Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 318 (2004) 1–3
BBRC
www.elsevier.com/locate/ybbrc

Potent isothiocyanate inhibitors of carbonic anhydrase: synthesis and evaluation

Moussa Khobzaoui,[a] L.M. Viranga Tillekeratne,[a,c] and Richard A. Hudson[a,b,c,*]

[a] *Department of Chemistry, College of Arts and Sciences, University of Toledo, Toledo, OH 43606, USA*
[b] *Department of Biological Science, College of Arts and Sciences, University of Toledo, Toledo, OH 43606, USA*
[c] *Department of Medicinal and Biological Chemistry, College of Pharmacy, University of Toledo, Toledo, OH 43606, USA*

Received 19 March 2004

Abstract

The reversible hydration of carbon dioxide by carbonic anhydrase (CA) regulates pH and carbon dioxide concentrations in diverse biological systems. Potent irreversible inhibition of CA would facilitate study of the dynamics of CA turnover as well as therapeutic effects due to long-term inhibition of the enzyme. We have synthesized isothiocyanate-containing sulfonamide inhibitors of CA from the corresponding aminosulfonamides. Significant increases in apparent binding of some of the isothiocyanate inhibitors over the amine analogues were consistent with covalent inhibition of the enzyme.

Since sulfanilamide was observed to be an inhibitor of carbonic anhydrase (CA) [1], sulfonamide derivatives have been widely used to establish the physiological functions of the enzyme [2]. These studies also led to the early use of sulfonamides as therapeutic agents based on carbonic anhydrase inhibition. Most presently known CA inhibitors are reversible in nature and cannot provide long-lasting inhibition. Irreversible or slowly reversed inhibitors would provide long-term inhibition that would have therapeutic implications and further, could be useful also in studying CA regulation of physiologic systems. We synthesized several examples of a class of CA inhibitors that incorporate an isothiocyanate functional group in place of the amino group in known arylamino-sulfonamide inhibitors. The synthesis, characterization, and evaluation of these new inhibitors are described herein.

Materials and methods

All chemicals were of reagent grade from Aldrich and were used without further purification. NMR measurements were performed on a 200 or 400 MHz Varian spectrometer in acetone-d_6. FT-IR spectroscopy was performed on a Perkin Elmer 1600 spectrophotometer. Pure bovine carbonic anhydrase II was purchased from Worthington. Aromatic aminesulfonamides used as starting materials were synthesized based on known procedures or were purchased from Aldrich. The microchemical enzyme assay method of Maren [3] was used to determine the activity of the enzyme in the presence and absence of inhibitor.

*3-Isothiocyanatobenzenesulfonamide (**1**).* To 3-aminobenzenesulfonamide (50 mg, 0.3 mmol), maintained on salt-ice-bath at −10 to 0 °C, was added carbon disulfide (1 mL, 16.7 mmol) followed by pyridine (0.5 mL), and triethylamine (0.5 mL). The mixture was stirred for 1 h under nitrogen. Dicyclohexylcarbodiimide (50 mg) was added and the mixture was stirred for additional 3 h at −10 to 0 °C and then overnight at room temperature. The solvent was evaporated in vacuo. Acetone was added and the resulting solid was separated by filtration. The filtrate was evaporated to dryness and chromatographed on silica in acetone/hexane (1:4) to obtain a white solid (10 mg), mp 130 °C; ^{1}H NMR (400 MHz) δ 6.78 (br, 2H, NH_2) 7.59–7.87 (4H, aromatic); ^{13}C NMR (100 mHz) δ 123.60, 125.12, 129.01, 130.86, 146.22; IR (KBr) 3300, 3200, 2200 cm^{-1}; MS $m/z = 214$, 150, 134, 90; Anal. Calcd. for $C_7H_6N_2O_2S_2$: C 39.27, H 2.80, N 13.07, S 29.90, found C 39.67, H 2.91, N 12.71, S 29.37.

*4-Isothiocyanatobenzenesulfonamide (**2**).* This was synthesized from 4-aminobenzenesulfonamide (50 mg, 0.3 mmol) as described for the synthesis of **1** above. The product obtained as a yellowish solid was washed with acetone and filtered. The filtrate was evaporated to dryness and the residue was chromatographed on silica in acetone/hexane (1:4) to obtain a white solid (7 mg), mp 135 °C; ^{1}H NMR (400 MHz) 6.76 (br, 2H, NH_2) 7.55 (d, 2H, Ar, $J = 10$ Hz), 7.96 (d, 2H, Ar, $J = 10$ Hz); IR (KBr) 3400, 3300, 2100 cm^{-1}; MS $m/z = 214$, 198, 134.

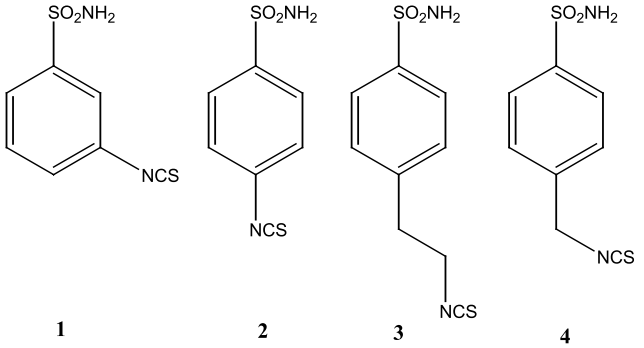
*4-(2-Isothiocyanatoethyl)benzenesulfonamide (**3**).* To 4-(2-aminoethyl)benzenesulfonamide (500 mg, 2.5 mmol) in a round-bottomed

* Corresponding author. Fax: 1-419-530-7946.
E-mail address: richard.hudson@utoledo.edu (R.A. Hudson).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.181

flask on a salt-ice-water bath at a temperature between −10 and 0 °C was added carbon disulfide (2 mL, 33 mmol), with pyridine (1 ml) and dicyclohexylcarbodiimide (500 mg). The solution was stirred overnight at room temperature. The solvent was removed in vacuo. Repeated recrystallizations from acetone gave white solid (450 mg, 90%), mp 146 °C; ^{1}H NMR (400 MHz) δ 3.15 (t, 2H, CH_2, $J = 6.4$ Hz), 3.97 (t, 2H, CH_2, $J = 6.4$ Hz), 6.59 (brs, 2H, NH_2), 7.53 (d, 2H, $J = 8.0$ Hz), 7.87 (d, 2H, $J = 8.0$ Hz); ^{13}C NMR (100 MHz) δ 35.71, 45.94, 126.56, 129.66, 142.43, 143.20, 143.25; IR (KBr) 3300, 3200, 2200, and 2100 cm^{-1}; MS *m/z* 242, 170, 106, 72; Anal. Calcd. for $C_9H_{12}N_2O_2S_2$: C 44.63, H 4.13, N 11.57, S 26.45, found C 44.58, H 4.22, N 11.52, S 26.60.

*4-(Isothiocyanatomethyl)benzenesulfonamide (**4**).* To 4-aminomethylbenzenesulfonamide hydrochloride (500 mg, 2.3 mmol) maintained at −10 to 0 °C in an salt-ice-bath were added carbon disulfide (2 mL, 33 mmol), pyridine (1 mL), and triethylamine (1 mL), and then dicyclohexylcarbodiimide (500 mg.). The mixture was allowed to warm to RT and stirred overnight. The solvent was removed in vacuo and the residue was recrystallized from acetone several times to obtain a white solid (300 mg, 60%), mp 155 °C; ^{1}H NMR (400 MHz) δ 5.04 (s, 2H, CH_2), 6.65 (s, 2H, NH_2), 7.62 (d, 2H, $J = 8.0$ Hz), 7.95 (d, 2H, $J = 8.0$ Hz); ^{13}C NMR (100 MHz) δ 48.04, 126.89, 127.80, 139.30, 144.36, 144.39; IR (KBr) broad peaks at 3400, 3300, 2200, and 2100 cm^{-1}; MS $m/z = 228$, 170, 106, and 89; Anal. Calcd. for $C_8H_{10}N_2O_2S_2$: C 42.11, H 3.51, N 12.28, S 28.01, found C 42.38, H 3.65, N 12.28, S 27.93.



1 **2** **3** **4**

Results and discussion

The substitution of an isothiocyanate group for the amine function in several known aminosulfonamide CA inhibitors has the potential to produce major changes in the nature of the inhibition. The isothiocyanate group is both electron withdrawing and hydrophobic while the amino group is electron-donating and hydrophilic. Further, the strategy employed could produce irreversible inhibition of the enzyme.

A simple chemical transformation employing carbon disulfide in combination with dicyclohexylcarbodiimide (DCC) in pyridine and triethylamine was used to synthesize 3-isothiocyanatobenzenesulfonamide (**1**), 4-isothiocyanatobenzenesulfonamide (**2**), 4-(2-isothiocyanatoethyl)benzenesulfonamide (**3**), and 4-(isothiocyanatomethyl)benzenesulfonamide (**4**). Chemical yields were mitigated by the need to crystallize the isothiocyanate products from the thiourea mixture, a separation which was more difficult for the aromatic (**1** and **2**) than for the alkyl (**3** and **4**) isothiocyanates. The amino analogues were also used to compare directly their enzyme binding affinities with those of the corresponding isothiocyanates.

The isothiocyanates prepared were analyzed for their CA inhibitory activity using a modified colorimetric assay based on the procedure of Maren [3] to determine their binding affinity. IC_{50} was determined from plots of inhibitor concentration vs. time required to hydrate carbon dioxide to a common end point. From the IC_{50}, the apparent dissociation constant (K_I) was calculated from the relationship of Cheng and Prusoff [4] ($K_I = IC_{50} - E_0/2$). Previous studies have shown that inhibition of hydration and neutralization kinetics can also be measured by pH-stat methods [5]. Also, inhibition of esterase activity of the enzyme can be used to determine IC_{50} [6]. When more exact pH-dependent kinetic studies are required, these methods may be preferable to the simpler procedures used here. However, the Maren procedure as adopted here was quite satisfactory for determining IC_{50} as illustrated here. The method accurately reproduces known K_d's. For instance, we determined the K_I of acetalozamide and found it to be $K_I = 10 \pm 7$ nM. This was consistent with the range of K_I values (7–25 nM) reported elsewhere [7,8]. The IC_{50} values were determined for both the amino and isothiocyanate analogues and are reported in Table 1. The isothiocyanates are bound about 2–3 orders of magnitude more strongly to the enzyme when compared with the corresponding amines.

From the results shown, the isothiocyanates appear to be far better inhibitors of CA than the corresponding amines, suggesting that these new inhibitors may form covalent bond between the enzyme active site and the isothiocyanate. The isothiocyanate NCS has been used extensively in protein modification studies. The isothiocyanate functional group reacts readily under mild aqueous conditions with amino acid side chain nucleophiles like amino, hydroxyl, sulfhydryl, tyrosyl, and histidyl [9]. However, not all of the adducts are stable. The resulting thiourea or thioimidate linkages are not always stable as the covalent linkage is often slowly hydrolyzed. The hydrophilic, electron-donating amine is converted to the large, electron-withdrawing but hydrophobic isothiocyanate. The strength of the reversible complex initially formed with the enzyme can be possibly quite different for the two classes of inhibitors

Table 1
IC_{50} values for the isothiocyanates **1**–**4** and corresponding amines

	IC_{50} (isothiocyanate) (nM)	IC_{50} (amine) (μM)
Compound **1**	7 ± 4	2 ± 0.8
Compound **2**	0.7 ± 0.2	0.5 ± 0.3
Compound **3**	20 ± 7	2 ± 0.9
Compound **4**	30 ± 20	1.4 ± 0.3

notwithstanding the possibility of reaction of the thiocyanate inhibitors with protein nucleophiles.

Further, the potential for covalent reaction with the enzyme in the case of the different isothiocyanates prepared can be affected by: (1) differences in reactivity of the alkyl and aryl isothiocyanates, (2) the stability of reaction products formed between isothiocyanate and active site nucleophiles, and (3) the steric placement of the isothiocyanate group in relation to potentially reactive active-site nucleophiles subsequent to the initial reversible interaction of the sulfonamide functional group with the divalent zinc in the enzyme. The results reported here suggest but do not prove that the inhibitors bind covalently with the enzyme or form transiently stable adducts. Further experimentation is necessary to prove the irreversible nature of the binding for these strong inhibitors.

Acknowledgments

We wish to thank Astra-Hassel (now Astra-Zeneca) for financial support. Moussa Khoubzaui wishes to thank the University of Toledo for a graduate assistantship.

References

[1] T. Mann, D. Keilin, Sulphanilamide as a specific inhibitor of carbonic anhydrase, Nature (Lond.) 146 (1940) 164–165.
[2] B.C. Tripp, K. Smith, J.G. Ferry, Carbonic anhydrase: new insights into an ancient enzyme, J. Biol. Chem. 276 (2001) 48615–48618.
[3] T.H. Maren, A simplified micromethod for the determination of carbonic anhydrase and its inhibitors, J. Pharmcol. Exp. Ther. 130 (1960) 26–29.
[4] Y. Cheng, W.H. Prusoff, Relationship between the inhibitor constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22 (1990) 3099–3108.
[5] J.E.A. McIntosh, Assay of carbonic anhydrase by titration at constant pH, Biochem. J. 109 (1968) 203–207.
[6] Y. Pocker, J.T. Stone, The catalytic versatility of erythrocyte carbonic anhydrase. III Kinetic studies of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate, Biochemistry 6 (1967) 668–678.
[7] T.H. Maren, Direct measurements on the rate constants of sulfonamides with carbonic anhydrase, J. Pharmacol. 41 (1991) 419–426.
[8] C.T. Supuran, A. Nicolae, A. Popescu, Carbonic anhydrase inhibitors. Part 35: synthesis of Schiff bases derived from sulfanilamide, J. Med. Chem. 31 (1996) 431–438.
[9] A.H. Maddy, A fluorescent label for the outer component of the plasma membrane, Biochim. Biophys. Acta 88 (1964) 390–399.