Journal of Medicinal Chemistry

Volume 51, Number 11 June 12, 2008

Miniperspective

Recent Developments of Carbonic Anhydrase Inhibitors as Potential Anticancer Drugs

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Received December 7, 2007

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes present in prokaryotes and eukaryotes, being encoded by four distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, bacteria, algae, and cytoplasm of green plants), the β-CAs (predominantly in bacteria, algae, and chloroplasts of both mono- and dicotyledonds), the γ-CAs (mainly in archaea and some bacteria), and the δ-CAs, present in some marine diatoms.[1–7] In higher vertebrates, including humans, 14 α-CAs isozymes have been discovered and numbered from I to XIV. Recently, an additional CA isozyme (CA XV) has been highlighted in several animal species except in humans and chimpanzees.[2] CA isozymes are distinguished, on one hand, by different subcellular and tissue distribution and, on the other hand, by a modulation of the catalytic activity (CO_2 hydration) due to structural differences present in their active site.[4–7] CAs are cytosolic (isozymes I, II, III, VII, and XIII), mitochondrial (isozymes VA and VB), membrane-bound (isozymes IV, IX, XII, XIV, and XV) or secreted in the saliva (isozyme VI). Other isozymes, called CARPs (carbonic anhydrase related proteins) are acatalytic and are present in the cytosol. The loss of classical CA catalytic activity is the consequence of a lack of histidine residues required to bind the zinc ion. CAs catalyze a very simple reaction, the interconversion between carbon dioxide and bicarbonate ion ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). They are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolytes secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, and calcification. Moreover, some recent evidence suggests the role of CAs in pathological processes (i.e., tumorigenicity, obesity, epilepsy). Many of these isozymes are targets of inhibitors with different therapeutic applications. Clinical CA inhibitors include acetazolamide, methazolamide, and ethoxzolamide.[4,5] Recently, it has been shown that two CA isozymes are prominently associated with cancer, where they are involved in pH regulation and tumor progression.[8] Recent advances in understanding their exact role led to the development of new carbonic anhydrase inhibitors as therapeutic and/or diagnostic agents.[9]

2. Cancer-Associated CA Isozymes: Structure and Function

The first CA found to be associated with cancer was CA IX, as reported in 1992 by Pastoreková and colleagues.[10] CA IX exhibits a particular expression pattern because of its relatively limited presence in normal tissue (especially the gastrointestinal tract) and its overexpression in a large variety of cancer cells.[11] The expression of CA IX is strongly induced by hypoxia, a common feature of solid tumors involving the binding of HIF-1 (hypoxia inducible factor) to a hypoxia response element in the CA9 promoter. CA IX has thus been proposed as a reliable intrinsic marker of tumor hypoxia that will be helpful in clinical practice.[8,11]

The second one, CA XII, was subsequently shown to be coexpressed with CA IX in several tumor tissues and was also found in a wider range of normal tissues. The CA XII expression is also induced by hypoxia, but the underlying molecular mechanism remains unknown.[11]

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10.1021/jm701526d CCC: $40.75
Published on Web 03/19/2008

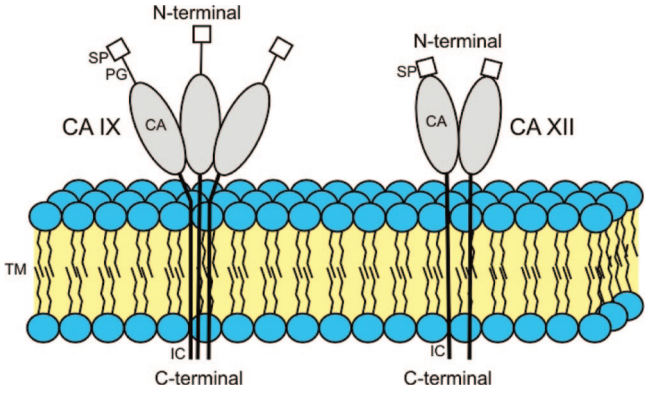

Figure 1. Schematic representation of CA IX and CA XII structures: signal peptides (SP), proteoglycan-related regions (PG), carbonic anhydrase domains (CA), transmembrane regions (TM), and intracellular tails (IC).

Unlike CA XII, CA IX forms a trimer linked by disulfide bonds and has a unique proteoglycan-like region located at the N-teminus (Figure 1).[11] This last domain contains an imperfect repetitive sequence of six amino acids that has been implicated in cell adhesion and differentiation.[8,12,13]

The proposed role of CAs is in sensing and maintaining the acidic tumor environment, a fundamental property of solid tumors, by their CO_2 hydration catalysis.[11] Low extracellular pH has been associated with tumor progression through several pathways (i.e., chromosomal rearrangements, extracellular matrix breakdown, migration, invasion, induction of cell growth factors, and protease activation.).[8,11,14] Acidic pH is also related to a chemoresistance by a decrease in uptake of weakly basic anticancer drugs. Most anticancer drugs are transported by either active transport or passive diffusion into cells, where they frequently undergo further metabolism.[8,14] Because all of these processes are pH sensitive, the cytotoxic activity of anticancer drugs could depend on both intracellular and extracellular pH. Moreover, the fact that bicarbonate is required in the synthesis of pyrimidine nucleotides also suggests that the tumor-associated isozymes are probably involved in providing bicarbonate used as a substrate for cell growth.[6,8]

3. CA Inhibitors as Potential Anticancer Drugs

Targeting the tumor-associated isozymes with specific inhibitors is a promising strategy in the cancer therapy.[8,9] It should contribute, on one hand, to enhance the action of weakly basic anticancer drugs and, on the other hand, to reduce the acquisition of metastatic phenotypes by controlling the pH (im)balance in the tumor cells.

Aromatic sulfonamide compounds (the classical CA inhibitors, Figure 2) have been shown (i) to reverse the effect of tumor acidification (compound **1**),[15] (ii) to inhibit the growth of cancer cells with GI_{50} values (corresponding to the molarity of inhibitor producing a 50% inhibition of tumor cell growth after a 48 h exposure to the drug) in the micromolar range (methazolamide, ethoxzolamide, indisulam, compounds **2**−**4**),[16−19] (iii) and to suppress the tumor invasion mediated by the cancer-associated CAs (acetazolamide).[8,12,20] Teicher and colleagues reported that acetazolamide reduced tumor growth when given alone and delayed the development of the tumor when administrated in combination with various chemotherapeutics agents.[21] Among the interesting molecules developed as anticancer agents, indisulam is characterized by a complex mechanism of action involving CA inhibition among others.[8,16,22] Indisulam is currently in phase II clinical trials.

Unfortunately, all these classical CA inhibitors do not selectively target CA IX and XII. Thus, they are also able to inhibit other CA isozymes (i.e., CA I and II) that have a physiological relevance.[16] Therefore, several strategies have been proposed to improve the CA-isozyme specificity. Isozymes CA IX and XII share a global topology that distinguishes them from the physiologically dominant CA I and II: isozymes IX and XII are transmembrane proteins that orient their CA catalytic domain extracellularly, while isozymes I and II are soluble proteins located in the cytosol.[16] Preparation of aromatic sulfonamides with an impaired ability to diffuse through the

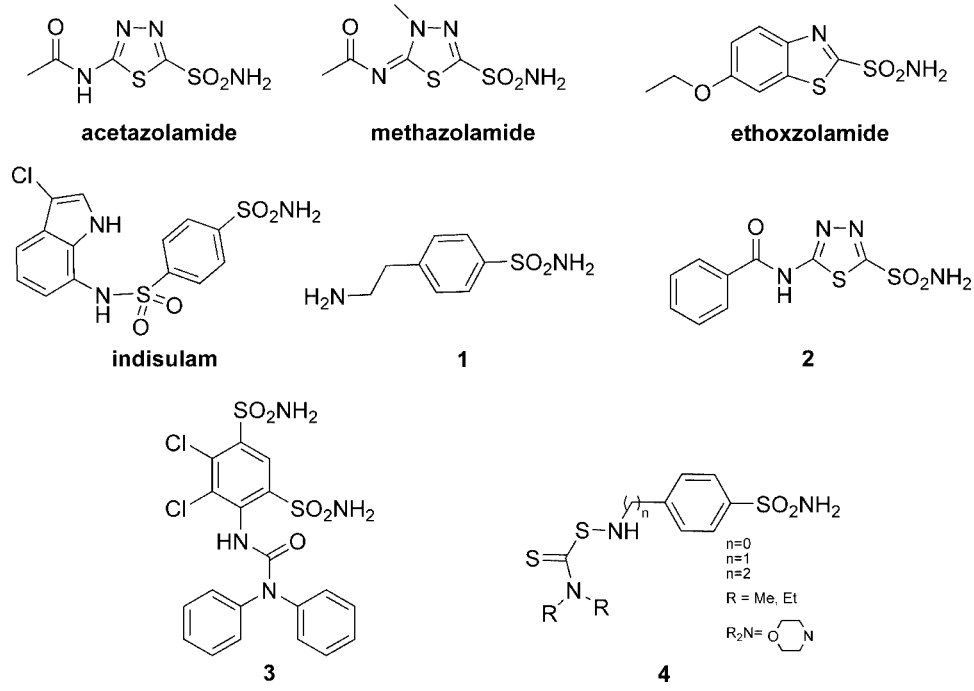

Figure 2. Structures of classical carbonic anhydrase inhibitors.

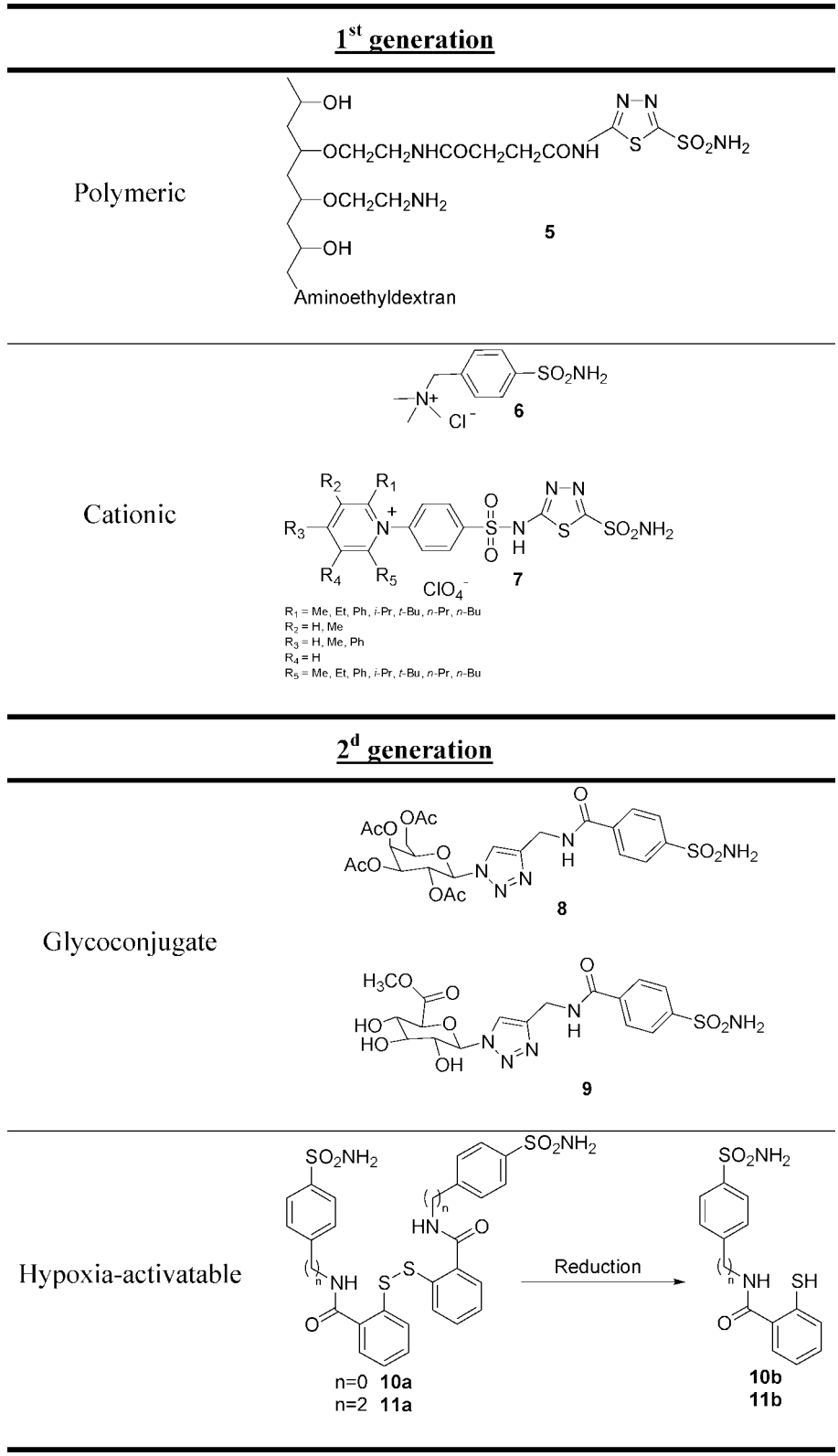

Figure 3. Structures of membrane-impermeant carbonic anhydrase inhibitors.

lipid membranes is therefore one possible strategy to reach CA specificity of the cancer-associated isozymes.

The first historical approach for inducing membrane impermeability to CA inhibitors consisted of the design of polymeric (high-molecular weight) inhibitors (compound **5**, Figure 3).[16] Nevertheless, the in vivo effects of such compounds were disappointing because of the usual concerns connected with polymers (i.e., allergic reactions or bioavailability problems). The development of cationic sulfonamides was then initiated by reacting aromatic/heterocyclic sulfonamides containing free NH_2 groups with pyrilium salts, affording pyridinium derivatives.[16,23] The quaternary ammonium sulfanilamide **6** was one of the lead molecules in the design of cationic inhibitors. Compounds derived from **7** were correlated with a low nanomolar affinity for the tumor-associated CA IX (K_I in the range of 3−45 nM).[23] Ex vivo studies showed that such compounds

Table 1. Inhibition Activities and K_I Ratio Data for the Best Second-Generation Membrane-Impermeant Carbonic Anhydrase Inhibitors against Human Isozymes I, II, and IX (Determined Using a Stopped-Flow Technique)

	K_I (nM)			K_I ratio[a]		
compd	hCA I	hCA II	hCA IX	K_I(hCA I)/K_I(hCA IX)	K_I(hCA II)/K_I(hCA IX)	ref
8	8700	470	76	114.5	6.2	25
9	2400	378	23	104.3	16.4	25
10a	4350	4975	653	6.7	7.6	27
10b (reduced form of **10a**)	276	16	9.1	30.3	1.7	27
11a	4265	4860	724	5.9	6.7	27
11b (reduced form of **11a**)	85	29	9.0	9.4	3.2	27

[a] The K_I ratios are indicative of the inhibition selectivity. A weak selective inhibitor is characterized by low K_I ratios.

can discriminate membrane-bound CA isozyme versus the cytosolic isozyme. However, in vivo, those compounds were unable to cross the plasma membranes.[16,23,24]

As it is well-known that carbohydrates have a compromised ability to diffuse across the cell membranes, Wilkinson and colleagues recently prepared a series of glucoconjugate benzene sulfonamides by "click-tailing" sugar moieties to the classical high-affinity aromatic sulfonamide pharmacophore (Ar-SO_2-NH_2).[25,26] Two compounds (**8**, **9**) were found to be both highly active and selective against the CA IX (Figure 3 and Table 1). Sugar tails therefore prove to be a valuable approach to generate CA isozyme selective compounds.

As hypoxia constitutes hallmarks of many solid tumors, another strategy to target the tumor-associated isozymes is the design of bioreductive, hypoxia-activatable CA inhibitors (compounds **10** and **11**, Figure 3).[27–29] The rationale is based on exploiting the reducing conditions present in such tumors where oxygen is generally less than 1%.[29] Moreover, this type of reduction can eventually be mediated by the redox protein thioredoxin-1, which is found at high levels in many human cancers.[30] Disulfide derivatives of aromatic/heterocyclic sulfonamides were thus synthesized by De Simone and colleagues.[27] In principle, such disulfide-containing sulfonamides should be bulky enough and thus unable to bind within the restricted space of the CA active site, which normally accommodate only one benzensulfonamide/heterocyclic sulfonamide moiety. However, bioreduction in hypoxic tumors of such dimeric sulfonamides would generate thiols that are much less bulky and should bind in the active site of the cancer-associated isozyme found in hypoxic tumors.[27] Such corresponding thiols were indeed found to be potent inhibitors of hCA I, II, and IX (K_I in the range of 3.2–18 nM). Table 1 indicates the inhibitory potency of two such compounds (**10a**, **11a**, and their corresponding reduced forms **10b** and **11b**).[27]

4. CA Inhibitors in the Cancer Diagnosis

As previously mentioned, the CAs IX and XII are expressed in cancer cells and may be used as markers for a broad spectrum of solid hypoxic tumor types. Several fluorescent sulfonamides were thus designed as interesting tools for imaging and further investigating of hypoxic tumors.[31] One of the most promising compound developed so far is derivative **12** ((4-sulfamoylphenylethylthioureido)fluorescein) prepared by reaction of fluorescein isothiocyanate (FITC) with amino-substituted aromatic homosulfanilamide (Figure 4). It was shown to bind only to hypoxic tumor overexpressing the cancer-associated isozymes, which makes it an important candidate for imaging purposes of this type of cancer.[15,31,32] This compound presents a K_I against the CA IX of 24 nM and shows a membrane-impermeant property assessed through an ex vivo model of red blood cells membranes.[31] Compound **12** was also able to reduce the extracellular acidification of Madin−Darby canine kidney CA IX (MDCK-CA IX) cells in hypoxia, and their effect on the normoxic extracellular pH was negligible. At the present time, it is developed in clinical studies as a diagnostic tool for imaging hypoxic tumors.[31,33]

5. Perspectives

The tumor-associated CA IX is clearly an attractive target in both diagnosis and therapy because of its overexpression in cancer cells and its absence from the normal tissues. Elsewhere, the CA XII isozyme is also associated to cancer, but because it is found in normal cells, inhibitors of CA XII are less attractive as anticancer agents. Consequently, targeting CA XII does not seem really promising in the development of anticancer drugs.

The main goal in the further development of CA IX inhibitors is reaching a high selectivity in order to avoid any side effects by inhibiting the other CA isozymes that play physiological roles. Several pathways have been discussed in this review to meet this challenge such as the development of cationic sulfonamides, glycoconjugate, or hypoxia-activatable derivatives. Promising data from our laboratory may lead to increased selectivity.[34] As the three-dimensional structure of CA IX has not been solved, a homology model of CA IX has been built and the active site of CA IX has been characterized and compared to other CA isozymes.[34] Specific amino acid residues have been identified that could be exploited to enhance the selectivity of CA IX inhibitors (Figure 5). The presence of Asp132 residue in hCA IX could be interesting for the design of new inhibitors. In fact, the incorporation of a positively charged side chain could form an ionic interaction with this moiety and would enhance the selectivity. Other CA IX specific amino acid residues have been highlighted and can also be targeted (i.e., Thr69 located at the hydrophilic pocket and Arg130 located at the entrance of the CA IX active site).[33,34] Moreover, bulky groups should be chosen to avoid any

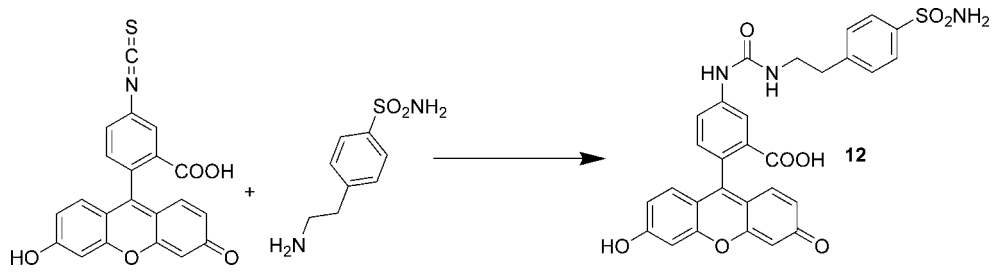

Figure 4. Preparation of the fluorescent sulfonamide **12** from the fluorescein isothiocyanate (FITC) used as a diagnostic tool for imaging hypoxic tumor.

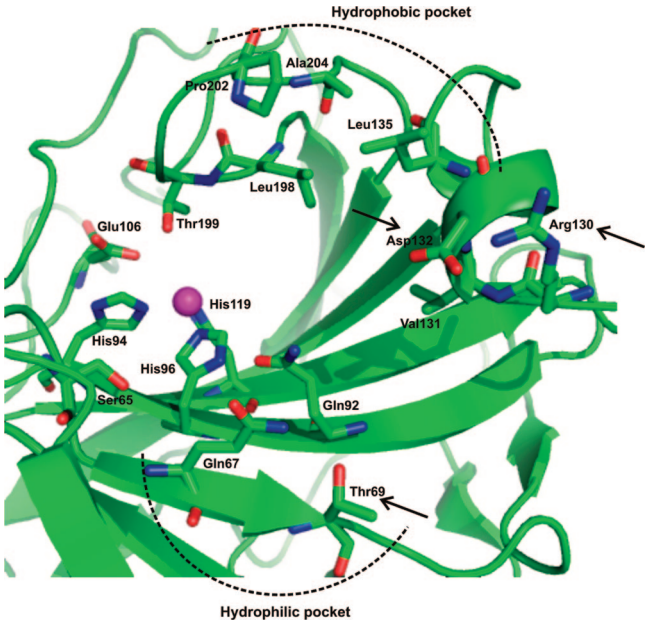

Figure 5. View of the CA IX active site. The specific amino acid residues (indicated by black arrows) should be targeted to enhance the selectivity of CA IX inhibitors.

interaction with Phe131 present in CA II (and changed by a Val residue in CA IX).

The design of boron-containing inhibitors with a high affinity for the tumor-associated CA IX is another promising direction. Indeed, this may lead to important advances in boron neutron capture therapy applications targeting hypoxic tumors, which are nonresponsive to classical chemoradiotherapy.[35]

Finally, modulating the extratumoral pH by using CA IX inhibitors alone will probably not be sufficient to cure cancer. However, the use of CA IX inhibitors in combination with other anticancer drugs would help to manage drug resistance developed by cancer cells. Further experiments are required to demonstrate the real benefit of CA IX inhibitors in cancer therapy.

Biographies

Anne Thiry received her Pharm.D. degree from the Université Catholique de Louvain in 2004. She received her Masters degree in Drug Design from the Ecole Nationale Supérieure de Chimie at Lille (France) in 2005. She has an award from the Belgian F.R.S-FNRS for a research fellowship in 2006. She is acquiring a Ph.D. degree in Pharmaceutical Sciences at the University of Namur-FUNDP (Belgium) under the supervision of Prof. Masereel and Prof. Dogné. Her research interests in medicinal chemistry include the design, the synthesis, and the pharmacological evaluation of carbonic anhydrase inhibitors.

Claudiu T. Supuran received his B.Sc. in Chemistry from the Polytechnic University of Bucharest, Romania (1987) and a Ph.D. in Chemistry at the same university in 1991. He was Assistant Professor of Chemistry at the University of Bucharest. Since 1995 he is Research Fellow and Contract Professor of Chemistry at the University of Florence, Italy. His main research interests include medicinal chemistry, design of enzyme inhibitors and activators, heterocyclic chemistry, chemistry of sulfonamides, sulfamates, and sulfamides, biologically active organoelement derivatives, QSAR studies, X-ray crystallography of metalloenzymes, metal complexes with biologically active ligands (metal-based drugs), carbonic anhydrases, cyclooxygenases, serine proteases, matrix metalloproteinases, bacterial proteases, and amino acid derivatives, among others. He has published more than 500 original research papers in these fields, two patents, and two books.

Bernard Masereel received his Pharm.D. degree (1987) and his Ph.D. (1993) in Pharmaceutical Sciences from the University of Liège, Belgium. After a postdoctoral fellowship at the CNRS-INSERM Research Centre of Montpellier (France), he became Industrial Pharmacist (1990) and he received a Masters in Drug Design (1991, University of Lille II, France). In 1987, he became Assistant at the Laboratory of Medicinal Chemistry (University of Liège). In 1997 he moved to the University of Namur, Belgium, where he is currently a Full Professor and Director of the Department of Pharmacy. He is teaching medicinal chemistry, pharmaceutical analyses, and biochemistry. His main research topics include drug design, molecular modeling, heterocyclic synthesis, enzymology, and pharmacology. He is author or coauthor of more than 100 original research papers and several patents.

Jean-Michel Dogné received his Pharm.D. degree in 1996 and his Ph.D. degree in Pharmaceutical Sciences in 2000 from the University of Liège. After postdoctoral research in Chemistry at the University of Florence, he was appointed Senior Researcher in the Laboratory of Medicinal Chemistry of the University of Liège in 2003. In 2006, he moved to the University of Namur where he is currently a Full Professor of Medicinal Chemistry and Human Biochemistry. His research interests are mainly in heterocyclic chemistry and pharmacology. He has made major scientific contributions in the discovery and development of modulators of the cyclooxygenase pathway in cardiovascular diseases and of carbonic anhydrase inhibitors in epilepsy and cancer. He is author or coauthor of more than 100 original research papers and several patents.

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