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Carbonic anhydrase inhibitors: inhibition of the membrane-bound human isozyme IV with anions

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Abstract—The membrane-associated human isozyme of carbonic anhydrase, hCA IV, has been investigated for its interaction with anion inhibitors, for the CO_2 hydration reaction catalyzed by this enzyme. Surprisingly, halides were observed to act as potent hCA IV inhibitors, with inhibition constants in the range of 70–90 μM, although most of these ions, and especially fluoride, the best hCA IV inhibitor among the halides, are weak inhibitors of other isozymes, such as hCA I, II and V. The metal poisons cyanate, cyanide and hydrogen sulfide were weaker hCA IV inhibitors (K_i's in the range of 0.6–3.9 mM), whereas thiocyanate, azide, nitrate and nitrite showed even weaker inhibitory properties (K_i's in the range of 30.8–65.1 mM). Sulfate was a good hCA IV inhibitor (K_i of 9 mM), although it is a much weaker inhibitor of isozymes I, II, V and IX. Excellent hCA IV inhibitory properties showed sulfamic acid, sulfamide, phenylboronic acid and phenylarsonic acid, with K_i's in the range of 0.87–0.93 μM, whereas their affinities for the other investigated isozymes were in the millimolar range. The interaction of some anions with the mitochondrial isozyme hCA V has also been investigated for the first time here. It has been observed that among all these isozymes, hCA V has the lowest affinity for bicarbonate and carbonate (K_i's in the range of 82–95 mM), which may represent an evolutionary adaptation of this isozyme to the rather alkaline environment (pH 8.5) within the mitochondria, where hCA V plays important functions in some biosynthetic reactions involving carboxylating enzymes (pyruvate carboxylase and acetyl coenzyme A carboxylase). There are important differences of affinity for anions between the two membrane-associated isozymes, hCA IV and hCA IX.

1. Introduction

Among the 14 different α-carbonic anhydrase (CA, EC 4.2.1.1) isozymes isolated up to now in higher vertebrates, four are membrane-bound (CA IV, CA IX, CA XII and CA XIV), one (CA V) is mitochondrial and another one, CA VI, is secreted in saliva.[1–3] These enzymes, similarly to the better studied cytosolic isoforms CA I, II, III, VII and XIII,[4] are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate, but at least some of them possess an interesting catalytic versatility, being able to catalyze different other hydrolytic processes.[1,2]

The membrane-associated isoform CA IV was the first membrane-type CA to be isolated,[5,6] being originally purified from bovine lung as a 52 kDa glycoprotein with high CO_2-hydrase activity.[5] It was subsequently shown that the human isozyme, hCA IV, has many similar properties with the bovine enzyme (bCA IV), but in contrast to this, hCA IV is smaller (around 35 kDa) and contains no carbohydrate residues in its molecule.[6] Furthermore, CA IV is anchored to membranes in a unique way among the membrane-type CAs, by means of glycosylphosphatidylinositol moieties, from which it may be released by treatment with phosphatidylinositol-specific phospholipase C.[6,7] hCA IV is quite abundant in a multitude of tissues, such as nasal mucosa, oesophageal epithelium, kidneys, pancreas, salivary glands, heart muscle (endothelial and muscle cells), eyes, lungs, brain capillaries and colon, playing important physiological functions related to the nasal chemosensitivity to CO_2, the antireflux defense, bicarbonate reabsorption, NH_4^+ output,

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pH regulation, production of ocular fluid, gas exchange, etc., only to mention the most important ones.[8]

The catalytic properties of hCA IV have been studied in detail by Baird et al.[9] who showed that this is a high activity isoform both for the CO_2 hydration as well as the bicarbonate dehydration reactions. Furthermore, for the last reaction, hCA IV is even more active than hCA II, one of the best studied CAs, and also the isozyme known to play a host of critical physiological and physiopathological functions.[1–3,6,8] In this valuable paper,[9] Sly's group also investigated the inhibition of hCA IV with some anions, such as halides, bicarbonate, formate, acetate, sulfate and phosphate, for the bicarbonate dehydration reaction catalyzed by this isozyme. On the other hand, we have recently studied the inhibition of various CA isozymes (such as the α-CA isozymes V[10] and IX,[11] the γ-CA from a methanogenic archaeon, Cam,[12] etc.) with different classes of inhibitors, among which also the inorganic anions, but for the CO_2 hydration reaction.[10–12] In most published papers on CA inhibition with anions available up to now[13] the inhibition studies were done for the CO_2 hydration reaction (except the study of Baird et al.[9] mentioned above). Since hCA IV inhibition with this class of inhibitors is largely unexplored,[13] we report here an exhaustive such study, as well as a new procedure for obtaining with high yields recombinant hCA IV. Both physiological anions (such as chloride, bicarbonate) as well as 'metal poisons' (cyanide, cyanate, thiocyanate, azide, etc.) were included in this study, together with sulfamide and sulfamate (as sodium salt), the two simplest anions incorporating the sulfonamide moiety present in the other class of potent CA inhibitors with clinical applications, the aromatic/heterocyclic sulfonamides.[1–3] Inhibition data for two completely novel classes of CA inhibitors, the boronic acids and the phosphonic acids, are also presented for the first time here.

2. Chemistry

Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite and sulfate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid are also commercially available. Recombinant human isozymes hCA I, II, V and IX were used for comparison in the inhibition studies, which were obtained as reported earlier,[14–18] whereas hCA IV has been obtained by a new procedure described here.[19]

3. CA inhibition

Inhibition data against five CA isozymes, that is, hCA I, II, IV, V and IX, with anions as well as sulfamic acid, sulfamide, phenylboronic and phenylarsonic acids are shown in Table 1. Data of hCA I, II and IX have previously been published,[11,13] and are presented here because they are useful for the discussion, whereas on

Table 1. Inhibition constants of anionic inhibitors against human isozymes hCA I, II, IV and V, for the CO_2 hydration reaction, at 20 °C[20]

Inhibitor	$K_i^{\#}$ (mM)				
	hCA I[a]	hCA II[b]	hCA IV	hCA V[c]	hCA IX[f]
F^-	>300	>300	0.07	241	48
Cl^-	6	200	0.09 (36[d])	156	33
Br^-	4	63	0.09 (52[d])	50	16
I^-	0.3	26	0.08 (11[d])	25	7
CNO^-	0.0007	0.03	0.61	0.028	0.043
SCN^-	0.2	1.6	39.0	0.74	0.13
CN^-	0.0005	0.02	0.77	0.015	0.004
N_3^-	0.0012	1.5	65.1	0.30	0.005
HCO_3^-	12	85	6.6 (44[d])	82	13
CO_3^{2-}	15	73	5.7	95[e]	NT
NO_3^-	7	35	58.7	16	46
NO_2^-	8.4	63	30.8	16[e]	NT
HS^-	0.0006	0.04	3.9	0.023	0.007
HSO_3^-	18	89	13.2	65[e]	NT
SO_4^{2-}	63	>200	9.0 (44[d])	680[e]	NT
H_2NSO_3H[g]	0.021	0.39	9.3×10^{-4}	0.12	NT
$H_2NSO_2NH_2$	0.31	1.13	8.8×10^{-4}	0.84	NT
$PhB(OH)_2$	58.6	23.1	8.8×10^{-4}	4.5[e]	NT
$PhAsO_3H_2$[g]	31.7	49.2	8.7×10^{-4}	7.4[e]	NT

[#] Errors were in the range of 3–5% of the reported values, from three different assays.
[a] From Refs. 11 and 13.
[b] From Refs. 11 and 13.
[c] From Ref. 10.
[d] From Ref. 9, for the bicarbonate dehydration reaction (very high errors, generally ± 50% of the reported value).
[e] Data reported here for the first time.
[f] From Ref. 11.
[g] As sodium salt.

CA V these data are somehow more incomplete,[10] so that the inhibition with carbonate, nitrite, bisulfite, sulfate as well as phenylboronic and phenylarsonic acids are presented here for the first time, as they were not available in the literature.

As seen from data of Table 1, the membrane-associated isozyme hCA IV is inhibited by anions, with affinities for this class of inhibitors, which are quite different from those of hCA I (an isozyme quite susceptible to this class of inhibitors) and hCA II or hCA V (isozymes more resistant to inhibition by anions, but very susceptible to be inhibited by sulfonamides) and hCA IX (a membrane-bound isozyme more similar to hCA I and II regarding its affinity for anion inhibitors).[1–3,10,13] Thus, halides showed excellent hCA IV inhibitory properties, with inhibition constants in the range of 70–90 μM. This is rather surprising, especially for fluoride, the best hCA IV inhibitor among these anions, which is a very ineffective CA inhibitor for other investigated isozymes, such as CA I, II, V and IX (K_i's higher than 200 mM)—see Table 1. Chloride and bromide showed the same level of hCA IV inhibition for the CO_2 hydration reaction (all discussion here refer to this reaction, if not otherwise specified), although as reported earlier by Baird et al.,[9] these anions showed a diverse inhibitory capacity for the bicarbonate dehydration reaction. Iodide also was an excellent hCA IV inhibitor, with a potency intermediate between that of fluoride on one part, and chloride/bromide on the other one. It should be noted the important difference of affinity of these anions for the two membrane-bound CAs investigated here: hCA IV has a high affinity for such anions, whereas hCA IX a much lower one (Table 1).

Some other anions, such as cyanate, cyanide, bicarbonate, carbonate and hydrogen sulfide, showed a rather efficient hCA IV inhibition (but less as compared to that showed by halides), with inhibition constants in the range of 0.61–6.6 mM. Some of these anions are well known for their ability to complex metal ions present in metalloenzymes,[1–3] and for their strong inhibition of hCA I (K_i's of 0.5–0.7 μM for cyanate, cyanide and hydrogen sulfide) and hCA IX. Thus, it is interesting to note that their efficiency towards hCA IV is inferior as compared to that against isozymes I, but also II and V, which are all much more susceptible to be inhibited by them. The same situation is true for other 'metal-poison' anions, such as thiocyanate and azide, which are quite weak hCA IV inhibitors (K_i's of 39–65 mM), although they inhibit much better the other investigated isozymes. Inefficient inhibitors were also nitrate and nitrite (K_i's in the range of 30–58 mM), whereas bisulfite and especially, sulfate, behaved as much better hCA IV inhibitors as compared to hCA I, II or V inhibitors, with inhibition constants against isozyme IV in the range of 9–13 mM. The data of sulfate are indeed remarkable for two reasons: (i) this anion is a much weaker inhibitor of other isozymes, including hCA V, for which the data were not available up to now (a K_i of 680 mM against hCA V, see Table 1), and (ii) sulfate may be formally considered the anion from which other very potent CA inhibitors derive, such as the sulfamates (sulfamic acid), sulfamides and sulfonamides. Indeed, all these simple molecules were previously shown to act as CA inhibitors, and the X-ray crystal structure of the adduct of hCA II with sulfamic acid and sulfamide were reported by our group.[21] It is thus really amazing to observe that these two compounds are low micromolar inhibitors of hCA IV (K_i's of 0.88–0.93 μM), whereas their affinity for other isozymes, such as hCA I, II and V is much lower (K_i's in the range of 0.02–1.13 mM). These differences of affinity are tremendous, and difficult to explain for the moment, but may lead to the obtaining hCA IV-specific inhibitors. Indeed, all these isozymes possess the same ligands for the Zn(II) ion from their active site (His 94, His 96, His 119 and a water molecule/hydroxide ion, which participates in a hydrogen bond network with the OH moiety of the conserved residue Thr 199, which in turn is anchored to the carboxylate group of Glu 106, another conserved residue in all these isozymes).[1–3]

It should also be mentioned that other anions, which have been tested for the first time as hCA V inhibitors, such as carbonate, nitrite and bisulfite, have rather low affinity for this isozyme, with K_i's in the range of 16–95 mM (Table 1). It may be observed that bicarbonate is the worst CA V inhibitor among all the investigated isozymes, which may be put into relationship with the subcellular localization of this isozyme, present only in the mitochondria, at pH 8.5, where probably high amounts of this anion are present as a buffering system (carbonate/bicarbonate) for assuring this rather elevated pH value of this organellae. Thus, the relative insensivity at inhibition of hCA V with bicarbonate/carbonate, may represent an evolutionary adaptation of this isozyme to the relatively alkaline environment within the mitochondria, while still maintaining a good CO_2 hydration activity, necessary for the participation of this isozyme into biosynthetic reactions involving pyruvate carboxylase and acetyl coenzyme A carboxylase among others.[22]

Two other compounds, phenylboronic acid and phenylarsonic acid were investigated for the first time for their interaction with all these CA isozymes. It may be seen that both compounds are very weak hCA I and hCA II inhibitors (K_i's in the range of 23–58 mM), weak hCA V inhibitors (K_i's in the range of 4.5–7.4 mM), but very effective hCA IV inhibitors (K_i's in the range of 0.87–0.88 μM). We presume that these compounds bind to the zinc ion of the CA active site by coordinating to the metal ion, as outlined in Figure 1. Indeed, boronic acids are known to act as inhibitors of other metalloenzymes, such as for example the binuclear, manganese-containing arginase,[23] and that the trigonal planar boronic acid moiety undergoes nucleophilic attack from the metal ion bound hydroxide ion, with formation of a boronate anion, shown schematically in Figure 1A. As Zn(II) may also have a trigonal-bipyramidal geometry in CAs, this type of binding may also be considered, as shown schematically in Figure 1C. In addition to the Zn(II) ligands (His 94, 96 and 119), the gate-keeping residue Thr 199 is also proposed to participate in the stabilization of the hCA IV–phenylboronic acid complex, since it is well established the role this residue plays

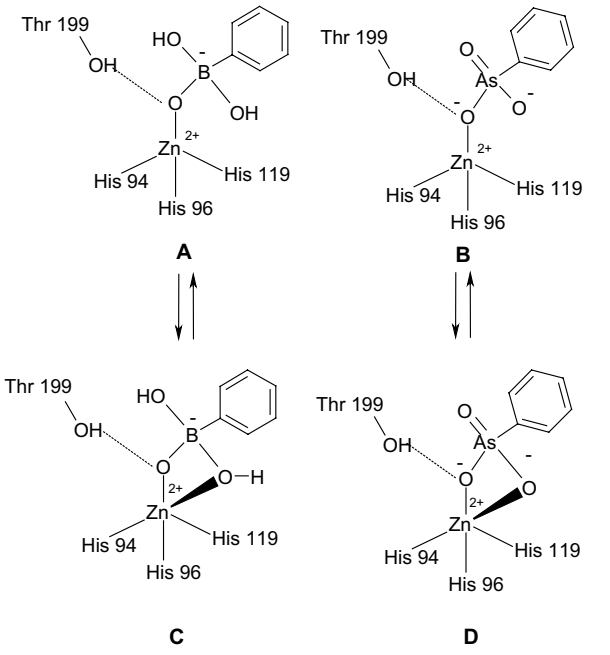

Figure 1. Proposed binding of phenylboronic acid and phenylarsonic acid to hCA IV: adduct with phenylboronic acid, Zn(II) ion in tetrahedral (A) or trigonal-bipyramidal (C) geometry. Adduct with phenylarsonic acid, Zn(II) ion in tetrahedral (B) or trigonal-bipyramidal (D) geometry. The protein zinc ligands (His 94, 96 and 119) and the gate-keeper residue Thr 199 are also shown schematically.

for the binding of inhibitors to the various CA isozymes.[1–3,20] In the case of phenylarsonic acid, again a binding with Zn(II) in tetrahedral (Fig. 1B) or trigonal-bipyramidal geometry (Fig. 1D) has been proposed, with the same role of Thr 199 as for the phenylboronic acid complex mentioned above.

It is rather difficult to rationalize at this moment, from a structural point of view, these results, but work is in progress in our laboratory to obtain X-ray crystal structures of some of these adducts. It is thus clear that CA IV-selective inhibitors should be obtained either starting from sulfamide/sulfamic acid, or from phenylboronic/phenylarsonic acids as lead molecules. In fact, preparation of isozyme-selective CA inhibitors targeting the many CAs involved in diverse physiological/physiopathological processes, is the ultimate goal of medicinal chemists involved in this fascinating field. A goal that remained much unfulfilled for the moment.

References and notes

1. *Carbonic Anhydrase–Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton (FL), USA, 2004; pp 1–363, and references cited therein.
2. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146–189; (b) Supuran, C. T.; Scozzafava, A. *Exp. Opin. Ther. Patents* **2002**, *12*, 217–242.
3. (a) Supuran, C. T. Carbonic anhydrases: catalytic mechanism, distribution and physiological roles. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, J., Conway, J., Eds.; CRC: Boca Raton FL, USA, 2004; pp 1–24; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Exp. Opin. Ther. Patents* **2004**, *14*, 667–702.
4. Lehtonen, J.; Shen, B.; Vihinen, M.; Casini, A.; Scozzafava, A.; Supuran, C. T.; Parkkila, A. K.; Saarnio, J.; Kivela, A. J.; Waheed, A.; Sly, W. S.; Parkkila, S. *J. Biol. Chem.* **2004**, *279*, 2719–2727.
5. Whitney, P. L.; Briggle, T. V. *J. Biol. Chem.* **1982**, *257*, 12056–12059.
6. Sly, W. S.; Hu, P. Y. *Annu. Rev. Biochem.* **1995**, *64*, 375–401.
7. Zhu, X. L.; Sly, W. S. *J. Biol. Chem.* **1990**, *265*, 8795–8801.
8. Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzym. Inhib. Med. Chem.* **2004**, *18*, 199–229.
9. Baird, T. T., Jr.; Waheed, A.; Okuyama, T.; Sly, W. S.; Fierke, C. A. *Biochemistry* **1997**, *36*, 2669–2678.
10. Franchi, M.; Vullo, D.; Gallori, E.; Antel, J.; Wurl, M.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2857–2861.
11. Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *J. Enzym. Inhib. Med. Chem.* **2003**, *18*, 403–406.
12. Innocenti, A.; Zimmerman, S.; Ferry, J. G.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4563–4567.
13. Ilies, M. A.; Banciu, M. D. Nonsulfonamide carbonic anhydrase inhibitors. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC: Boca Raton (FL), USA, 2004; pp 209–242.
14. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group (Lindskog, S.; Behravan, G.; Engstrand, C.; Forsman, C.; Jonsson, B. H.; Liang, Z.; Ren, X.; Xue, Y. In *Carbonic anhydrase—From Biochemistry and Genetics to Physiology and Clinical Medicine*; Botrè, F., Gros, G., Storey, B. T., Eds.; VCH: Weinheim, 1991; pp 1–13). Cell growth conditions were those described in Ref. 15 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[16] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\,mM^{-1}cm^{-1}$ for CA I and $54\,mM^{-1}cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.3 kDa for CA II, respectively.[11,18] hCA V has been obtained as described in Ref. 10.
15. Behravan, G.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1990**, *190*, 351–357.
16. Khalifah, R. G.; Strader, D. J.; Bryant, S. H.; Gibson, S. M. *Biochemistry* **1977**, *16*, 2241–2247.
17. Lindskog, S.; Coleman, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *70*, 2505–2508.
18. Steiner, H.; Jonsson, B. H.; Lindskog, S. *Eur. J. Biochem.* **1975**, *59*, 253–259.
19. Construction of bacterial expression vector containing hCAIV: truncated human carbonic anhydrase 4 (M83670, Genbank) was isolated from a human lung cDNA library by PCR using oligonucleotides F1 and R1 (F1, 5′-TGCAGAGTCACACTGGTGCT-3′; R1, 5′-TCATGCCTAAAGTCCCACCT-3′). The purified PCR product was used for the cloning of DNA encoding the mature protein (102–899bp) by PCR with oligonucleotides MP-F and MP-R (MP-F, 5′-GGGAATTCCATATGGCAGAGTCACAC-3′; MP-R, 5′-CCGCTCGAGGGACTTTATCACCGTGCG-3′). The resulting product was cut with *Nde*I and *Xho*I and ligated into the bacterial

expression vector pET24 (Novagen) using the *Nde*I and *Xho*I cloning sites to create pET24-hCAIV. Expression and purification of recombinant human CAIV: *Escherichia coli*, strain BL21-CodonPlus (DE3)-RIL (Stratagene), transformed with pET24-hCAIV was grown at 37 °C in 50 mL LB medium containing 25 μg/mL kanamycin. The overnight cultures were diluted 50 times into 0.5–1 L LB medium containing 25 μg/mL kanamycin and grown at 37 °C to OD_{600} 0.5–0.7. The expression of human CAIV was induced by adding 0.5 mM IPTG and the cells were grown at 37 °C for another 4 h. The bacterial cells were recovered by centrifugation at 5000*g* for 20 min at 4 °C and stored overnight at −80 °C. For purification the cells were thawed on ice for 15–30 min and resuspended in 5 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) per gram of cells containing 200 μg/mL lysozyme. The suspension was incubated on ice for 30 min and treated with a sonicator (six times for 10 s with 10 s cooling time in between). After centrifugation at 10,000*g* for 30 min at 4 °C the cleared lysate was incubated with Ni-NTA suspension (Qiagen). The expressed protein was purified according to the manufacturer's protocol. The isolated hCA IV was further purified by Sephadex G25 chromatography (Amersham). The purified enzyme had a catalytic activity very similar to that described in Ref. 9.

20. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA I, II, IV and V 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, 10 mM Na_2SO_4 (for maintaining constant the ionic strength—this anion is not inhibitory for most isozymes, except hCA IV, case in which it has been replaced by 10 mM $NaNO_3$, which is much less inhibitory, see Table 1), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. Saturated CO_2 solutions in water at 20 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10–50 mM (in the assay buffer) and dilutions up to 0.01 μM done with the assay buffer mentioned above. Enzyme concentrations were 0.09 μM for CA I, 0.06 μM for CA II, 1 μM for CA V and for hCA IV. Inhibition constants were calculated as described by: Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561–2573.
21. Abbate, F.; Supuran, C. T.; Scozzafava, A.; Orioli, P.; Stubbs, M. T.; Klebe, G. *J. Med. Chem.* **2002**, *45*, 3583–3587.
22. Supuran, C. T. *Exp. Opin. Ther. Patents* **2003**, *13*, 1545–1550.
23. Cama, E.; Shin, H.; Christianson, D. W. *J. Am. Chem. Soc.* **2003**, *125*, 13052–13057.