over too soon for two dozen frames a second to do it full Technicolor justice. Yet for me this filmic scene of surgical improvisation was an epiphany. For the first time I had a sense of how our medicinal drugs have all the user-friendliness, the appeal, of the cowboy’s scream-stifling stick. Pure wood, not laced with an active ingredient or complex medicinal product, no microneedle drug delivery, no painkiller. Nothing. Yet this stick, this object, acquired a genuine therapeutic dimension. It channeled the cowboy’s anguish and fear to a single point, thus shifting his attention and soothing his pain. The snapping of the stick brought treatment to a close and marked the end of suffering. Suppose for a moment that this stick contains an active ingredient. Imagine that it releases an analgesic in anticipation of the painful removal of the arrow. And that on being snapped it releases an antibiotic. In short, what if this stick were really a drug, or rather that our drugs were as smart as this stick?

This artless thought prompted my interest in the pharmaceutical world for my end-of-year design project in 2001. My aim was to invest with meaning the process of getting well and to involve the patient in the treatment, as an ally.

For another approach to the patient, to treatment, to well-being

Half of all prescription drugs are not taken correctly, ergo treatment efficacy is suboptimal in fifty percent of cases. Constant advances—in drug efficacy, targeted treatments, the stability of active ingredients—should not befuddle us into forgetting the behavior of patients (failure to adhere to treatment, irregular dosing, overdosage...).

This is because drugs today are presented to patients in terms of their chemical nature, overlooking the patient’s psychology. What do we really do about reassuring patients? Reminding them to take their medication? To make them feel they are not alone? To help them forget sometimes that they need treatment?

These questions, and many more besides, are crucial if we are to keep the promise to care for the patient. Instinctively, the physician will adapt a discourse to the patient. The disease may be the same, the treatment too, but not the words: firm or soft, friendly or professorial. All this has a point, a purpose: to build a physician-patient relationship.

As a designer, I am expected to devise and fashion objects, things that are inert. The majority of users and consumers, most designers themselves, see this as the designer’s purview. Political science, geopolitics, finance, medicine, nature and ecology, all have their specialists. We designers are seen simply as specialists in things. I beg to differ. If this is how I viewed my profession, my mission, I would feel quite useless. Instead, I see my vocation of designer as a creator of relations. As such, the designer naturally uses things, but these are never the end in itself, but a means to an end. Here is a concrete example.

In working on my drug design project, I was interested, for instance, in antibiotic treatments. We know that all too often patients will not take the full treatment. For two main reasons.
Therapeutic Objects. The principle of this “antibiotic in layers” is for the patient to remove and consume one layer every day as one would peel an onion, starting with the darkest layer and progressing to the lightest, to reach the center where the final “recovery” capsule is found. © Studio Mathieu Lehanneur.

Andrea: Best Invention Award, Popular Science magazine, USA (2008). Filtration system of interior air by plants, in collaboration with Harvard University. Photo © Véronique Huyghe.
First, because they feel better. The symptoms subside after three days and they believe it makes no sense to continue taking the tablets. Second, patients have been told time and again that excessive use of antibiotics has the devastating effect of increasing bacterial resistance. So they think: “The sooner I stop my treatment, the better.” Naturally, physician and pharmacist alike have said otherwise, have reminded patients that the key to getting better is scrupulous observance of the doctor’s instructions. But words and advice fade with time.

My idea then was to lend meaning to dosage. A medicine carries the promise of a cure, or at least an improvement. Over time, pending a turn for the better, from the first day to the last. So I thought of an antibiotic treatment as a succession of layers. Like an onion, the layers of antibiotic are peeled away day by day. As they diminish, the layers become lighter in color towards the heart, the last stage—the cure. This creates a direct, obvious, and intuitive relation between patient and treatment: my drug decreases as my disease resolves. So, after three days, even if my symptoms have abated, I no longer think in terms of stopping my treatment midway. In the end, I am proposing a thing, a therapeutic object, but above all a relationship between the patient, the treatment, and the disease. In my eyes, the key to a successful design resides not in the quality of its lines or the harmony of its colors, but in its capacity to change the world around it by creating new relations.
Palliative care: designing a sense of “tomorrow”

More recently, the head of palliative care at the Hôpital des Diaconesses in Paris came to my agency and explained how his department’s expert medical teams and equipment underpin its efficiency. I later saw this with my own eyes. It would be going too far to say that it’s a good place to live, but it has everything needed to accompany patients on their last journey. The professor’s request was clear and open: “Help us do even better!” He felt that beyond the unquestioned experience and skills of his care teams, the department lacked a soul, another way of caring. Perhaps without voicing it, he felt that a relationship was missing.

After a few months of reflection and visits to the hospital, I suggested installing a new object in each room. My reasoning was that from birth onwards each day is followed by another. Each day save one: the last. In this end-of-life setting, every day potentially becomes this day with no tomorrow. This may be blindingly obvious, but it’s less of a commonplace than it seems. Heedlessly, we spend our time and conversations speaking of what is to come, sooner or later: “in a while,” “this evening,” “tomorrow,” “this weekend,” “next summer,” “when I grow up,” “when I retire.” We constantly project into the future. (Strangely, this future continues to frighten us, but that’s another debate.)

In a palliative care unit there is no future, no exit, projection is not possible, there is just the present, drawn out, slow, painful. That was why I suggested bringing into each room a “future” and an “elsewhere.” Thus it was that each room was fitted out with a large, convex screen that seems to float on the wall. Data are gathered from international meteorological websites and analyzed to create a real-time video animation of the next day’s forecast. Around the clock the screen shows how the sky will be at the same time tomorrow. A realistic sky that has yet to be. Clouds drifting across a pale blue sky, overcast in myriad grays threatening rain, shining heavens flooding the room with light. When a patient arrives, the screen shows the following day’s sky over his or her birthplace. Or any other sky that takes the patient’s fancy: the family’s, a sky of childhood or of dreams. In an empty room the screen is just an object, but when the patient enters it becomes an open “window.” An object no longer, but a sky, a relationship, a step ahead of death itself. It is called Tomorrow Is Another Day.

My interests are not limited to illness and end-of-life care, but these special situations clearly shed light on what design can achieve when it does not focus solely on the shape of things.

Interior design for a Roman church: a liturgy in white marble

One of the most unexpected commissions I was entrusted with came from a priest, Father Lefebvre, who asked me to design the church choir, altar, and pulpit in a Roman church, a listed UNESCO World Heritage site, the Saint-Hilaire Church at Melle, in the Deux-Sèvres department, roughly midway between Nantes and Bordeaux in western France. The project was carried out in partnership with the French Ministry of Culture and Communication, the Poitou-Charente Regional Council, and the town of Melle. When I asked Father Lefebvre why he had chosen me for the project, since I do not specialize in liturgical furniture nor even am a believer, he replied: “Because I know that your project will be specifically for this place, for what it has been, for what it is today, and for those that in...
Choir of Saint-Hilaire Church in Melle, with “telluric” platform plates, altar, and pulpit. Photo © Felipe Ribon.
habit it. I know that what you design will not be your project, but our project.” The church is built on a slope from which it appears to emerge, partly buried, firmly rooted into the ground, as a spiritual projection of mysterious telluric forces. To reflect this, I planned the choir as a multilayered mineral construction in white Carrare marble, evocative of topographic contour lines forming a mound-like rising structure with a sunken pool-shaped baptistery, the latter reminiscent of ancient baptisteries by immersion and of the river flowing at the foot of the slope on which the church rests. The altar and pulpit, also in marble, are like two columns echoing the church’s Romanesque columns, both massive and at the same time, thanks to their beveled base, seeming about to rise as if participating in the ascending movement of the liturgy. The mineral whiteness and minimalist lines, in full harmony with the surrounding architecture, are conducive to meditation and producing a state of peace and calm, which, whether one is a believer or not, are in themselves therapeutic in this hectic age of ours.

And what of your work colleagues around the boardroom table? Have you seen how their posture depends on the distance from the bosses, how they move away from the table as the meeting goes on? They lean back, lift their hands from the table, unobtrusively type text messages. No stakes, no excitement, no concentration.

Whence my aim to create a space for exciting meetings brimming with ideas. For Pullman Hotels around the world, I designed a new meeting area: the Business Playground. No need for details, one will suffice. The table is encircled by a comfortably upholstered padded leather rim, like those around gaming tables. Comfort is found not by leaning against the chair back (there is none), but on the table itself. This may seem like a trifling matter, but it wholly changes the posture. It encourages everyone to be present physically, to be involved, to take interest in the meeting and what is at stake.

Restroom etiquette restored by a small fly
A textbook example of how precise observation and dissection of human behavior helps design to create relations is provided by an unlikely venue—public restrooms. The sharpest minds have for years been seeking a solution to that ancestral male failing: unreliable aim. Urinals designed for this purpose have failed to solve the problem. Experts in the geometry of liquid flow have applied themselves, ergonomists have scrutinized the structural geometry of the human-object relation, applied sociologists have urged good manners with words on restroom walls around the globe: “Please help keep this restroom clean.” All to no avail. Until a nameless genius working for Duravit, the famous German producer of industrial and home sanitary ceramics and bathrooms, looked elsewhere. No leafing through geometry books or guides to etiquette. Instead he took a piercing look inside the human brain, or rather into the mind of the male of the species. And what did he find? That Mr Everyman thinks of his male organ as a gun of sorts and so sees himself as a kind of hunter. His brilliant idea, for which I confess I felt great jealousy, was to bake into the ceramic of the urinal, at just the right spot, an image of a black fly. Prettily drawn and life-size. I can assure you, Mr Everyman takes up the challenge, wants to show his mettle: he aims at the fly, slave to the atavistic instincts of the hunter. And even when he realizes that this is one fly that will not fall stunned into the bowl or take to the air, he continues to aim, and well!

Andrea: designing a living air-purifying system with NASA
A few years ago I imagined and designed a domestic air purifier. It was developed through various studies and experiments conducted in the 1980s by NASA. After the first long stays in space, the American space agency realized that the very materials of the shuttle were poisoning their astronauts. Benzene, trichloroethylene, formaldehyde were found in high concentrations in their blood. For years NASA scientists stud-
ied the capacity of plants to metabolize toxins in the atmosphere inside the shuttle craft. In our everyday lives we are exposed to the same materials and hence the same pollutants, at home and in the workplace. With the help of Professor David Edwards of Harvard University, I developed a mass-produced purifier that filters air by means of a plant and a fan that circulates the air through the soil and around the plant’s roots, thus amplifying the plant’s air-cleaning capacity.

Tests during development showed that the purifier was highly effective, but the aesthetic constraints of a space shuttle are not those of interior design. Beyond tangible proof of the scientific relevance of our plant-based purifier, I had to imagine how to make it into an acceptable, or rather desirable, product. At the design stage, I set myself two objectives: one, make crystal-clear the coexistence of nature and technology; two, create an object that sits well near a couch or desk (to avoid the frequent fate of such devices being relegated to the corner of a room).

To achieve the first objective, I designed the object as a sort of large-scale pharmaceutical capsule with an upper transparent half to house the plant and a lower opaque half to hide the workings. Visually therefore the plant took pride of place and symbolically represented the human brain inside its skull. Without being an exotic species, the plant was thus crowned with a special aura, like a jewel in a display case.

To achieve the second objective, I shaped the object with my hands. I mimed in the air the place and shape that I wanted beside an imaginary couch. I stroked the top of the purifier as I would the head of a seated faithful Labrador. This movement, this caress, defined the final form and my pencil rendered it in three dimensions.

Lastly, I used what could be called microsigns to put the finishing touches to the aesthetic thinking behind the object. A grid of thin slats in front of the fan directly conjures the sensation of airflow, as we have all seen with industrial air conditioners. A button to adjust the airflow speed serves as a formal allusion to a stereo system, both precise and technological. A back drawer supplies water to the plant, thus breaking with the traditional watering can in a procedure akin to that used with an espresso machine.

The choice of a product, the aesthetic decisions too, must be as precise as the lines of a sonnet. Weighed, assayed, tempered. This complex assembly of signs has as its sole voca-
Chocolate Flagship: The flagship store of Swiss chocolatier Maison Cailler. Photo © Vincent Duault.
tion the creation of the desired image within the user's brain. On its release, this filtration system, which I named after my son, Andrea, won a Best Invention Award from the American magazine Popular Science. I am naïve enough to think that the image created in the minds of the prize committee members was not unrelated to the choice of the winning product.

“Form is substance that rises to the surface” (Victor Hugo)

Other projects, among many others, have included:

◆ the Wiser collection of electronic devices for Schneider Electric, to be attached to house electric appliances such as washing machines, refrigerators, dishwashers, boilers, etc, whose purpose it is to reduce energy consumption while providing maximum comfort.

◆ The building and interior design of the Swiss chocolatier Maison Cailler's flagship store in Broc, in the canton of Fribourg, intended to resemble a giant wrapped chocolate with a bite taken out of it.

◆ dB, the roving “white noise” speaker. This is a loudspeaker that moves around like a rolling ball and, when it detects an excessive (in volume or frequency) source of sound, will emit what is called “white noise,” which is the sum of all the frequencies audible to the human ear brought to the same level of intensity, thus creating a sound barrier perceived as far more preferable than the initial offensive noise.

The recurring theme of all my projects, their common denominator, is not the object itself, but the relation it engenders. The object becomes a means, a lure, a spark that acts on and for the individual. Naturally, these relational therapeutic or intelligent objects have to be given form: they must become incarnate and not remain solely in the world of ideas. In writing “Form is the substance that rises to the surface,” Victor Hugo gave us what is arguably one of the best definitions of design. A maxim that could be chiseled into the pediment of all the world’s schools of design.

A few months ago at the forum “Osons la France,” (“Dare to France”) the great French mathematician Cédric Villani and I were of one voice in the optimistic observation that France’s USP (unique selling proposition) goes beyond luxury goods and gastronomy, to science and design. It is perhaps not beyond the realms of possibility that design may save the world.

If today we had to rethink the cowboy’s wooden stick, to produce a less outlandish application, we would need to meld the relational, symbolic, and aesthetic. By concentrating on purpose and equipping it with functions and signs, we can heighten understanding of the stick and spur its adoption. If tomorrow you see one on a shelf in your local pharmacy, spare a thought for the cowboy who inspired it.

Further readings

– Website: www.mathieulehanneur.fr

Une révolution dans le design: réinventer notre rapport aux objets et à nos intérieurs

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