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N-Hydroxyurea—A versatile zinc binding function in the design of metalloenzyme inhibitors

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Abstract—*N*-Hydroxyurea binds both to carbonic anhydrase (CA) and to matrix metalloproteinases (MMPs). X-ray crystallography showed *N*-hydroxyurea to bind in a bidentate mode by means of the oxygen and nitrogen atoms of the NHOH moiety to the Zn(II) ion of CA, participating in a network of hydrogen bonds with a water molecule and Thr199. A derivatized *N*-hydroxyurea showed low-micromolar affinity for several CAs. This simple zinc binding function may be exploited for obtaining potent metalloenzyme inhibitors, due to its versatility of binding to the metal ion present in the active site of such enzymes.

Inhibitors of zinc enzymes need precise structural requirements in order to tightly bind to the metal ion(s) present in their active sites.[1] For example, primary sulfonamides (RSO_2NH_2), sulfamates ($ROSO_2NH_2$) or sulfamides ($RNHSO_2NH_2$) show high affinity for binding the Zn(II) ion present in α-carbonic anhydrases (CAs, EC 4.2.1.1), acting as potent inhibitors with clinical applications as antiglaucoma, diuretic, antiobesity or antitumor drugs.[2–6] Various CA isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target in fact quite different isozymes of the 15 presently known in humans.[2–6] In all of them, the sulfonamide/sulfamate/sulfamide drug binds in deprotonated form to the catalytically critical Zn(II) ion, also participating in extensive hydrogen bond and van der Waals interactions with amino acid residues both in the hydrophobic and hydrophilic halves of the enzyme active site, as shown by X-ray crystallographic work of enzyme–inhibitor complexes.[7–9] Although the Zn(II) coordination of CAs is identical to that of the matrix metalloproteinases (MMPs) (Fig. 1),[1] a family of zinc endopeptidases degrading extracellular matrix (and many other substrates),[1] being constituted of three histidine ligands and a water molecule/hydroxide ion acting as nucleophile in the hydrolytic processes, sulfonamides, sulfamates or sulfamides do not bind to MMPs with high affinity.[1,10] Instead, the main classes of MMP inhibitors are constituted by compounds incorporating hydroxamate or carboxylate zinc binding functions.[1,10–14] Indeed, X-ray crystallographic studies showed both the carboxylate as well as the hydroxamate deprotonated moieties to be coordinated in bidentate fashion to the catalytic Zn(II) ions present in many of the more than 25 MMPs presently known.[1,10–14] On the other hand, hydroxamates do show weak CA inhibitory properties,[10] whereas some structurally related zinc binding groups, such as the *N*-hydroxyurea one (a derivatized hydroxamate), seem to be appropriate for designing both CA[15] as well as MMP[13] inhibitors.

N-Hydroxyurea **1** was previously shown to act as a weak CA inhibitor,[15] similar to the isosteric acetohydroxamic acid **2**[10b] (Table 1).[16] The X-ray crystal structure of the adduct of isozyme hCA II with **2** has been reported, being shown that the hydroxamate moiety (deprotonated at the nitrogen atom) is coordinated in monodentate fashion to the Zn(II) ion, whereas its OH and CO groups participate in two hydrogen bonds with the OH moiety of Thr199 and another hydrogen bond with the backbone NH of the same residue.[10b] However, the X-ray crystal structure and thus the precise binding mode of **1** to CA II was not known up to now. Here we report the X-ray crystal structure of the adduct of hCA II with **1**, and its consequences for the design of metalloenzyme inhibitors (Table 2).

Keywords: Zinc enzyme; Carbonic anhydrase; MMP; *N*-Hydroxyurea; X-ray crystallography; Enzyme–inhibitor.
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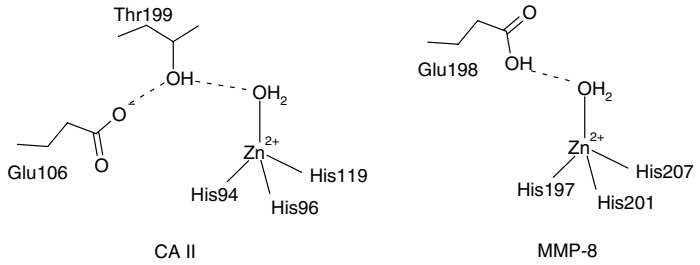
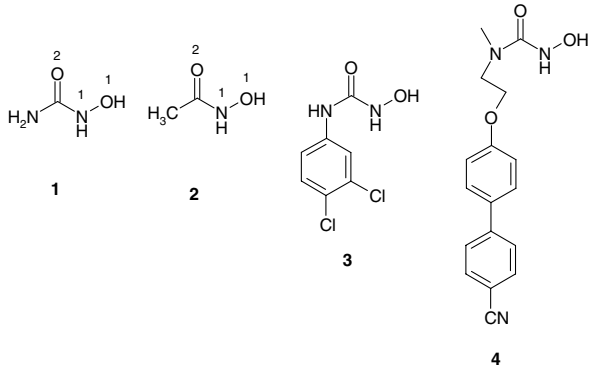


Figure 1. Zn(II) coordination sphere in CAs and MMPs.

Table 1. CA inhibition data with compounds **1**–**3**



Inhibitor	K_I[a] (μM)			
	hCA II[c]	hCA IV[c]	hCA VA[c]	hCA IX[d]
1	28	113	31	23
2	47[b]	nt	nt	nt
3	0.31	0.89	0.91	0.54

Compound **4** is a MMP inhibitor.[13]
nt, not tested.
[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] IC_{50} value, from Ref. 10b.
[c] Human (cloned) isozymes, by the CO_2 hydration method.[16]
[d] Catalytic domain of human, cloned isozyme, by the CO_2 hydration method.[16]

Table 2. Crystallographic parameters and refinement statistics

Parameter	Value
X-ray source	Enhance Ultra
Wavelength (Å)	1.54
Cell parameters	a = 42.20 Å
	b = 41.45 Å
	c = 72.26 Å
	β = 104.4°
	$\alpha = \gamma$ = 90°
Space group	$P2_1$
Water molecules	177
No. of unique reflections	16,238
Completeness[a] (%)	97.3 (93.9)
No. of reflections [$>2\sigma(F_o)$]	16,061
$\langle I/\sigma(I)\rangle$	10.0 (4.9)
Resolution range (Å)	10.0–2.0
R-merge (%)	14.0 (22.5)
Multiplicity	3.3 (2.8)
R-factor (%)	19.8
R-free[b] (%)	22.9
rmsd of bonds from ideality (Å)	0.012
rmsd of angles from ideality (°)	1.38

[a] Values in parentheses relate to the highest resolution shell (2.11–2.0).
[b] Calculated using 10% of data.

Analysis of the three-dimensional structure of the hCA II–**1** complex[17] revealed that the overall protein structure remained largely unchanged upon binding of the inhibitor. As a matter of fact, an rmsd value of 0.26 Å was calculated over the entire Cα atoms of hCA II–**1** complex, with respect to the unbound enzyme. The electron density of the inhibitor **1** bound in the neighborhood of the zinc ion is well defined (data not shown). Unexpectedly, as shown in Figure 2 and Table 3, *N*-hydroxyurea binds in a bidentate fashion to the Zn(II) ion within the hCA II active site, by means of both the nitrogen and oxygen atoms belonging to the NHOH moiety, with distances of these atoms from zinc in the range of 2.00–2.07 Å. Thus, unlike the isosteric hydroxamate **2**, which binds in monodentate fashion, only by means of the nitrogen atom of the NHOH moiety (with a distance Zn–N of 2.1 Å),[10b] *N*-hydroxyurea **1** is the first inhibitor showing this very exotic, bidentate binding illustrated in Figure 2 (a superposition of the two adducts, that is, hCA II–**1** and hCA II–**2**, is shown in Figure 3). The OH moiety of **1** (which is presumably deprotonated) also participates in a strong hydrogen bond (of 2.50 Å) with the OH moiety of Thr199 (the same bond was also observed in the adduct of **2** with hCA II). In addition, the oxygen of the carbonyl moiety of **1** makes another hydrogen bond with the backbone NH group of Thr199. This bond was also observed in the hCA II–**2** adduct.[10b] A water molecule (wat84) also participated in a network of two hydrogen bonds involving the OH and NH groups of the inhibitor molecule bound to zinc ion, as shown in Figure 3 and Table 3. Thus, a network of four hydrogen bonds and the bidentate coordination to the metal ion assure the binding of *N*-hydroxyurea to the CA active site. Figure 3 shows that although compounds **1** and **2** bind in the same region of the hCA II active site, near the zinc ion, they are not superposable, with *N*-hydroxyurea **1** binding much closer to the metal ion, so that the above-mentioned bidentate interaction can be achieved. Indeed, data of Table 3 clearly show that for **1**, in its complex with hCA II, both the N1 and O1 atoms of the NHOH moiety are at a distance of 2.00–2.07 Å from the Zn(II) ion. However, the corresponding oxygen atom (O1) of the hydroxamate **2** in the complex with hCA II is much further away from zinc, at a distance of 2.84 Å (whereas the nitrogen N1, which is coordinated to Zn(II), is at 1.96 Å, a distance compara-

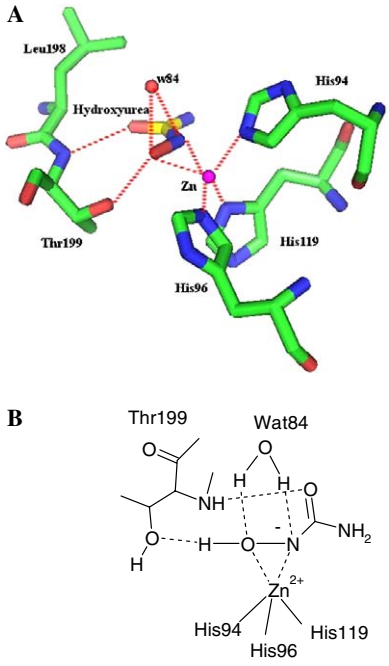


Figure 2. (A) *N*-Hydroxyurea interactions when bound to the active site of hCA II. (B) Schematic representation of the interactions between the inhibitor **1** and the hCA II active site. Distances between the Zn(II) ion and the inhibitor atoms, and hydrogen bonds in which the inhibitor is involved (dotted lines) are shown in Table 3.

Table 3. Hydrogen bonds and contacts of the hCA II–*N*-hydroxyurea **1** adduct and the hCA II–acetohydroxamic acid **2** adducts

	hCA II residue	Distance (Å)
Hydroxyurea 1		
O1	Zn	2.07
O1	Oγ Thr199	2.50
O1	w84	2.60
N1	Zn	2.00
N1	w84	2.92
O2	NH Thr199	2.75
Hydroxamate 2		
O1	Zn	2.84
N1	Zn	1.96
O2	w1	2.75
O2	N Thr199	2.80

ble to the corresponding distance in the hCA II–**1** adduct). These data clearly explain the better hCA II inhibition observed with **1** (K_I of 28 μM) as compared to **2** (an IC_{50} of 47 μM has been reported for this derivative),[10b] and also the fact that the inhibitor **1** is coordinated in a truely bidentate fashion to the metal ion, whereas the inhibitor **2,** like many other CA inhibitors investigated earlier, is bound in monodentate fashion to Zn(II). It should be also observed that various CA isozymes show quite different affinity for **1**, with CA II, VA, and IX being inhibited with K_Is in the range of 23-31 μM, whereas CA IV is less prone to be inhibited (K_I of 113 μM). But are these data relevant for the design of potent, medicinally useful metalloenzyme inhibitors?

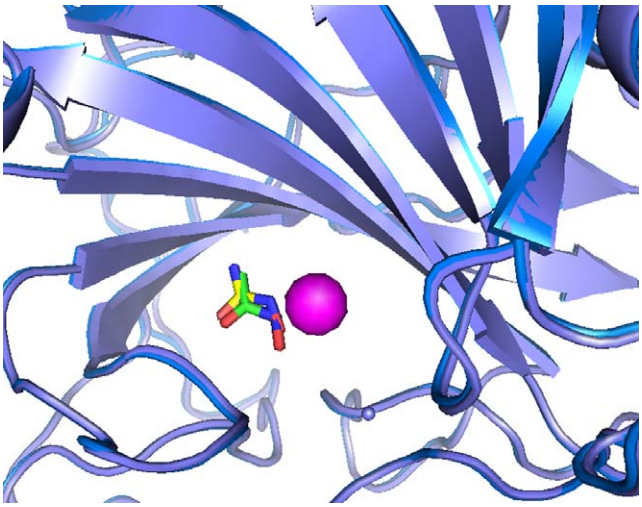


Figure 3. Superposition of the hCA II–$H_2NCONHOH$ **1** (in yellow) and hCA II–$CH_3CONHOH$ **2**[10b] (in green) adducts.

For replying to this question, we have prepared the substituted *N*-hydroxyurea **3**, by reacting 3,4-dichlorophenyl isocyanate with hydroxylamine, as reported in the literature.[22] Compound **3** was then tested comparatively with **1** for the inhibition of four physiologically relevant CA isozymes, CA II, IV, VA and IX (Table 1). It was observed that **3** acts as a low micromolar inhibitor of all isozymes, being 34–127 times more effective as CA inhibitor as compared to the lead **1**. Thus, using **1** as lead, much more potent CA inhibitors can be designed, as exemplified by this quite simple derivative **3**. Presumably, the zinc anchoring function of **3** binds in the same manner as **1** to the metal ion, but its organic scaffold may participate in supplementary interactions with amino acid residues present in the hydrophobic and hydrophilic halves of the hCA II active site (as evidenced in adducts of hCA II with sulfonamide or sulfamate inhibitors),[23] these favorable interactions explaining the enhanced inhibition of **3** against various CA isozymes are reported in Table 1.

Figure 4 also shows the binding of another substituted *N*-hydroxyurea derivative, compound **4**, to the Zn(II) ion in MMP-8, as recently reported by Campestre et al.[13] In this case, the oxygen atom of the NHOH moiety of **4** is coordinated to zinc, with a Zn–O distance of 2.3 Å.

In conclusion, this study proves that *N*-hydroxyurea constitutes a highly versatile ligand for the design of metalloenzyme inhibitors. The parent derivative acts as a weak inhibitor of several CA isozymes, and binds in bidentate fashion, by means of its oxygen and nitrogen atoms of the NHOH moiety to the Zn(II) ion of the active site, participating in a network of hydrogen bonds, similarly with the binding of the monodentate inhibitor acetohydroxamic acid. A slightly more complex *N*-hydroxyurea derivative, that is, 3-(3,4-dichlorophenyl)-1-*N*-hydroxyurea, showed on the other hand low micromolar affinity for several physiologically relevant CA isoforms, such as CA II, IV, VA, and IX, some of which are targets for the design of anti-obesity or anti-

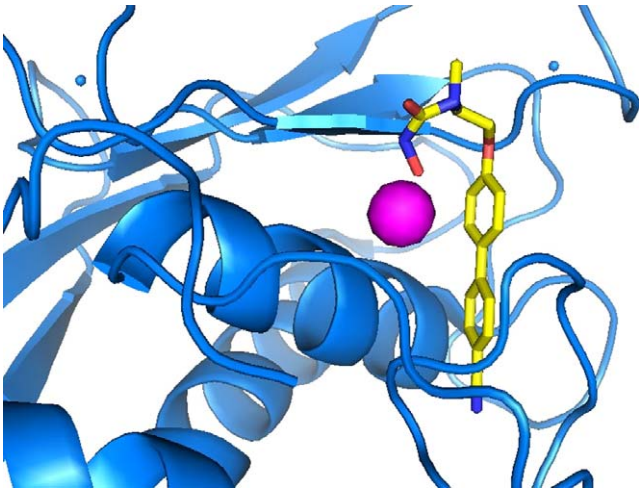


Figure 4. Binding of the *N*-hydroxyurea derivative **4** to MMP-8.[13]

tumor therapies. *N*-Hydroxyurea derivatives bind in a monodentate mode to MMPs, by means of the oxygen atom belonging to the NHOH moiety. This simple zinc binding function may be thus exploited for obtaining interesting and potent metalloenzyme inhibitors, due to its versatility of binding to the metal ion present in the active site of such enzymes.

Acknowledgments

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References and notes

1. Supuran, C. T.; Scozzafava, A. Matrix metalloproteinases (MMPs). In *Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development*; Smith, H. J., Simons, C., Eds.; Taylor & Francis: London & New York, 2002; pp 35–61.
2. Supuran, C. T.; Scozzafava, A.; Conway, J. *Carbonic Anhydrase—Its Inhibitors and Activators*; CRC: Boca Raton, New York, London, 2004, p 1–363.
3. (a) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199; (b) Supuran, C. T.; Scozzafava, A.; Casini, A. Development of Sulfonamide Carbonic Anhydrase Inhibitors. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; pp 67–147.
4. (a) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Expert Opin. Ther. Patent* **2004**, *14*, 667.
5. Winum, J. Y.; Scozzafava, A.; Montero, J. L.; Supuran, C. T. *Med. Res. Rev.* **2005**, *25*, 186.
6. Winum, J.-Y.; Scozzafava, A.; Montero, J.-L.; Supuran, C. T. *Expert Opin. Ther. Patent* **2006**, *16*, 27.
7. (a) Weber, A.; Casini, A.; Heine, A.; Kuhn, D.; Supuran, C. T.; Scozzafava, A.; Klebe, G. *J. Med. Chem.* **2004**, *47*, 550; (b) Di Fiore, A.; Pedone, C.; D'Ambrosio, K.; Scozzafava, A.; De Simone, G.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 437.
8. (a) De Simone, G.; Di Fiore, A.; Menchise, V.; Pedone, C.; Antel, J.; Casini, A.; Scozzafava, A.; Wurl, M.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2315; (b) Casini, A.; Antel, J.; Abbate, F.; Scozzafava, A.; David, S.; Waldeck, H.; Schafer, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 841.
9. (a) Abbate, F.; Coetzee, A.; Casini, A.; Ciattini, S.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 337; (b) Abbate, F.; Casini, A.; Owa, T.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 217.
10. (a) Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2000**, *43*, 3677; (b) Scolnick, L. R.; Clements, A. M.; Liao, J.; Crenshaw, L.; Hellberg, M.; May, J.; Dean, T. R.; Christianson, D. W. *J. Am. Chem. Soc.* **1997**, *119*, 850.
11. Santos, M. A.; Marques, S.; Gil, M.; Tegoni, M.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 233.
12. (a) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735; (b) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305.
13. Campestre, C.; Agamennone, M.; Tortorella, P.; Prezioso, S.; Biasone, A.; Gavazzo, E.; Pochetti, G.; Mazza, F.; Hiller, O.; Tschesche, H.; Consalvi, V.; Gallina, C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 20.
14. (a) Grams, F.; Crimmin, M.; Hinnes, L.; Huxley, P.; Pieper, M.; Tschesche, H.; Bode, W. *Biochemistry* **1995**, *34*, 14012; (b) Grams, F.; Reinemer, P.; Powers, J. C.; Kleine, T.; Pieper, M.; Tschesche, H. *Eur. J. Biochem.* **1995**, *228*, 830.
15. Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2003**, *11*, 2241.
16. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An applied photophysics 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 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 μM 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.[9–11]
17. The hCA II-**1** complex was co-crystallized at 4 °C by the hanging drop vapor diffusion method. Drops containing 5 μl of 10–20 mg/ml CAII in 50 mM Tris–HCl buffer, pH 7.7–7.8, were mixed with 5 μl of precipitant buffer (2.4–2.5 M $(NH_4)_2SO_4$ in 50 mM Tris–HCl, pH 7.7–7.8, and 1 mM sodium 4-(hydroxymercury)benzoate) with added 50 mM **1** and equilibrated over a reservoir of 1 ml of precipitant buffer. Crystals were transferred into a cryo-protectant solution (20% ethylene glycol) and mounted in nylon loop and exposed to a cold (100 K) nitrogen stream. Diffraction data were collected on a CCD Detector KM4

CCD/Sapphire using CuKα radiation (1.5418 Å). Data were processed with MOSFLM and CCP4 suite.[18,19] The structure was analyzed by difference Fourier technique, using the PDB file 1BV3 as starting model for refinement. Electron density maps ($2F_o - F_c$) and ($F_o - F_c$) were calculated with REFMAC5 program[20] and displayed using the graphic program O.[21] The final model of the complex had an *R*-factor of 19.8%, *R*-free 22.9%, for 16,061 reflections at $F > 2\sigma$ (F_o) in the resolution range 10.0–2.0 Å with a rmsd from standard geometry of 0.012 Å in bond lengths and 1.38° in bond angles. Crystallographic parameters and refinement statistics are summarized in Table 2. The X-ray coordinates of the hCA II–*N*-hydroxyurea adduct are available in PDB, with the ID 2GEH.

18. Leslie, A. G. W. *MOSFLM Users Guide*; MRC-LMB: Cambridge, UK, 1994.
19. Collaborative Computational Project, Number 4. *Acta Crystallogr.* **1994**, *D50*, 760.
20. Brunger, A. T.; Adams, P. D.; Clore, G. M.; Delano, W. L.; Gros, P.; Grosse-Kunsteleve, R. W.; Fiang, J.; Kuszewsky, J.; Niles, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simmonson, T.; Warren, G. L. *Acta Crystallogr.* **1998**, *D54*, 905.
21. Jones, T. A.; Zhou, J. Y.; Cowan, S. W.; Kjeldoard, M. *Acta Crystallogr.* **1991**, *A47*, 110.
22. Supuran, C. T.; Scozzafava, A.; Jurca, B. C.; Ilies, M. A. *Eur. J. Med. Chem.* **1998**, *33*, 83.
23. (a) Abbate, F.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2357; (b) Abbate, F.; Winum, J.-Y.; Potter, B. V. L.; Casini, A.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 231; (c) Di Fiore, A.; De Simone, G.; Menchise, V.; Pedone, C.; Casini, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1937; (d) Menchise, V.; De Simone, G.; Alterio, V.; Di Fiore, A.; Pedone, C.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 5721.