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Bioorganic & Medicinal Chemistry Letters 15 (2005) 367–372
Bioorganic & Medicinal Chemistry Letters

# Carbonic anhydrase inhibitors. Novel sulfanilamide/acetazolamide derivatives obtained by the tail approach and their interaction with the cytosolic isozymes I and II, and the tumor-associated isozyme IX

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Received 24 September 2004; revised 18 October 2004; accepted 21 October 2004
Available online 13 November 2004

**Abstract**—A series of sulfonamides has been obtained by reacting sulfanilamide or 5-amino-1,3,4-thiadiazole-2-sulfonamide with ω-chloroalkanoyl chlorides, followed by replacement of the ω-chlorine atom with secondary amines. Tails incorporating heterocyclic amines belonging to the morpholine, piperidine and piperazine ring systems have been attached to these sulfonamides, by means of an alkanoyl-carboxamido linker containing from two to five carbon atoms. The new derivatives prepared in this way were tested as inhibitors of three carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic isozymes CA I and II, and the catalytic domain of the transmembrane, tumor-associated isozyme CA IX. Several low nanomolar CA I and CA II inhibitors were detected both in the aromatic and heterocyclic sulfonamide series, whereas the best hCA IX inhibitors (inhibition constants in the range of 22–35 nM) all belonged to the acetazolamide-like derivatives.


## 1. Introduction

Inhibition of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) with sulfonamides may be exploited clinically for the treatment and prevention of a multitude of diseases.[1,2] With the early report that sulfanilamide **1**[3] acts as an inhibitor of CA, a great scientific adventure initiated, leading to the development of several classes of drugs based on the sulfonamide motif, such as the anti-hypertensives of the benzothiadiazine and high-ceiling diuretics types, the CA inhibitors (CAIs) initially used as antiglaucoma agents, some anti-thyroid drugs, hypoglycemic sulfonamides and ultimately to some novel types of anticancer and antiviral agents.[1,2,4–8]

Several sulfonamide CAIs are used clinically, such as acetazolamide **2**, methazolamide **3**, dichlorophenamide **4**, ethoxolamide **5**, dorzolamide **6** and brinzolamide **7**.[1,2]

Recently, the interest in this class of pharmacological agents has been revived by the finding that some of the 15 presently known CA isozymes in humans, are mainly found in tumors,[1,2,4–8] and that their inhibition may lead to tumor cell growth inhibition by complex mechanisms, that involve change of the tumor pH among others.[4–9] Very recently, Robertson et al.[9c] demonstrated the critical role played by CA IX in tumor growth and survival by using RNA interference protocols. One of our groups on the other hand reported inhibitors of one of the isozymes involved in tumorigenesis, that is, CA IX,[4–8] based both on the sulfonamide[4–9] as well as sulfamate types of compounds,[10,11] showing that such agents may be useful for the imaging of tumors or for inhibiting their growth both alone, or in combination with other anticancer agents.[9] Thus, CA IX represents a new and attractive target for the development of novel anti-tumor agents possessing different

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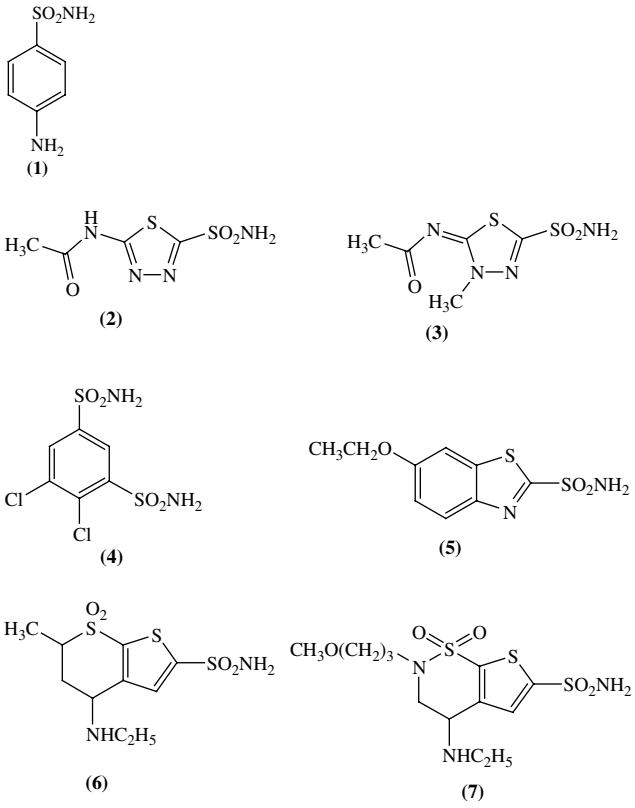
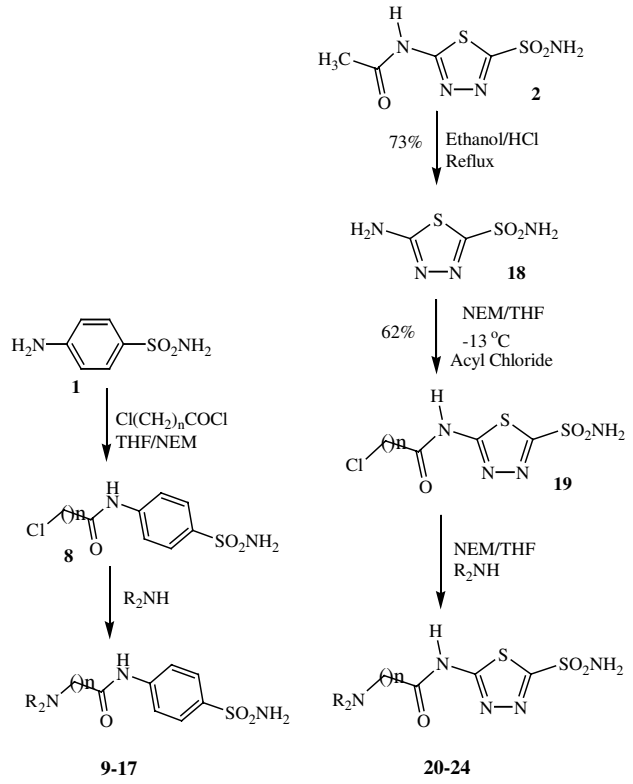
doi:10.1016/j.bmcl.2004.10.070




**Scheme 1.** Synthesis of the new derivatives **9**–**17** and **20**–**24** reported in the paper.

mechanisms of action.[1,2,9] Here we extend this research in the design of such enzyme inhibitors, and present the synthesis and CA IX inhibition data for two series of sulfonamides based on the sulfanilamide and acetazolamide motifs. These compounds have also been tested for the inhibition of the major cytosolic isozymes I and II, widely distributed in the human body, and participating in important physiological/pathological processes.[1,2]

## 2. Chemistry

In previous work[12] from one of our groups, some analogues of acetazolamide and α-fluorobenzyl sulfonamides were synthesized. In vitro inhibitory activity of such compounds showed them to be potent inhibitors of isozyme CA II. Considering the fact that a general approach for the synthesis of strong CAIs with different applications both as antiglaucoma or anti-tumor agents among others has recently been described, that is, the tail approach,[13] in this paper we report the application of the tail approach for the synthesis of CAIs incorporating protonatable heterocyclic moieties in the sulfanilamide and acetazolamide scaffolds, in order to design putative CA IX inhibitors with enhanced affinity for the enzyme and eventually increased water solubility.

Our first aim was to synthesize novel sulfanilamide derivatives of type **9**–**17** (Scheme 1). Sulfanilamide **1** was reacted with 3-chloropropionyl chloride (or the congeneric derivatives with one, two or four carbon atoms in their molecule) in the presence of triethylamine (TEA) or *N*-ethyl-morpholine (NEM). The chloro-substituted amide key intermediates **8** were then treated with secondary amines such as methylpiperazine, morpholine, benzyl-piperidine, benzyl-piperazine and methyl-piperidine in dry THF, in order to obtain the desired tertiary amines **9**–**17** (Table 1).

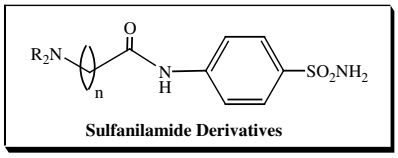
Our second aim was to synthesize novel acetazolamide derivatives of type **20**–**24**, by the same approach described above for the corresponding sulfanilamide compounds **9**–**17** (Scheme 1). Acetazolamide **2** was used as starting material, being deacetylated with concentrated hydrochloric acid, when 5-amino-1,3,4-thiadiazole-2-sulfonamide **18** was obtained.[14] The second step of the synthesis consisted in the reaction of amine **18** with chloroacyl chlorides (i.e., 2-chloroethanoyl chloride or 3-chloropropanoyl chloride), leading to the second key intermediates, of types **19**, which have been further derivatized as shown above for the corresponding sulfanilamide compounds. The new sulfonamides **20**–**24** were thus obtained in good yield (Table 2 and Scheme 1).[15]

## 3. CA inhibition

Inhibition data against three CA isozymes, that is, hCA I, II and IX, with the new sulfonamides reported here and some standard CAIs, of types **1**–**6**, are shown in Table 3.[16]

The following should be noted regarding data of Table 3: (i) derivatives **9**–**17** and **20**–**24** reported here generally

**Table 1.** Sulfanilamide derivatives **9**–**17** prepared in this study



Sulfanilamide Derivatives

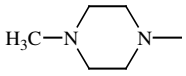
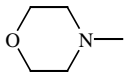
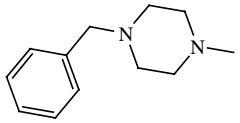
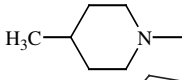
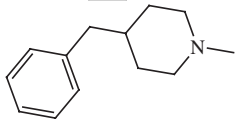
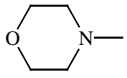
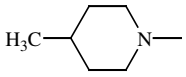
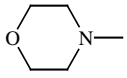
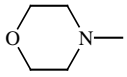
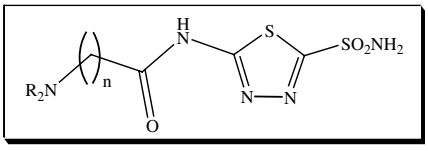
| *n* | Compd no | $R_2N$– | Yield% |
|---|---|---|---|
| 2 | **9** |  | 15 |
| 2 | **10** |  | 75 |
| 2 | **11** |  | 65 |
| 2 | **12** |  | 72 |
| 2 | **13** |  | 62 |
| 1 | **14** |  | 70 |
| 3 | **15** |  | 64 |
| 3 | **16** |  | 65 |
| 4 | **17** |  | 65 |

**Table 2.** Acetazolamide-like derivatives **20**–**24** synthesized in this study



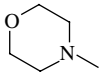
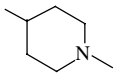
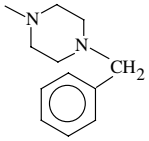
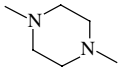
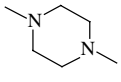

| *n* | Compd no | $R_2N$– | Yield% |
|---|---|---|---|
| 1 | **20** |  | 70 |
| 1 | **21** |  | 60 |
| 1 | **22** |  | 60 |
| 1 | **23** |  | 50 |
| 2 | **24** |  | 65 |

**Table 3.** Inhibition data for sulfonamides **9**–**24** investigated in the present paper and standard sulfonamide CAIs **1**–**6**, against isozymes hCA I, II and IX[16]

| Inhibitor | $K_I$[a] (nM) | | | Selectivity ratio |
|---|---|---|---|---|
| | hCA I[b] | hCA II[b] | hCA IX[c] | $K_I$ (hCA II)/ $K_I$ (hCA IX) |
| **1**[d] | 28,000 | 300 | 294 | 1.02 |
| **2**[d] | 900 | 12 | 25 | 0.48 |
| **3**[d] | 780 | 14 | 27 | 0.52 |
| **4**[d] | 25 | 8 | 34 | 0.23 |
| **5**[d] | 1200 | 38 | 50 | 0.76 |
| **6**[d] | 50,000 | 9 | 52 | 0.05 |
| **9** | 644 | 165 | 235 | 0.70 |
| **10** | 9.4 | 82 | 184 | 0.44 |
| **11** | 549 | 234 | 240 | 0.97 |
| **12** | 381 | 173 | 197 | 0.87 |
| **13** | 752 | 258 | 248 | 1.04 |
| **14** | 9.6 | 87 | 181 | 0.48 |
| **15** | 820 | 265 | 90 | 2.94 |
| **16** | 371 | 104 | 246 | 0.42 |
| **17** | 7.9 | 77 | 163 | 0.47 |
| **18**[d] | 8600 | 60 | 41 | 1.46 |
| **20** | 14.0 | 0.9 | 22 | 0.04 |
| **21** | 8.2 | 3.8 | 35 | 0.10 |
| **22** | 7.6 | 1.8 | 67 | 0.02 |
| **23** | 9.6 | 1.6 | 31 | 0.05 |
| **24** | 7.1 | 1.9 | 33 | 0.05 |

[a] Errors in the range of 5–10% of the reported value (from three different assays).
[b] Human (cloned) isozymes, by the $CO_2$ hydration method.
[c] Catalytic domain of human, cloned isozyme, by the $CO_2$ hydration method.[23]
[d] From Ref. 24.

act as better inhibitors against all three investigated isozymes, as compared to the parent sulfonamides from which they were prepared, that is, sulfanilamide **1** in the case of the first derivatives, and aminothiadiazole-sulfonamide **18**, in the case of the acetazolamide-like derivatives **20**–**24**; (ii) against hCA I, three sulfanilamides, that is, **10**, **14** and **17**, and all the acetazolamide-like derivatives **20**–**24**, act as very potent inhibitors, with inhibition constants in the range of 7.1–14.0 nM, being much more effective than the two lead compounds **1** and **18**, which showed $K_I$ values in the range of 8600–28,000 nM. These compounds are also much better hCA I inhibitors as compared to the clinically used derivatives **1**–**6** (Table 3). It is very interesting to note that for the sulfanilamide derivatives **10**, **14** and **17**, all these potent inhibitors incorporate the morpholine moiety in their tail, whereas for the heterocyclic series **20**–**24**, the corresponding morpholine derivative (**20**) was the least active. It is quite difficult to rationalize these results at the present moment. The number of aliphatic carbon atoms separating this heterocyclic amine moiety from the sulfanilamide scaffold in derivatives **10**, **14** and **17** seems to be less important for these aromatic CAIs, since both the mono-, two- or four-carbon atoms derivatives showed quite comparable enzyme inhibitory activity. On the other hand, the other aromatic derivatives, that is, **9**, **11**–**13**, **15** and **16**, showed moderate hCA I inhibitory properties, with $K_I$ values in the range of 371–820 nM; (iii) against hCA II, the five heterocyclic sulfonamides **20**–**24** showed excellent

inhibitory properties, with $K_I$ values in the range of 0.9–3.8 nM, being thus much better inhibitors than the clinically used compounds **1**–**6** or the lead molecule **18**. In the aromatic subseries, again **10**, **14** and **17** were the best inhibitors, with $K_I$ values in the range of 77–87 nM, whereas the other derivatives (**9**, **11**–**13**, **15** and **16**) showed weaker inhibition, with $K_I$ values in the range of 104–265 nM. Thus, SAR is relatively simple, since heterocyclic, acetazolamide-like derivatives act as excellent inhibitors irrespective of the heterocyclic moiety incorporated into the tail, whereas for the sulfanilamide-like compounds **9**–**17**, best CA inhibitory properties are induced by morpholine-containing tails. Other heterocyclic tails (piperazine, benzyl/methyl-piperazine, methylpiperidine, etc.) and diverse length of the spacer between the heterocyclic moiety and the sulfanilamide scaffold (from C1 to C4), influence less the biological activity of these derivatives; (iv) against the tumor-associated isozyme hCA IX, best inhibitory activity was shown by four of the heterocyclic compounds, that is, **20**, **21**, **23** and **24**, which possessed $K_I$ values in the range of 22–35 nM, being more effective inhibitors than the lead molecule **18** ($K_I$ of 41 nM) and having activity in the same range as the clinically used derivatives **2**–**6** (Table 3). Weaker inhibitors were then **22** and the aromatic sulfonamide **15** ($K_I$ values in the range of 67–90 nM), whereas the other sulfanilamide-like compounds (i.e., **9**–**14** and **16**, **17**) showed moderate hCA IX inhibitory properties, with inhibition constants in the range of 163–248 nM; (v) very few of the new derivatives reported here showed any selectivity for the tumor-associated over the cytosolic isozyme II. Thus, only compound **15** has a selectivity ratio close to 3, derivative **13** has this parameter close to the unity, whereas all other derivatives, similarly to the clinically used sulfonamides, act as better CA II than CA IX inhibitors (Table 3).

## 4. Conclusions

A series of sulfonamides has been obtained by applying the tail approach to the sulfanilamide and 5-amino-1,3,4-thiadiazole-2-sulfonamide scaffolds. Tails incorporating heterocyclic amines belonging to the morpholine, piperidine and piperazine ring systems have been attached to such sulfonamides, by means of an alkanoylcarboxamido linker containing from two to five carbon atoms. The new derivatives prepared in this way were tested as inhibitors of three CA isozymes, the cytosolic major isozymes I and II; and the transmembrane, tumor-associated isozyme IX. Several low nanomolar CA I and CA II inhibitors were detected both in the aromatic and heterocyclic sulfonamide series, whereas the best hCA IX inhibitors (inhibition constants in the range of 22–35 nM) all belonged to the acetazolamide-like derivatives.

## Acknowledgements

This research was financed in part by a 6th frame EU project (EUROXY), by the Turkish Government Planning Organisation (DPT Project No: 2003K120590) and by grants from Harran University Research Financial Centre (HUBAK Project No: 348, 295 and 505). We would like to thank Prof. Dr. Nurettin Yayli (KTU, TURKEY) for $^{1}H/^{13}C$ NMR spectral analyses. Some of us are grateful to Prof. Dr. G. M. Blackburn (Sheffield University, UK) for helpful discussions. Special thanks are addressed to Professor Raffaello Giannini and Dr. Cristina Vettori (CNR, IGV Department, Florence, Italy) for their invaluable help.

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15. An example of the new compounds prepared: The key intermediate *4-(3-Chloropropionylamino)-benzenesulfonamide* (**8**): Sulfanilamide **1** (5.40 g, 0.03 mol) and NEM (3.80 g, 0.03 mol) were stirred in THF (200 mL) until most of the starting material had dissolved. 3-Chloropropanoyl chloride (7.71 g, 0.06 mol) in THF was slowly added to the reaction mixture. The reaction was stirred at −15 °C for 4 h under anhydrous conditions. After warming to room temperature the white precipitate of NEM·HCl salt filtered off. The THF was removed in vacuo and the resulting white solid dissolved in ethyl acetate. The organic extract was washed with 3 M hydrochloric acid (20 mL) then with saturated sodium bicarbonate solution (20 mL) and finally with brine. Drying over magnesium sulfate and evaporation yielded a white solid (**8**) which was recrystallized from water to give the title compound. (70%), mp 228–230 °C; $\lambda_{max}$ (KBr, $cm^{-1}$) 1672 (NHCO), 1186 ($SO_2NH_2$), 1532 (C═N), 661 (C–Cl); $\delta_H$ (DMSO-$d_6$) 10.3 (1H, s, –CONH), 7.8 (4H, m, –Ar–H), 7.0 (2H, s, $SO_2NH_2$), 3.87 (2H, t, *J* 6 Hz, Cl$CH_2$), 2.88 (2H, t, *J* 6 Hz, –$CH_2$CO); $\delta_C$ (DMSO-$d_6$) 172.84 (C═O), 140.75 (CNH–), 136.92 (C–$SO_2NH_2$), 125.7 (C-2 Aryl), 125.7 (C-2 Aryl), 117.99 (C-3 Aryl), 117.99 (C-3 Aryl), 38.7 ($CH_2$Cl), 37.87 ($CH_2$CO); *m/z* $EI^+$ 264 $[M]^+$. *4-(3-Methylpiperazinopropionylamino)-benzenesulfonamide* (**9**): To a stirred solution of an excess of methylpiperazine (3 equiv) in tetrahydrofuran (20 mL) was added compound **8** (1.00 g, 3.80 mmol) over 30 min at 0 °C. The reaction was allowed to warm to room temperature and stirred at 40 °C for 48 h. The impurities were removed by flash column chromatography (ethyl acetate/methanol, 6:1) to give the title compound (65%), mp 199–200 °C; $\lambda_{max}$ (KBr, $cm^{-1}$) 1681 (NHCO), 1152 ($SO_2NH_2$); $\delta_H$ (DMSO-$d_6$) 10.78 (1H, s, –CONH), 7.84 (4H, m, –Ar–H) 7.7 (2H, s, $SO_2NH_2$), 2.74 (2H, t, *J* 6 Hz, –N$CH_2$), 2.58 (4H, t, *J* 6 Hz, C$H_2$N$CH_2$), 2.55 (4H, t, *J* 6 Hz, C$H_2$N$CH_2$), 2.28 (2H, s, *J* 6 Hz, C$H_2$CO), 2.15 (3H, s, C$H_3$N–); $\delta_C$ (DMSO-$d_6$) 172.27 (C═O), 141.14 (CNH–), 134.99 (C–$SO_2NH_2$), 126.12 (C-2 Aryl), 126.12 (C-2 Aryl), 118.12 (C-3 Aryl), 118.12 (C-3 Aryl), 53.97 ($CH_2$N$CH_2$), 52.55 ($CH_2$N$CH_2$), 51.27 ($CH_2$N–), 44.87 ($CH_3$N–), 32.47 ($CH_2$CO); *m/z* $EI^+$ 327 $[M]^+$.
16. 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 et al.[17] Cell growth conditions were those described in Ref. 18 and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[19] 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.[20,21] A variant of the previously published[9,10] CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.[22]) was amplified by using PCR and specific primers for the glutathione *S*-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *E. coli* BL21 Codon Plus bacterial strain (from Stratagene). The bacterial cells were sonicated, then suspended in the lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8, 150 mM NaCl and 0.2% Triton X-100). After incubation with lysozime (approx. 0.01 g/L) the protease inhibitors Complete™ were added to a final concentration of 0.2 mM. The obtained supernatant was then applied to a prepacked Glutathione Sepharose 4B column, extensively washed with buffer and the fusion (GST-CA IX) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The advantage of this method over the previous one,[9,10] is that CA IX is not precipitated in inclusion bodies from which it has to be isolated by denaturing–renaturing in the presence of high concentrations of urea, when the yields in active protein were rather low, and the procedure much longer. The obtained CA IX was further purified by sulfonamide affinity chromatography,[19] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with $CO_2$ as substrate.[23] The specific activity of the obtained enzyme was the same as the one previously reported,[9,10] but the yields in active protein were 5–6 times higher per litre of culture medium. An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed $CO_2$ hydration activity.[23] 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 for a period of 10–100 s. Saturated $CO_2$ solutions in water at 20 °C were used as substrate.[23] Stock solutions of inhibitor (1 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 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. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The activity of these enzyme preparations seem to be rather similar with the in vivo activity of CA IX in tumors as recently proven by CA inhibitor binding studies and disturbance of tumor pH following CA IX inhibition as compared to normal tissue pH[9].
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