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The protein tyrosine kinase inhibitors imatinib and nilotinib strongly inhibit several mammalian α-carbonic anhydrase isoforms

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ABSTRACT

The protein tyrosine kinases (PTKs) are essential enzymes in cellular signaling processes that regulate cell growth, differentiation, migration and metabolism. Their inhibition was recently shown to constitute a new modality for treating cancers. Two clinically used PTK inhibitors (PTKIs), imatinib (Glivec™/Gleevec™) and nilotinib (Tasigna™) were investigated for their effects on the zinc enzymes carbonic anhydrases (CAs, EC 4.2.1.1). The two PTKIs inhibited all 13 catalytically active mammalian isoforms CA I–XV with K_Is in the range of 4.1 nM–20.2 μM. CA I and CA II were the most potently inhibited isoforms (K_Is of 4–32 nM), whereas CA VA and VB showed the lowest affinity for these drugs (K_Is of 5.4–20.2 μM). In cancer cells, these inhibitors may interact with CAs in addition to the targets for which they were designed, the PTKs.

The control of cellular processes, such as cell growth, division, and death, involves signal transduction, which commonly involves the transfer of the terminal phosphate moiety of adenosine triphosphate (ATP) to tyrosine residues on substrate proteins, assisted by the protein tyrosine kinase (PTK) family of enzymes.[1–3] Protein phosphorylation and dephosphorylation play a pivotal role in intracellular signaling; and for regulating such signal transduction pathways. There are 518 protein kinases and approximately 100 protein phosphatases encoded within the human genome.[1–3] A major focus of cancer research in recent years has been to identify oncogenic molecules and the signal transduction pathways in which they are involved, in order to develop specifically targeted drugs. In cancer, as well as in other proliferative diseases, unregulated cell proliferation, differentiation and survival frequently results from abnormal protein phosphorylation. Receptor and non-receptor PTKs are essential enzymes in cellular signaling processes that regulate cell growth, differentiation, migration and metabolism.[1–3] Aberrant catalytic activity of many PTKs, via mutation or overexpression, plays an important role in numerous pathological conditions, the most important of which is cancer. PTKs associated with platelet-derived growth factor (PDGF) receptors, Abelson (ABL) protein, KIT protein (also known as stem cell factor [SCF] receptor), protein kinase AI (PKAI), bcl-2/bcl-xL, FLT3 (fms-related tyrosine kinase/Flk2/Stk-2)—a receptor tyrosine kinase primarily expressed on hematopoietic cells, epidermal growth factor receptor (EGFR), and ErbB-2 transmembrane tyrosine kinases are currently being targeted by various compounds/drugs in the treatment of cancer.[1–4]

The first tyrosine kinase inhibitor to be used clinically, imatinib **1** (as mesylate salt) (Glivec™/Gleevec™, Novartis Pharmaceuticals) blocks activity of the Bcr-Abl oncoprotein and the cell transmembrane tyrosine kinase receptor c-Kit, and was recently approved for several indications in the treatment on chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST).[3–9] In both of these examples the target protein was identified by an oncogenic, activating mutation. Imatinib **1** is also a potent inhibitor of PDGFR kinase and is currently being employed for the treatment of chronic myelomonocytic leukemia and is being evaluated in glioblastoma multiforme, based upon evidence in these diseases of activating mutations in PDGFR.[4–9] The molecular pathogenesis of CML in particular, depends on formation of the Bcr-Abl oncogene, leading to constitutive expression of the tyrosine kinase fusion protein, Bcr-Abl. Based on these observations, imatinib was developed as a selective inhibitor of the Bcr-Abl protein tyrosine kinase. The extraordinary success of imatinib in CML and GIST represents a model for molecularly targeted therapy for tumors, whereas the molecular basis and the detailed mechanisms of action of this drug are still not completely understood at this moment.[10]

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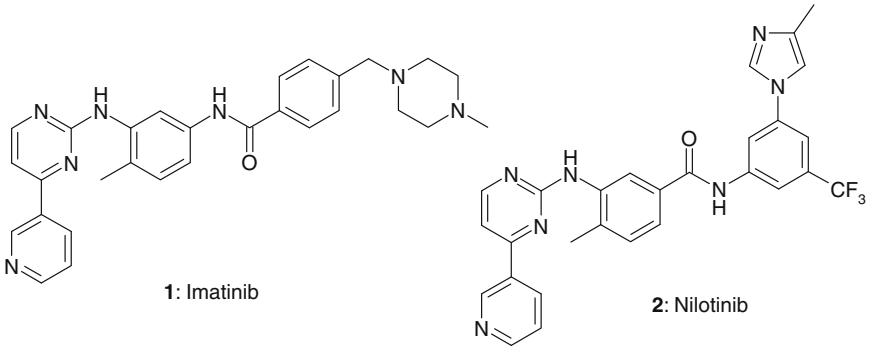

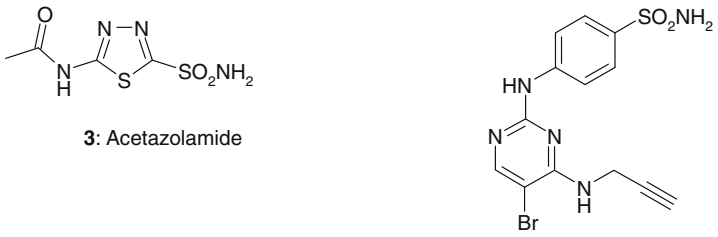

Nilotinib **2**, is a second-generation PTK inhibitor (PTKI) and was approved in 2007, for the treatment of adult patients with chronic-phase and accelerated-phase Philadelphia chromosome-positive (Ph+) CML, resistant to or intolerant of prior treatment that included imatinib.[11] The compound is also being investigated for the treatment of patients with GIST.[11]

There is only one paper in the literature[12] showing that some isoforms of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1)[13] are over-expressed in GIST. Indeed, in mammals, this family of metalloenzymes comprises 16 different isoforms, of which several are cytosolic (CA I–III, CA VII, and CA XIII), five are membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial (CA VA and VB), and one (CA VI) is secreted into saliva/milk.[13] Three acatalytic forms are also known, that is, CA VIII, CA X and CA XI.[13] These enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and several other physiologic/pathologic processes.[13] Many CAs are well-established drug targets.[13–16]

The main class of CA inhibitors (CAIs) is constituted by sulfonamides, such as acetazolamide **3**,[13] and this compound is also known to exert antitumor activity in vitro and in vivo.[13–16] A very recent report[17] showed that a novel generation PTKI from Bayer-Schering (compound **4**), is a potent, multitarget PTKI, also showing submicromolar affinity for a CA isoform (i.e., CA II). All these data, regarding the possible cross-reactivity between PTKs and CA inhibition, prompted us to investigate the potential of imatinib **1** and nilotinib **2** to act as inhibitors of all the catalytically active mammalian CA isoforms, that is, the human (h) or murine (m) CA I–XV.[18–20]

The following should be noted regarding the inhibition data of Table 1:[21]

(i) The PTKIs imatinib **1** and nilotinib **2** acted as very potent inhibitors of two CA isozymes, that is, human CA I (hCA I) and II (hCA II), with inhibition constants in the range of 4.1–31.9 nM. The isoform with the highest affinity for these drugs was the ubiquitous, physiologically dominant hCA II. In fact, the clinically used sulfonamide inhibitor par excellence, acetazolamide **3**,[13] has a K_I of 12 nM against hCA II, intermediate between that of nilotinib (K_I of 4.1 nM) and

Table 1
Inhibition of mammalian isozymes CA I–XV (h = human, m = murine isoform) with compounds **1–3** and **5b**, by a stopped-flow, CO_2 hydration assay method[21]

Isozyme[a]	K_I[c] (nM)			
	1	**2**	**3**	**5b**[d]
hCA I	31.9	29.3	250	78
hCA II	30.2	4.1	12	59
hCA III	528	443	200,000	>10^5
hCA IV	4553	446	74	380
hCA VA	20,200	5485	63	9,600
hCA VB	17,005	14,920	54	17,700
hCA VI	392	461	11	35,700
hCA VII	109	99	2.5	27,900
hCA IX[b]	75.7	41.9	25	54,500
hCA XII[b]	980	302	5.7	48,600
mCA XIII	7450	4665	17	790
hCA XIV	468	223	41	780
mCA XV	78	79	72	93,100

[a] h = human; m = murine isozyme.
[b] Catalytic domain.
[c] Errors in the range of ±5 % of the reported data from three different assays.
[d] IC_{50} from Ref. 13c.

imatinib (K_I of 30.2 nM). The sulfonamide PTKI **4** was a weaker hCA II inhibitor (K_I of 331 nM),[17] but it should be mentioned that an esterase assay with 4-nitrophenylacetate as substrate has been used for determining its inhibitory properties.[17] This method generally leads to higher K_Is as compared to the CO_2 hydrase assay.[22] The second cytosolic isoform, hCA I, also showed high affinities for both drugs (K_Is of 29.3–31.9 nM), although the K_I values were an order of magnitude lower than that of acetazolamide. Importantly, the results showed that nilotinib was a slightly better hCA I and II inhibitor compared to imatinib.

(ii) Effective inhibition with imatinib **1** and nilotinib **2** was also observed against the cytosolic isoform, hCA VII, the tumor-associated, transmembrane enzyme, hCA IX, and the membrane-anchored enzyme mCA XV (Table 1). These isoforms were inhibited by the two compounds with K_Is in the range of 41.9–109 nM. The two PTKIs showed K_Is of 99–109 nM against the preponderantly brain-associated hCA VII, being less active than acetazolamide (K_I of 2.5 nM). hCA IX is one of the most promising new anticancer drug targets as shown recently by this and other groups.[13–15,23] The development of agents targeting this isozyme may have clinical and diagnostic significance for the management of hypoxic tumors in which CA IX is generally overexpressed.[23] It may be observed that imatinib **1** and nilotinib **2** significantly inhibited this isoform, with inhibition constants of 41.9–75.7 nM, in the same range as the sulfonamide **3** (K_I of 25 nM). We are thus tempted to hypothesize that part of the excellent anticancer effects of these drugs may also be due to their interaction with this or other CA isoforms involved in carcinogenesis. The affinity of the two drugs **1** and **2** for mCA XV on the other hand is very similar to that of acetazolamide **3**, the three compounds showing K_Is of 72–79 nM.

(iii) A third group of CA isozymes, including hCA III (cytosolic), VI (secreted in saliva and milk), XII (transmembrane, present in some tumors among other tissues) and XIV (transmembrane) were moderately inhibited by imatinib and nilotinib, with K_Is in the range of 223–980 nM. The membrane-bound hCA IV was also inhibited moderately by nilotinib (K_I of 446 nM) but much less by imatinib (K_I of 4553 nM, Table 1). It may be observed that all these isoforms (except for CA III) are generally much better inhibited by the sulfonamide **3** than by **1** and **2**, but sometimes inhibition constants of 200–300 nM (e.g., **2** against hCA XII and XIV) may have significance in vivo. It may also be observed, that as for all other isoforms discussed here, nilotinib **2** was a more efficient CAI compared to imatinib **1**.

(iv) The mitochondrial isoforms hCA VA and VB, together with the slow cytosolic isoform mCA XIII showed the weakest inhibition with the PTKIs **1** and **2**, with inhibition constants in the range of 4665–20,200 nM. As shown above, nilotinib was a better CA I compared to imatinib for a given isoform, and the sulfonamide **3** showed quite efficient, usually nanomolar affinity for these CAs.

Unlike the clinically used CAIs, possessing a zinc-binding group of the sulfonamide, sulfamate or sulfamide type, and interacting thus with the catalytically crucial Zn(II) ion of the enzyme,[13a,b,16] the PTKIs investigated here do not contain moieties normally associated with CA inhibition. Furthermore, they do not contain labile bonds which might be hydrolyzed by the CAs. In fact, some CA isoforms possess potent esterase or phosphatase activities, whereas no peptidase activity has been detected so far for any CA isoform.[22,23] Thus, the amide bond present in **1** and **2** is not hydrolyzed under the catalytic effect of these enzymes, since even after incubation of **1** and **2** with CA I/II for 24–48 h, the original PTKIs were evidenced unchanged by means of thin layer chromatography and/or HPLC (data not shown). The only hypothesis we can thus suggest for explaining the potent CA inhibitory effect of **1** and **2** against some isoforms is that they bind to the enzyme active site in a manner completely different from that of the classical inhibitors (sulfonamides and their bioisosteres), which substitute the Zn(II)-bound water molecule/hydroxide ion.[13a,b] We hypothesize two possible binding modes for **1** and **2** within the CA active site:

(1) similarly to the phenols, investigated in detail by this and other groups,[25] imatinib and nilotinib may be anchored to the CA active site, and more precisely to the zinc-bound water molecule by means of the pyridine nitrogen atom which may form a strong hydrogen bond with the water molecule (Fig. 1A). The rest of the scaffold can then participate in polar or hydrophobic interactions with the active site. Alternatively, the pyridine nitrogen of **1** and **2** might be directly coordinated to the Zn(II) ion, by substituting the water molecule/hydroxide ion, but we think this second hypothesis is less probable due to steric hindrance in the neighborhood of the metal ion within the active site cavity.

(2) recently, we have demonstrated that coumarins such as the rather bulky, natural product derivative **5a**, constitute a completely novel class of CAIs,[13c] binding to the enzyme active site in a non-zinc dependent manner. In fact the coumarins initially act as substrates for the esterase activity of CAs, being transformed to *cis*-2-hydroxy-cinammic acids (**5a** is thus transformed to **5b**), which then bind towards the external part of the active site, making several favorable interactions with amino acid residues and water molecules present there.[13c] Indeed, the affinity of many coumarin derivatives to various CA isozymes is in the same range as that of the two PTKIs investigated here, that is, from the nano- to the micromolar range (see also Table 1 where data for inhibition of CA I–XV with **5b** are presented).[13c] Thus, it is not unlikely that these two compounds **1** and **2** investigated here, possessing a bulky scaffold and an extended conformation, may bind similarly to the substituted-*cis*-2-hydroxy-cinammic acids (coumarin hydrolysis products) towards the external part of the CA active site (i.e., without interaction with the catalytic Zn(II) ion), plugging it and not allowing the substrate to bind within the cavity (Fig. 1B). The binding of the coumarin **5a** hydrolysis product, compound **5b**, is shown in Figure 2 for comparison, as determined by X-ray crystallography, and proving thus that bulky, non-sulfonamide compounds may effectively bind within the CA active site.[13c] It should be observed that even if **5b** possesses two bulky side chains, it binds to the entrance of the CA active site, plugging it effectively and leading to a completely novel inhibition mechanism of this type of enzymes. It may be observed that the first three aromatic rings of **1** and **2** have less bulky side chains compared to **5b**, which makes this type of interaction with the CA active site not improbable. Work is in progress in our laboratories to find out which of the two hypotheses is the correct one for explaining the efficient inhibition of some CA isozymes with imatinib and nilotinib.

Given the systemic exposures achieved at the standard recommended doses of both imatinib (steady state C_{max}/C_{min} 5.2 and 2.5 μM at 400 mg q.d.) and nilotinib (steady state C_{max}/C_{min} 4.0 and 1.8 μM at 400 mg b.i.d.), it seems likely that the inhibition of at least some of the CAs, alone or in concert with one another, by these drugs might be physiologically relevant. Thus for example, CA IX and CA XII promote tumor cell survival within the hypoxic

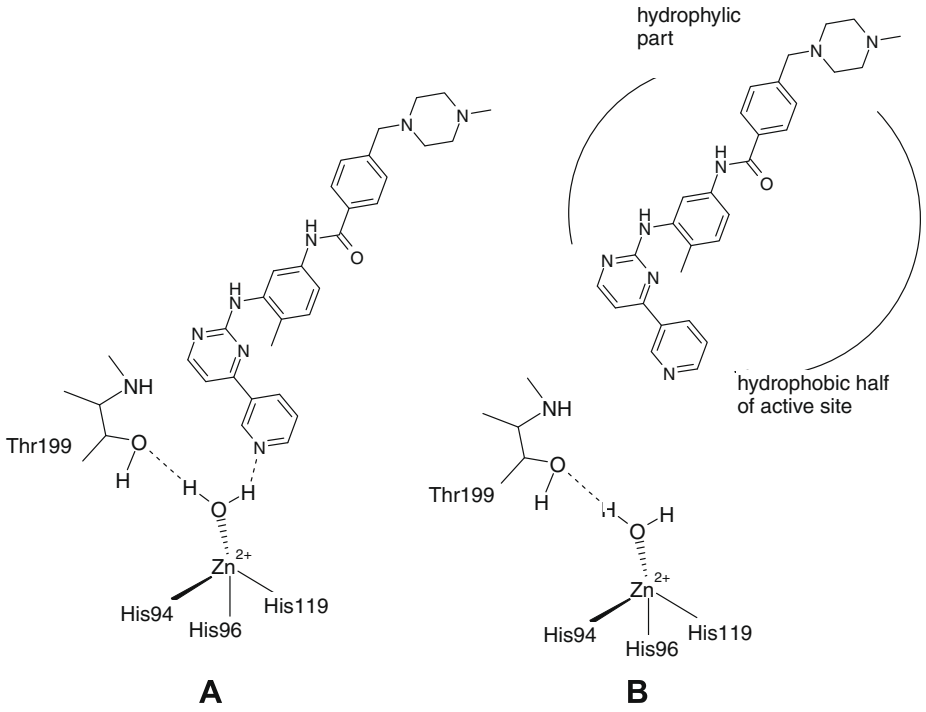

Figure 1. Proposed CA inhibition mechanism with the PTKI imatinib **1**. (A) The inhibitor is anchored to the Zn(II) coordinated water molecule from the hCA II active site. (B) The inhibitor does not interact with the metal ion or its non-protein ligand, but is anchored towards the entrance of the active site, similarly to the coumarin CAIs.[13c] The Zn(II) ion is coordinated by three His residues (His94, 96 and 119). The gate-keeper residues Thr199 (making a hydrogen bond with the fourth zinc ligand) is also shown.

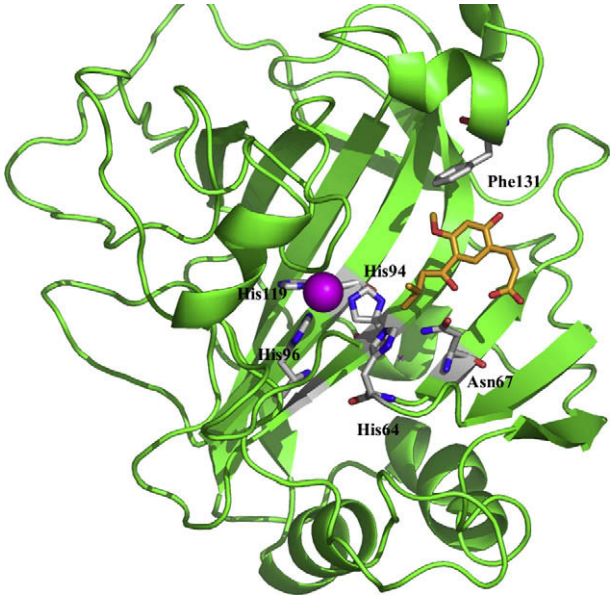

Figure 2. Binding of **5b** to hCA II as determined by X-ray crystallography.[13c] The protein is represented as green ribbon, the catalytic Zn(II) ion as the violet sphere and its three protein ligands (His94, 96 and 119) in CPK colors, as stick models. The inhibitor **5b** (in gold) and amino acid residues interacting with it at the entrance of the active site cavity, Phe131, Asn67, are also evidenced, together with His64, a residue involved in the catalytic cycle.[13c]

tumor microenvironment,[23b] and therefore their inhibition by imatinib and nilotinib might contribute to the efficacy of these drugs in GIST. Some other effects of these drugs might also be related to CA inhibition. Thus, CAs are known to play a pivotal role in bone metabolism,[24] and acetazolamide inhibits bone resorption in vitro/ex vivo,[24b] being shown to be effective for the long-term therapy of osteoporosis.[24a] Therefore, the recently reported long-term effect of imatinib therapy in promoting bone formation in CML patients,[26] mimics quite well the action of acetazolamide observed in the same clinical settings, and might be related to CA II inhibition in osteoclasts and osteoblasts, in addition to the inhibition of kinases. It is in fact well-known that several CA isozymes (CA II, CA XII and XIV) are involved in the acidification processes in osteoclasts, leading to inorganic matrix dissolution that precedes enzymatic removal of the organic bone matrix.[24c] By inhibiting these CA isozymes with sulfonamides, the osteoclasts acidification and bone dissolution processes are also inhibited.[24]

In conclusion, the two clinically used PTKIs, imatinib and nilotinib were investigated as inhibitors of the CA isoforms possessing catalytic activity, CA I–XV. The two compounds inhibited all 13 catalytically active mammalian isoforms with K_Is in the range of 4.1 nM–20.2 μM. CA I and CA II were the most efficiently inhibited isoforms (K_Is of 4.1–31.9 nM), whereas CA VA and VB showed the lowest affinity for these drugs (K_Is of 5.4–20.2 μM). A recent paper showed that the oxidoreductase NQO2 was bound and inhibited by these two drugs at pharmacologically relevant concentrations.[27] The anticancer activity of the two PTKIs investigated here may also involve their interaction with CAs in addition to the targets for which they were designed originally, that is, the PTKs.

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21. Khalifah, R. G.. *J. Biol. Chem.* **1971,** *246*, 2561. An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nM, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water with 5–10 % (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations. CA isozymes were recombinant ones obtained as reported earlier.[18–20]
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