Inhibition of Carbonic Anhydrase-II by Sulfamate and Sulfamide Groups: An Investigation Involving Direct Thermodynamic Binding Measurements

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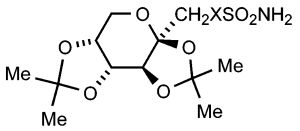
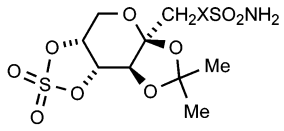
This paper examines the relative effectiveness of bioisosteric sulfamate and sulfamide derivatives for inhibition of human carbonic anhydrase-II (CA-II) by using a direct binding assay based on the ThermoFluor method (Matulis et al. *Biochemistry* **2005**, *44*, 5258). Compounds **1**−**10**, which represent five cognate sulfamate/sulfamide pairs, were studied by ThermoFluor to obtain binding affinities (K_a values). The corresponding dissociation constants, K_d, provide an independent measure of CA-II activity relative to commonly used K_i values from enzyme kinetics studies. There was a sizable difference in potency between the sulfamates and sulfamides, with the sulfamides being much less potent, by factors ranging from 25 (**7**/**8**) to 1200 (**3**/**4**). These results are consistent with our recent report that sulfamides tend to be much weaker inhibitors of CA-II than their corresponding sulfamates (Maryanoff et al. *J. Med. Chem.* **2005**, *48*, 1941). Additionally, for arylsulfamides **10**−**12** the K_d values determined by ThermoFluor and the K_i values determined from enzyme kinetics are consistent. It appears that the sulfamide group is less suitable than the sulfamate group for obtaining potent inhibition of CA-II.

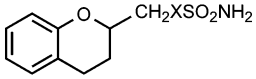
Carbonic anhydrase (CA) enzymes (EC 4.2.1.1), which are involved in catalyzing the hydration of carbon dioxide and the dehydration of bicarbonate [$CO_2 + 2\ H_2O \leftrightarrows HCO_3^- + H_3O^+$], are biochemically well characterized and clinically relevant.[1−4] CA-II, the most commonly studied isoform, is also the most prevalent isoform in many different organs and cell types.[2,4] For several decades, it has been widely recognized that potent inhibitors of CA-II can be derived by having a primary sulfonamide group (SO_2NH_2) on a suitable organic scaffold, such as benzenoid or heterocyclic structures.[3,5,6] This situation is not too surprising since CA-II contains a functional Zn(II) atom in its active site that is normally bound to the imidazole nitrogen atoms of three histidine residues (His-94, His-96, and His-119 in human CA-II).[6,7] Other Zn(II)-coordinating groups may also provide compounds that inhibit CA-II. However, it is important to note that inhibitors based on such Zn(II) ligands do not always operate with a high level of effectiveness (i.e., they can exhibit just moderate-to-weak potency).[3]



1 X = O
2 X = NH



3 X = O
4 X = NH



5 X = O
6 X = NH

Since our discovery of the antiepileptic drug topiramate (**1**),[8] we have developed an abiding interest in the pharmacological actions of compounds containing a primary sulfamate functionality (−OSO_2NH_2).[9] Coincident with our drug discovery efforts, we were prompted to investigate the inhibition of CA-II, as a possible mechanism of action and a potential source of additional pharmacology.[8,9a−c,10] In a recent article we reinforced the viewpoint that topiramate is a moderate inhibitor of CA-II (K_i value of ca. 500 nM),[9c] as opposed to being a potent inhibitor (K_i value <50 nM).[11] Interestingly, we also found that compounds containing a primary sulfamide group (−$NHSO_2NH_2$) can possess substantially weaker CA-II inhibitory potency than their corresponding sulfamates.[9c] To be more specific, a direct comparison of sulfamate/sulfamide bioisosteric pairs, such as **1**/**2**, **3**/**4**, and **5**/**6**, revealed dramatically different CA-II inhibition, with CA-II K_i values of 500/650000 nM for **1**/**2** and 12/20000 nM for **3**/**4**, and CA-II IC_{50} values of 130/71000 nM for **5**/**6** (by the pH-shift method).[9c] However, this divergence in CA-II inhibitory behavior between cognate sulfamates and sulfamides has not met with universal acceptance.[12] In 2003, Casini et al. reported that several sulfamides of relatively simple structure (e.g., $PhNHSO_2NH_2$) possess CA-II K_i values below 50 nM,[13] and other papers have also mentioned sulfamides as relatively potent CA-II inhibitors.[11a,12,14] Since the degree of CA-II inhibition attainable with sulfamate and sulfamide groups is an important issue with respect to future drug design, we sought to identify a means to reconcile these disparate views.

Are corresponding pairs of sulfamates and sulfamides nearly the same or very different in CA-II inhibitory potency, and are the sulfamides much weaker? Almost all CA-II inhibition data in this field have emanated from enzyme kinetics studies, often based on two standard assay protocols ("pH-shift" and "esterase" methods).[3b,9c,11−14] Despite the fact that we have obtained consistent results with both of these methods,[9c] it is not unheard of for enzyme kinetics studies in different laboratories to experience discrepancies in outcomes because of differences in experimental techniques and/or reaction conditions. To resolve this conundrum, we decided to employ an independent methodology that does not rely at all on enzyme kinetics, but rather relies on the direct determination of binding affinity, in terms of thermodynamic dissociation constants (K_d values). Thus, we

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Table 1. CA-II Binding Affinity and Inhibition Data for Compounds **1**−**10**

compd	K_d (nM)[a]	fold diff[b]	K_i (nM)[c]	fold diff[b]
1	290	- -	500[d]	- -
2	25000	88	650000[d]	1300
3	10	- -	12[d]	- -
4	12500	1250	20000[d]	1670
5	140	- -	130[d]	- -
6	10000	70	71000[d]	550
7	2000	- -	1020 (650−1590)[e]	- -
8	50000	25	408000 (255000−654000)[e]	400
9	110	- -	36 (26−50)[e]	- -
10	4550	40	7960 (4880−13000)[e]	220

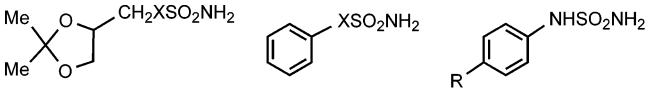
[a] The binding affinity to human CA-II was determined by the ThermoFluor method. [b] Fold difference in comparing the result for the sulfamide with that for its corresponding sulfamate. [c] Inhibition of human CA-II by measuring hydration of carbon dioxide via the shift in pH. [d] Data taken from ref 9c. [e] New data. The 95% confidence intervals are given in parentheses ($N = 2$ for **7** and **8**; $N = 3$ for **9**; $N = 4$ for **10**).

applied the specific thermochemical technique known as ThermoFluor,[15] which was used effectively to evaluate the affinity of six reference sulfonamide-based inhibitors of CA-II.[16] Herein, we report ThermoFluor results for five pairs of cognate sulfamates and sulfamides. The K_d values observed for these bioisosteric pairs are consistent with our K_i values from enzyme kinetics experiments and lend further support to the fact that the sulfamide group is not very effective for generating potent CA-II inhibitors.

Results and Discussion

CA-II Inhibition and Binding. To address the apparent inconsistencies in results for the inhibition of CA-II by sulfamide derivatives on the basis of enzyme kinetics studies,[9c,12−14] we conducted a thermodynamically oriented investigation based on the ThermoFluor method.[15,16] This method provides a thermal melting curve, derived from the change in fluorescence intensity for an environmentally sensitive dye, such as 1-anilino-8-naphthalenesulfonic acid (ANS), as a function of temperature. From this melting curve the midpoint transition temperature T_m, a measure of protein stability, can be determined. The binding affinity ($K_a = 1/K_d$) is directly related to the increase in protein stability, and T_m, in the presence of the test compound (L).[15] The magnitude of the change in T_m is proportional to both the ligand concentration, log[L], and the binding affinity. Since ThermoFluor is a plate-based technology, precise T_m values can be established rapidly under diverse experimental conditions, and with as many as 32 different inhibitor candidates in the same experiment. The binding affinity at the T_m, namely $K_{a,Tm}$, is calculated with the aid of calorimetrically measured parameters for protein stability, and is then extrapolated to 37 °C.[16] The ThermoFluor method was thoroughly validated for CA-II, as well as for CA-I, with six sulfonamide inhibitor ligands.[16]

By using ThermoFluor, we have determined the behavior of human carbonic anhydrase-II in the presence of compounds **1**−**10**, which represent five cognate sulfamate/sulfamide pairs, to derive K_d values (Table 1). The ThermoFluor-generated curves for **1**−**10** with CA-II, which depict the response of T_m relative to inhibitor concentration, are presented in Figure 1. The K_d values were derived from these concentration−response curves by using well-understood models of ligand-induced perturbations on the thermal stability of proteins.[16,17] To enable this process, we measured two, key protein-specific thermodynamic parameters for human CA-II by differential scanning calorimetry (DSC): the Gibbs free energy [$\Delta_U G_{(T)}$], which is a function of the calorimetric enthalpy [$\Delta_U H_{(T)}$], and the heat capacity of unfolding ($\Delta_U C_p$). Thus, the binding affinities (K_d values at 37 °C) for the sulfamate/sulfamide pairs were established as 290/25000 nM for **1**/**2**, 10/12500 nM for **3**/**4**, 140/10000 nM for **5**/**6**, 2000/50000 nM for **7**/**8**, and 110/4600 nM for **9**/**10**. The ratio of K_d values for the five sulfamate/sulfamide pairs ranged from 25 to 1250, with the sulfamide always having the lower affinity (Table 1). It should be especially noted that the K_d values for **1**/**2** and **3**/**4** are reasonably consistent with the K_i values that we determined by using enzyme kinetics: 500/650000 nM for **1**/**2** and 12/20000 nM for **3**/**4** (pH-shift method, 0 °C); 430/340000 nM for **1**/**2** and 38/25000 nM for **3**/**4** (esterase method, 23 °C).[9c] This trend also was applicable to the three other sulfamate/sulfamide pairs in Table 1: **5**/**6**, **7**/**8**, and **9**/**10**. The ratio of K_i values for the five sulfamate/sulfamide pairs ranged from 220 to 1670, with the sulfamide always having a much lower affinity. For topiramate (**1**), since the K_d value of 290 nM essentially recapitulates the K_i value of 500 nM,[9c] we indicate again that topiramate is just a moderate inhibitor of CA-II. It should be noted, however, that our K_d values for **1**, **9**, and **10** are not consistent with the K_i values reported by other researchers (esterase method): 5 nM for **1**,[11] 1.3 nM for **9**,[11b] and 12 nM for **10**.[13]



7 X = O **8** X = NH **9** X = O **10** X = NH **11** R = OMe **12** R = NO_2

Given our result with **10**, which departs markedly from the potent K_i value reported,[13] we decided to examine two analogous arylsulfamides, **11** and **12**. The interactions of **10**−**12** with CA-II were assessed by using the ThermoFluor method and two enzyme kinetics assays, hydration of CO_2 and hydrolysis of 4-nitrophenyl acetate (4-NPA) (Table 2). The ThermoFluor experiments were performed in duplicate under two different conditions, A and B (see Experimental Section). Compound **10** had K_d values in the range of 2000−5000 nM, **11** had K_d values in the range of 2000−5000 nM, and **12** had K_d values in the range of 1000−1700 nM (all at 37 °C). The K_i values for **10**−**12** from enzyme kinetics with the CO_2 hydration assay (at 0 °C) were 7960, 14400, and 892 nM, respectively, and with the 4-NPA hydrolysis assay (at 23 °C) were 8020, 18800, and 1740 nM, respectively. For sulfamate **9**, the ThermoFluor and kinetics data are equally consistent and serve as a reference for a more potent inhibitor of CA-II. In contrast to our observations, the K_i values reported by other researchers for **9**−**12** in the 4-NPA hydrolysis assay were 1.3, 12, 11, and 13 nM, respectively.[11b,13]

It is not clear what factors are behind the observed discrepancy between the K_i values for **1**, **9**, **10**, **11**, and **12** measured by the two different research groups. However, we have obtained fairly consistent results for enzyme kinetics-derived K_i values

Table 2. CA-II Inhibition and Binding Data for Arylsulfamides **10**−**12**

compd	CO_2 hydration K_i, nM[a]	4-NPA hydrol K_i, nM[b]	K_d (nM)[c]	K_d (nM)[d]
10	7960 (4880−13000)	8020 (5760−11200)	2000	3750
11	14400 (8790−23600)	18800 (13100−26900)	2650	5000
12	892 (546−1430)	1740 (1140−2660)	1000	1500

[a] Inhibition of human CA-II by measuring hydration of carbon dioxide via the shift in pH. The 95% confidence intervals are given in parentheses ($N = 4$ for **10**; $N = 3$ for **11** and **12**). [b] Inhibition of human CA-II by measuring the rate of hydrolysis of 4-nitrophenyl acetate (4-NPA) by absorption of light at 400 nm. The 95% confidence intervals are given in parentheses ($N = 5$ for **10**−**12**). [c] Binding affinity to human CA-II determined by ThermoFluor under the "A" conditions. [d] Binding affinity to human CA-II determined by ThermoFluor under the "B" conditions.

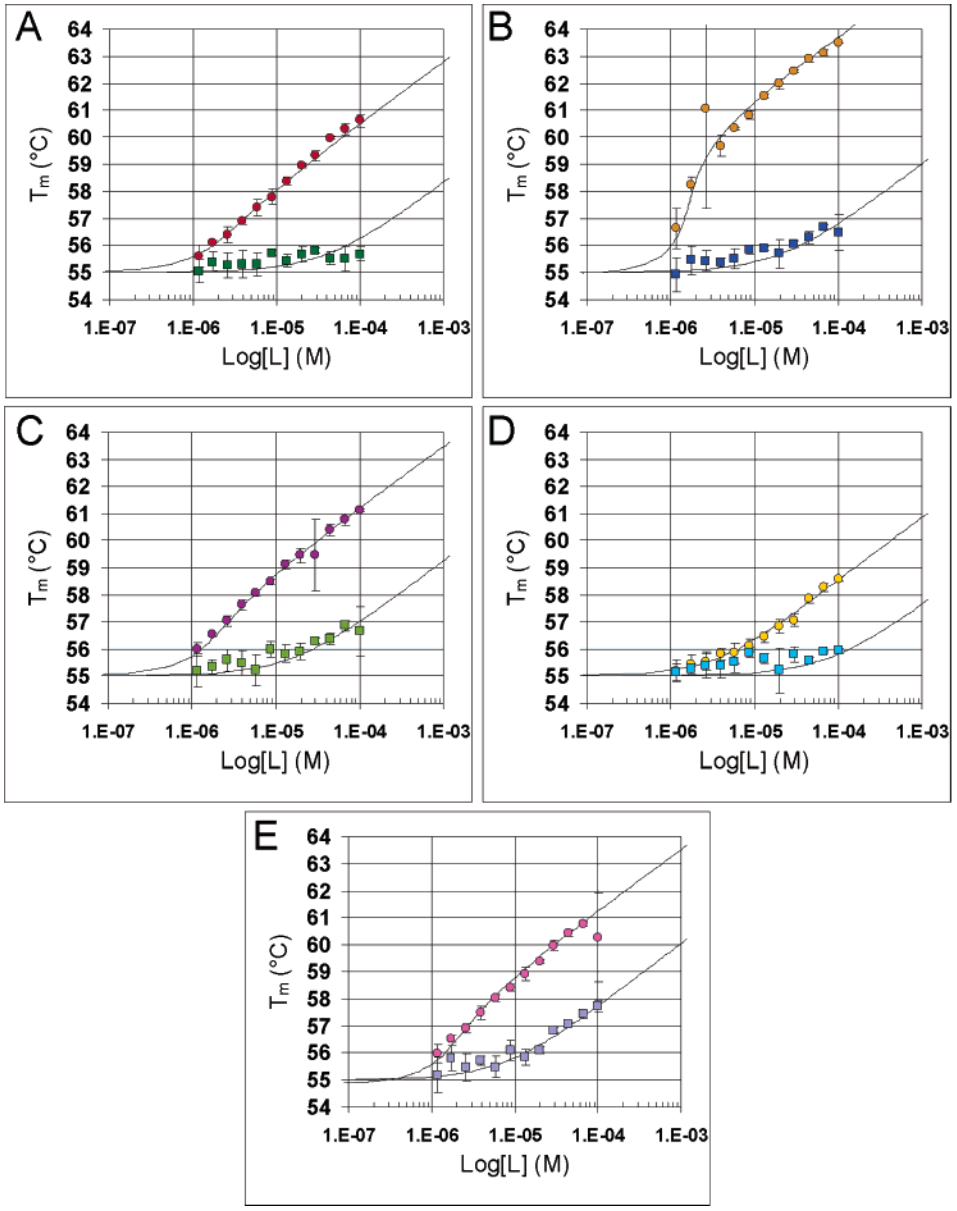


Figure 1. ThermoFluor concentration−response curves (T_m vs log[L]) for **1**−**10** (L = ligand). The data points for sulfamates are filled circles; the data points or sulfamides are filled squares; each set of data in Panels A−E is represented by different colors (see below). Experiments were performed in quadruplicate. Error bars represent the standard deviation of measured T_m values for each log[L]. Inhibitor dissociation constants (K_d) were derived from the concentration effect of ligand on T_m for each test compound. The solid lines are simulated (see Experimental Section[16]) by using $\Delta_U H$ = 197 kcal/mol, $\Delta_U C_p$ = 2.5 kcal mol^{-1}K^{-1}, and T_m = 55 °C with the determined K_d values. Legends for plots: Panel A, red circle **1** (290 nM), green square **2** (25000 nM); Panel B, yellow circle **3** (12 nM), blue square **4** (12500 nM); Panel C, purple circle **5** (100 nM), light green square **6** (10000 nM); Panel D, yellow circle **7** (2000 nM), light blue square **8** (50000 nM); Panel E, pink circle **9** (100 nM), violet square **10** (4500 nM).

with two assay systems,[9c] and there is agreement with the ThermoFluor-derived, direct binding parameters (K_d values). Under the circumstances, we must conclude that our measured levels of CA-II inhibition (K_i values) are the more accurate ones.

Sulfamates vs Sulfamides as CA-II Inhibitors. We have commented on the comparison of sulfamate and sulfamide molecules, which are *strict bioisosteres*, as inhibitors of CA-II.[9c] Compound **3** is a very potent inhibitor of human CA-II with a K_i value of 12 nM and a K_d value of 10 nM and thus serves as a benchmark for sulfamates. Its sulfamide isostere, **4**, is markedly less potent, with a K_i value of 20000 nM (1600-fold less potent) and a K_d value of 12500 nM (1200-fold less potent). A similar disparity exists for topiramate (**1**), with a K_i value of 500 nM and K_d value of 290 nM, and its sulfamide congener (**2**), with a K_i value of 650000 nM and a K_d value of 25000 nM. This pattern of substantially diminished CA-II inhibition/affinity for a sulfamide vs a sulfamate occurred for the five pairs of compounds that we studied, including the simple compounds **9** and **10** (K_d = 110 and 4600 nM, respectively). Considering the clear-cut potency difference for the sulfamate/sulfamide bioisosteric pairs, it would appear that the sulfamide moiety is not particularly desirable for obtaining potent inhibition of, or affinity to, CA-II.

The most straightforward explanation for the weaker potency of sulfamides relative to sulfamates could be their difference in pK_a. For sulfamates **1** and **3**, we had determined pK_a values of 8.66 and 8.51, respectively; whereas for sulfamide **2**, we had determined a pK_a value of 10.7.[9c] This difference of 2 orders of magnitude in pK_a is very significant. Since **2** is much less

acidic than **1**, it will have a much lower population of the anionic form that is required for binding to Zn(II) in the active site of CA-II.[6,7,18]

Conclusion

We have examined compounds **1**−**10**, which represent five sulfamate/sulfamide bioisosteric pairs, for their binding to human carbonic anhydrase-II by using the ThermoFluor method.[15,16] The resultant thermodynamically based binding parameters (K_d values) provide an independent measure of the effectiveness of the sulfamate and sulfamide groups compared with the inhibition of CA-II through enzyme kinetics studies (K_i values). The sulfamides were much less potent than the sulfamates by factors ranging from 25 (**7**/**8**) to 1200 (**3**/**4**). Additionally, we determined K_i values by enzyme kinetics and K_d values by ThermoFluor for **9**−**12** and obtained consistent results between each approach. Consequently, it would appear that the sulfamide group is not particularly suitable for obtaining potent CA-II inhibition.

Experimental Section

General Procedures. Reactions were conducted under an atmosphere of argon in solvents that were dried with molecular sieves (4A). Melting points were determined on a Thomas-Hoover apparatus calibrated with a set of melting point standards. ^{1}H NMR spectra were acquired on a Bruker Avance 300-MHz spectrometer (abbreviations used: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Thin-layer chromatography was conducted on Whatman silica gel GF plates (250 μm) with iodine staining. Column chromatography was performed on silica gel 60 (40−63 μm; EM Science). Unless otherwise specified, mass spectra were electrospray (ES) and were run on a Micromass Platform LC single quadrupole mass spectrometer in the positive or negative mode as indicated. Chemical-ionization (CI) mass spectra were recorded on a Finnigan 3300 mass spectrometer with ammonia as the reagent gas. Elemental analyses were obtained from Quantitative Technologies, Inc., Whitehouse, NJ; percentage of water was determined by the Karl Fischer method.

Materials. Compounds **1**−**7** were described previously by us.[8a,9c] Sulfamides **10**−**12** were prepared by reacting the commercially available amines with sulfamide, according to the method described in our preceding paper.[9c] Sulfamate **9** was prepared in the usual manner from phenol, sulfamoyl chloride, and NaH.[8a] Analytical data for **9**−**12** are presented in the Supporting Information.[19] Human carbonic anhydrase-II (purified from erythrocytes) and 4-nitrophenyl acetate (4-NPA) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Anilino-8-naphthalenesulfonic acid (ANS) was obtained from Molecular Probes at Invitrogen Corp. (Carlsbad, CA).

***N*-(2,2-Dimethyl-1,3-dioxolan-4-ylmethyl)sulfamide (8).** 2,2-Dimethyl-1,3-dioxolane-4-methanamine (5.24 g, 40 mmol; Aldrich Chemical Co.) was dissolved in 1,4-dioxane (100 mL), sulfamide (19.2 g, 200 mmol) was added, and the reaction was heated at reflux for 2 h. After concentration on a rotary evaporator, the residue was triturated with ethyl acetate, the solid was filtered off, and the filtrate was evaporated to give crude product, which was purified by flash-column chromatography (10% MeOH in CH_2Cl_2) to give an oil, which was redissolved in CH_2Cl_2. The solution was dried (Na_2SO_4) and evaporated in vacuo to afford the titled compound as a clear, colorless, viscous oil (5.46 g, 65%). ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3H), 1.45 (s, 3H), 3.27 (m, 2H), 3.77 (dd, J = 5,8, 8.5 Hz, 1H), 4.08 (dd, J = 6.5, 8.5 Hz, 1H), 4.34 (m, 1H), 5.22 (br s, 3H); CI-MS (NH_3) m/z 211 (MH^+), 228 ($M + NH_4^+$). Anal. Calcd for $C_6H_{14}N_2O_4S$•0.125 H_2O: C, 33.91; H, 6.76; N, 13.18; S, 15.09; H_2O 1.06. Found: C, 34.04; H, 6.70; N, 13.07; S, 15.06; H_2O 1.20.

ThermoFluor Studies. ThermoFluor[20] measurements were carried out by using available instruments (developed in house), according to the reported methodology.[16] Solutions (4 μL) of human CA-II (1 mg/mL) and test compound (0−100 μM) were dispensed into black 384-well polypropylene PCR microplates (Abgene) and overlaid with 1 μL of silicon oil (Fluka, type DC 200) to prevent evaporation. Measurements were made in quadruplicate on each plate. The medium was comprised of 10 mM HEPES (pH 7.5), 100 μM Na_2SO_4, 100 μM ANS, and 1.5% DMSO ("A" conditions). For **10**−**12**, measurements were also made by using 25 mM HEPES and no salt at pH 7.4 ("B" conditions). Plates were heated at a rate of 1 °C/min on a thermal block and illuminated with band-passed filtered UV light (380−400 nm). Fluorescence was measured by using an overhead CCD (charge-coupled device) camera that was filtered to detect wavelengths of 500 ± 25 nm.

To determine the K_d values from the melting curves, expressions were used that describe the total ligand concentration needed to raise the protein T_m values to a given value. Since these transcendental expressions cannot be solved explicitly, we simulated 12-point T_m concentration−response curves by calculating the concentration of total added ligand required to produce the experimentally observed T_m values. This simulation process was based on key protein-specific thermodynamic parameters for human CA-II that determine the temperature dependence of the Gibbs free energy of unfolding [$\Delta_U G_{(T)}$], which were measured by differential scanning calorimetry (DSC; with a VP-DSC calorimeter; Microcal, Inc., North Hampton, MA) according to reported methodology.[16] The calorimetric enthalpy [$\Delta_U H_{(T)}$] was 197 kcal mol^{-1} and the heat capacity of unfolding ($\Delta_U C_p$) was 2.5 kcal mol^{-1} K^{-1}. Since the dissociation binding constants determined by ThermoFluor are affinities at the melting temperatures, the K_d values were extrapolated to 37 °C by using established methods and previously measured binding enthalpy and heat capacity values. Under the standard ThermoFluor conditions ("A") with these computational methods, acetazolamide afforded a K_d value of 50 nM.[21]

Carbonic Anhydrase Inhibition. Carbon Dioxide Hydration and 4-Nitrophenyl Acetate Hydrolysis Assays. Purified human CA-II was used. Inhibition of CA-II was determined for both assays according to the procedures that were described earlier in detail.[9c] The temperatures for the CO_2 hydration and 4-NPA hydrolysis assays were 0−2 °C and 23 °C, respectively.[9c]

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Supporting Information Available: Analytical data for compounds **9**−**12**. ThermoFluor graphs of the original data for **10**−**12** that is represented in Table 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) See the paragraph at the end of this paper regarding Supporting Information.

(20) ThermoFluor is a registered trademark in the United States and certain other countries.

(21) For acetazolamide, we reported: CO_2 hydration $K_i = 3$ nM; 4-NPA hydrolysis $K_i = 18$ nM.[9c]

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