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Bioorganic & Medicinal Chemistry Letters 18 (2008) 1898–1903
Bioorganic & Medicinal Chemistry Letters

Carbonic anhydrase inhibitors: The very weak inhibitors dithiothreitol, β-mercaptoethanol, tris(carboxyethyl)phosphine and threitol interfere with the binding of sulfonamides to isozymes II and IX

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Received 13 December 2007; revised 4 February 2008; accepted 5 February 2008
Available online 9 February 2008

Abstract—The inhibition of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) with dithiothreitol, 2-mercaptoethanol, tris(carboxyethyl)phosphine (reducing agent frequently added to enzyme assay buffers) and threitol has been investigated. The agents were very weak inhibitors of isozymes CA II and CA IX, but unexpectedly, strongly influenced the binding of the low nanomolar sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide). Acetazolamide affinity for all investigated CAs diminished orders of magnitude with increasing concentrations of these agents in the assay system. DTT and similar derivatives should not be added to the assay buffers used in monitoring CA activity/inhibition, as they lead to under-estimation of the binding constants, by a mechanism probably involving the formation of ternary complexes.

Sulfonamide inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), such as acetazolamide **1**, have been used clinically as diuretics, antiglaucoma, or anticonvulsant agents for a long period,[1–7] whereas more recent drug design studies have evidenced some other CA inhibitors (CAIs) belonging to the sulfonamide/sulfamate/sulfamide classes as molecules of interest for developing novel therapies for obesity[8] and cancer[9–12] based on selective inhibition of CA isozymes involved in such pathologies, among the 16 presently known α-CAs in vertebrates.[1–12]

There are several assay methods used to monitor the activity and inhibition of the various CA isozymes from organisms all over the phylogenetic tree,[1–7] such as spectrophotometric stopped-flow[13] (or manual)[14] ones based on the CO_2 hydrase activity of these enzymes (i.e., monitoring the physiologic reaction in which CAs are involved: CO_2 hydration to bicarbonate and protons, and its inhibition by various classes of compounds); spectrophotometric methods based on the esterase activity of some CAs (with 4-nitrophenyl acetate being the most used substrate),[15] as well as fluorimetric, dansylamide competition binding assays,[16] which exploit the competition for the binding of a test inhibitor (usually a sulfonamide) and dansylamide (4-dimethylaminonaphthalene-1-sulfonamide) to the enzyme active site (obviously this last method can be used to study only inhibition, not catalytic activity of a given CA isozyme). More recently, a ThermoFluor method has also been reported for assaying CA inhibition.[17] This method monitors the protein thermal stability in the presence of various concentrations of ligands investigated as inhibitors in the presence of fluorescent dyes. All methods have advantages and disadvantages; for example the ones employing the hydratase activity use water solutions of CO_2 as substrate, which are difficult to be dealt with in order to maintain constant substrate concentra-

Keywords: Carbonic anhydrase; Inhibitor binding; Dithiothreitol; Threitol; Disulfide bridge; Isozyme II; Isozyme IX.
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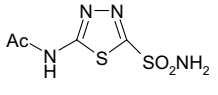
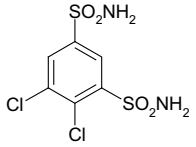
doi:10.1016/j.bmcl.2008.02.008

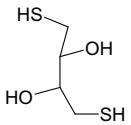
tions during the experiments, but their advantage is that they monitor the real physiologic reaction that these enzymes catalyze.[13,14] The esterase methods[15] are simpler as compared to the previous ones[13,14] (as 4-nitrophenyl acetate is an inexpensive, stable solid, in contrast to CO_2, which is a gas), but their main disadvantage is that most CAs are quite weak esterases (e.g., CAs I–III),[15] whereas other isoforms seem to be devoid of such an activity (e.g., CA VA and CA IX).[18] On the other hand, the dansylamide competition binding assay is an elegant but limited method, because it can be only used to monitor the inhibitors having an affinity for CAs of the same order of magnitude as dansylamide itself or 10–100 times less/more than dansylamide. Thus, very weak or very potent inhibitors are quite difficult to be investigated by this method, since the inhibitory activity of dansylamide for most CAs is in the micromolar range.[16] The Thermofluor method[17] monitors a quite complex process involving the thermal denaturation of CAs alone or in the presence of inhibitors. To date, it has been used to measure only CA I and CA II inhibition, with a very limited number of sulfonamides.[17] This method provided inhibition data[17] sometimes in disagreement with those obtained by all the other methods mentioned above. For example, dichlorophenamide **2**, a moderately-effective CA II inhibitor by all other assay methods, was the most potent inhibitor in the Thermofluor assay.[13–16] In fact, by the stopped-flow assay acetazolamide **1** is a 3.2-time more potent CA II inhibitor as compared to dichlorophenamide **2**, whereas by Thermofluor method[17] dichlorophenamide **2** is a 7-time more potent inhibitor as compared to acetazolamide **1**, a result difficult to interpret. Thus, although we may consider this new method of interest, only a very small number of compounds have been investigated up to now against only two isozymes (CA I and II).[17] Obviously, further investigations including other CA isozymes and inhibitors are warranted in order to demonstrate its applicability and reproducibility in determining CA inhibition profiles.



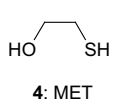
1: acetazolamide



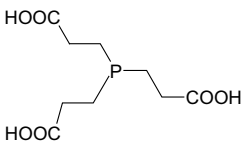
2: dichlorophenamide



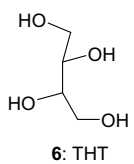
3: DTT



4: MET



5: TCP



6: THT

Another aspect encountered during our work in developing CAIs for various medical applications[1–6] is with regard to the use of reducing agents, such as dithiothreitol (DTT) **3** or other structurally related agents[19] in the assay buffer employed for enzyme activity and inhibition measurements.[20] Indeed, work from Maryanoff's group[20] used various assay methods (e.g., CO_2 hydrase, esterase and ThermoFluor ones) in which typically 1–10 mM DTT has been added to the buffer. Although heterocyclic mercaptans are known to act as rather potent (micromolar) CAIs,[21] no investigations regarding the effects of the aliphatic thiols on CA activity are available in the literature. Here we investigate the binding of **1** and some reducing agents, such as dithiothreitol DTT **3**, 2-mercaptoethanol MET **4**, and the non-mercaptan reducing agent tris(carboxyethyl)phosphine TCP **5** (each agent alone, or in combination) to three CA isozymes, including the cytosolic CA II (which does not contain S–S bridges, as it has only one Cys residue),[1–3] the catalytic domain of CA IX (which contains one intramolecular S–S bridge, not situated within the active site cavity, as well as one intermolecular disulfide bridge),[3,4,22] and the full-length extracellular domain of CA IX (i.e., the protein incorporating the catalytic plus proteoglycan (PG) domains), which is a trimer containing three active sites and three PG domains, bound by means of three interchain S–S bridges in addition to the intrachain/intermolecular S–S bridges present in the catalytic domain, mentioned above.[3,4,22,23] The thiol analogue of DTT **3**, threitol THT **6**, has also been investigated for its competing effects on the binding of acetazolamide to these three isoforms, although obviously, this is not a reducing agent in the conditions of our experiments.

The following should be noted regarding data of Table 1, in which the interaction of the three CAs with agents **3**–**6** is presented. DTT **3**, MET **4**, TCP **5** and THT **6** act as very weak inhibitors of the three investigated CA isoforms, the cytosolic CA II as well as the tumor-associated CA IX, of which two constructs have been used in our experiments: the catalytic domain and the full-length extracellular enzyme (i.e., the enzyme incorporating the PG domain). We chose to investigate the three enzymes because of their very different redox potential towards reducing agents: CA II is a cytosolic enzyme whose Cys residue is not involved in formation of S–S bridges (thus, this enzyme should not be affected by the reduction/oxidation mediated by agents **3**–**5**). The catalytic domain of CA IX (cdCA IX) on the other hand has three Cys residues which form an intrachain S–S bond and one intermolecular S–S bridge. These bonds are, however, outside the active site of the enzyme, and presumably should not interfere with the binding of inhibitors (but its reduction/oxidation can in theory influence the 3D conformation of the entire enzyme, and thus change affinity for inhibitors). Finally, the full-length CA IX (flCA IX) is a trimer in which the three polypeptide chains (incorporating the cdCA and the PG domains) are kept together by means of three interchain S–S bridges.[22,23] Obviously, as the enzyme incorporated three cdCA IX domains, it also has the three intrachain S–S bridges. Thus, both cdCA IX and flCA IX are in theory sensitive to reduction by **3**–**5**. Data of Table 1 prove that all three enzymes are weakly inhibited by compounds **3**–**6** (both the reducing agents **3**–**5** and the structurally related, non-reducing alcohol **6**). Thus, concentrations of DTT of 1–10 μM only slightly

Table 1. Inhibition data of CA II, IX (catalytic domain, cdCA IX) and IX (full-length extracellular enzyme, flCA IX) with DTT **3**, MET **4**, TCP **5** and THT **6**, by a stopped-flow, CO_2 hydrase assay at 25 °C[13]

	% CA activity*		
	CA II	cdCA IX	flCA IX
[DTT] (mM)			
0.001	92	98	97
0.01	85	97	94
0.1	81	93	85
1.0	77	85	76
2.5	75	71	65
10	73	58	54
[MET] (mM)			
0.001	84	100	100
0.01	74	99	100
0.1	66	96	97
1.0	55	90	91
2.5	41	87	89
10	36	84	86
[TCP] (mM)			
0.001	94	99	98
0.01	87	94	95
0.1	80	85	89
1.0	72	83	83
2.5	70	83	77
10	68	82	69
[THT] (mM)			
0.001	94	98	95
0.01	90	96	92
0.1	87	94	87
1.0	83	91	83
2.5	77	89	77
10	68	86	65

* CA activity in the absence of test compound taken as 100% (all values represent the mean from 3 different assays; errors were in the range of ±2%).

reduced the catalytic activity (which remains in the range of 85–97%), whereas higher concentrations of DTT led to an increased inhibition. However, no real inhibition constant of the compound could be measured, since even at concentrations as high as 10 mM, the CA activity remains higher than 50% (in the range of 54–78%). The least inhibited enzyme was CA II (at 10 mM DTT, a residual activity of 73% was still measured), whereas the most inhibited one was flCA IX (a residual activity of 54% was measured). The results suggest that either the intramolecular S–S bond or the oligomerization of CA IX is also important for the activity of this enzyme, since DTT clearly affects this enzyme more compared to CA II. It should be noted that both CA II and CA IX are very effective catalysts for the CO_2 hydration reaction monitored here.[18,22–25] Thus, from this data it is unclear whether DTT binds to the metal ion within the CA active site, as other weak inhibitors, such as for example phenol, were shown by means of X-ray crystallography to bind to the water molecule coordinated to the Zn(II) ion critical for catalysis.[7] What is clear from these data is that DTT **3** acts as a very weak inhibitor of the three investigated isoforms. Basically the same situation had been observed also when using MET or TCP as reducing agents, or THT added in the buffer for measuring the inhibitory activity of these compounds. Only MET was slightly more inhibitory as compared to DTT, but only against CA II, whereas the behavior of DTT and its alcohol analogue THT was very similar. This clearly proved that it is not the thiol which binds to the metal ion within the active site of these enzymes, since compounds possessing a good zinc binding group (ZBG) (such as the SH moiety present in DTT and MET) as well those without such a group (e.g., the reducing agent TCP and the non-reducing THT) had a similar behavior, of very weak CA inhibitor. We assume that this behavior is due to a hydrophobic interaction between the enzyme active site and the compounds **3**–**6**, which probably enter within the active site (see discussion later in the text) but does not coordinate to the catalytically critical metal ion.

However, the data in Tables 2–5 reveal a very interesting observation: DTT **3** and the related reducing agents MET **4**, as well as the phosphine TCP **5** and the alcohol THT **6**, had a dramatic effect on the inhibitory efficiency of the clinically used sulfonamide inhibitor acetazola-

Table 2. IC_{50} data for the inhibition of CA II, IX (catalytic domain, cdCA IX) and IX (full-length extracellular enzyme, flCA IX) by acetazolamide **1** in the absence and presence of various concentrations of DTT **3**, by a stopped-flow, CO_2 hydrase assay at 25 °C[13]

[DTT] (mM)	IC_{50}* of acetazolamide **1** (nM)		
	CA II[a]	cdCA IX[a,b]	flCA IX[b]
0	8.9	32	21
0.01	68	82	132
0.05	283	700	973
0.10	285	721	1520
0.25	351	735	1513
1.00	362	763	1520
10	441	775	1524

[a] Recombinant human isozyme produced in *E. coli*.[9b]
[b] Recombinant human isozyme produced in insect cells (Hilvo et al.,[25] submitted for publication).
* Mean from three different assays (errors were in the range of ±5% of the reported value).

Table 3. IC_{50} data for the inhibition of CA II, IX (catalytic domain, cdCA IX) and IX (full-length extracellular enzyme, flCA IX) by acetazolamide **1** in the absence and presence of various concentrations of MET **4**, by a stopped-flow, CO_2 hydrase assay at 25 °C[13]

[MET] (mM)	IC_{50}* of acetazolamide **1** (nM)		
	CA II[a]	cdCA IX[a,b]	flCA IX[b]
0	8.9	32	21
0.01	16.2	44.3	42.1
0.05	48.9	174.8	54.0
0.10	53.1	243.1	304.6
0.25	60.4	286.5	410.3
1.00	69.7	1297	622
10	98.3	1997	680

[a] Recombinant human isozyme produced in *E. coli*.[9b]
[b] Recombinant human isozyme produced in insect cells (Hilvo et al.,[25] submitted for publication).
* Mean from three different assays (errors were in the range of ±5% of the reported value).

Table 4. IC_{50} data for the inhibition of CA II, IX (catalytic domain, cdCA IX) and IX (full-length extracellular enzyme, flCA IX) by acetazolamide **1** in the absence and presence of various concentrations of TCP **5**, by a stopped-flow, CO_2 hydrase assay at 25 °C[13]

[TCP] (mM)	IC_{50}* of acetazolamide **1** (nM)		
	CA II[a]	cdCA IX[a,b]	flCA IX[b]
0	8.9	32	21
0.01	16.2	218.8	36.2
0.05	34.3	670.3	48.7
0.10	47.6	694.5	53.4
0.25	48.1	1024	65.2
1.00	48.3	1764	318.6
10	56.5	2134	746.9

[a] Recombinant human isozyme produced in *E. coli*.[9b]
[b] Recombinant human isozyme produced in insect cells (Hilvo et al.,[25] submitted for publication).
* Mean from three different assays (errors were in the range of ±5% of the reported value).

Table 5. IC_{50} data for the inhibition of CA II, IX (catalytic domain, cdCA IX) and IX (full-length extracellular enzyme, flCA IX) by acetazolamide **1** in the absence and presence of various concentrations of THT **6**, by a stopped-flow, CO_2 hydrase assay at 25 °C[13]

[THT] (mM)	IC_{50}* of acetazolamide **1** (nM)		
	CA II[a]	cdCA IX[a,b]	flCA IX[b]
0	8.9	32	21
0.01	18.5	183.3	36.7
0.05	36.0	200.6	52.5
0.10	68.6	236.1	56.7
0.25	77.8	999.8	123.6
1.00	89.9	1165	176.8
10	92.5	1294	208.1

[a] Recombinant human isozyme produced in *E. coli*.[9b]
[b] Recombinant human isozyme produced in insect cells (Hilvo et al.,[25] submitted for publication).
* Mean from three different assays (errors were in the range of ±5% of the reported value).

mide **1** toward the three investigated enzymes, when these compounds were present in the assay buffer together with the sulfonamide. In order to avoid artifactual results possibly obtained by using various transformations of the Michaelis–Menten equation for calculating K_Is,[20] we present the data of Table 2 as IC_{50} values, working at strictly the same concentration of enzymes in all our experiments.[13] It may be observed that acetazolamide **1** is a strong inhibitor of the three CAs investigated here, with IC_{50} values in the range of 8.9–21 nM, as reported earlier by our group (Table 2).[10–12] However, addition of DTT **3**, MET **4**, TCP **5** or THT **6**, in concentrations of 10 μM to 10 mM, leads to a constant and gradual decrease of acetazolamide affinity for the three enzymes. Thus, in the presence of DTT, this enhancement arrives in the range of 441–1524 nM (we stress again, all other parameters of the enzyme assay were the same, except for the presence of DTT in the buffer). The variation of only this parameter leads to an IC_{50} value of acetazolamide which is 49.5 times higher for CA II, 24.2 times higher for the catalytic domain of CA IX and 72.5 times higher for the full-length extracellular CA IX, as compared to the same values determined without the reducing agent (it is notable that DTT affects more flCA IX—where each monomer has two intramolecular disulfide bridges—than cdCA IX—where each monomer has one intramolecular disulfide bridge). The same effect was observed in the presence of MET **4** (Table 3), case in which (at the highest concentration of reducing agent) the IC_{50} increased 11-fold for CA II, 62.4-fold for cdCA IX, and 32.3-fold for flCA IX. The presence of TCP **5** in the assay buffer led to similar effects of increase in the IC_{50} of acetazolamide (Table 4), in the range of: 6.3-fold for CA II, 66.7-fold for cdCA IX, and 35.5-fold for flCA IX, respectively. The non-reducing agent THT **6** (structurally similar to DTT **3**) showed a similar effect, which represents another proof that the competing agent does not bind to the Zn(II) ion as the sulfonamide inhibitor. Thus, the IC_{50} of acetazolamide in the presence of increasing concentrations of THT **6** showed the same behavior as in the presence of reducing agents **3**–**5**, constantly increasing and reaching values 10.4 times higher for CA II, 40.4 times higher for cdCA IX, and 9.9 times higher for flCA IX, respectively, as compared to the same parameter without derivative **6** (Table 5).

The logical conclusion of all these data is that DTT **3** and the similar reducing/non-reducing agents **4**–**6** should not be present in the assay systems when measuring CA activity/inhibition, as they lead to errors in determining the inhibition constants for the binding of sulfonamides to various CA isozymes (if they contain or not S–S bridges in their polypeptide chain(s)), with IC_{50} or K_I data orders of magnitude higher than those normally measured when these agents are absent from the assay buffer. We do not know the precise mechanism by which DTT and the related compounds **4**–**6** interfere with the binding of acetazolamide to the CA active site, but we can more than surely speculate that a ternary complex between the enzyme, sulfonamide and reducing agent is formed, with both compounds bound to different regions of the enzyme cavity, which destabilize the CA–acetazolamide (very tight-binding)[10–12] adduct. Work is in progress in our laboratories to verify this hypothesis by means of X-ray crystallographic experiments. However, soaking experiments afforded only CA–acetazolamide adducts (even when a high excess of DTT was present in solution, as compared to acetazolamide), whereas cocrystallization of CA II–DTT–acetazolamide did not lead to the formation of crystals so far, although we worked in several different experimental conditions.

In conclusion, we investigated the inhibition of three CAs with dithiothreitol, 2-mercaptoethanol, tris(carboxyethyl)phosphine, all reducing agents frequently added to enzyme assay buffers, as well as the structurally related non-reducing agent threitol. All these agents behaved as very weak inhibitors of isoforms CA II (cytosolic) and CA IX (transmembrane, both full-length extracellular and catalytic domains), but they strongly influenced the binding of the low nanomolar-sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) to these enzymes. Indeed, acetazolamide affinity for all investigated CAs was diminished in the order of magnitude by increasing con-

centrations of these agents in the assay system (in the range of 10 μM–10 mM). DTT and the related agents MET, TCP or THT should thus not be added in assay buffers monitoring CA inhibition with sulfonamides, as they lead to an under-estimation of the binding constants, by a mechanism which remains to be investigated in more detail.

Acknowledgments

This research was financed in part by two grants of the 6th Framework Programme of the European Union (EUROXY and DeZnIT projects).

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