Eur. J. Med. Chem. 35 (2000) 31–39

S0223523400001021/FLA

Original article

Carbonic anhydrase activators – Part 21[#]. Novel activators of isozymes I, II and IV incorporating carboxamido and ureido histamine moieties

Andrea Scozzafava, Claudiu T. Supuran*

Università degli Studi, Laboratorio di Chimica Inorganica e Bioinorganica, Via Gino Capponi 7, I-50121, Florence, Italy

Received 29 March 1999; revised 16 June 1999; accepted 17 June 1999

Abstract – Reaction of histamine with tetrabromophthalic anhydride and protection of its imidazole moiety with tritylsulfenyl chloride, followed by hydrazinolysis, afforded N-1-tritylsulfenyl histamine, a key intermediate which was further derivatized at its aminoethyl moiety. Carboxamido derivatives were obtained by reaction of the key intermediate with carboxylic acid anhydrides, acyl chlorides or carboxylic acids in the presence of carbodiimides. Reaction of the same key intermediate with isocyanates, isothiocyanates, cyanamide or dicyandiamide afforded another series of compounds. Deprotection of the above-mentioned intermediates with hydrochloric acid in dioxane afforded two series of compounds, histamine derivatives possessing carboxamido, ureido, thioureido or guanidino moieties in their molecule. The new derivatives were assayed as activators of three carbonic anhydrase (CA) isozymes, hCA I, hCA II (cytosolic forms) and bCA IV (membrane-bound form, h = human, b = bovine isozyme). Efficient activation was observed against all three isozymes, but especially against hCA I and bCA IV, with affinities in the nanomolar range for the best compounds. hCA II was, on the other hand, activatable with affinities around 10–25 nM. This new class of CA activators might lead to the development of drugs/diagnostic agents for the CA deficiency syndrome, a genetic disease of bone, brain and kidneys. © 2000 Éditions scientifiques et médicales Elsevier SAS

carbonic anhydrase / histamine / carboxylic acid derivatives / isocyanates / isothiocyanates / enzyme activators

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors of the unsubstituted sulfonamide type RSO_2NH_2, are widely used drugs for the treatment or prevention of a variety of diseases such as: glaucoma [2], epilepsy [3], gastric and duodenal ulcers [4] or acid–base disequilibria [5] among others. In contrast to inhibitors, activators of this enzyme (for which at least eight different isozymes were isolated up to now in higher vertebrates) [6] were much less investigated. Only recently the X-ray crystallographic structures of the first adducts of the physiologically relevant isozyme II (hCA II) with the activators histamine [7] and phenylalanine (in this case a tertiary complex, in which azide is also bound to the Zn(II) ion) [8] have been reported by this group. Furthermore, few other QSAR [9–11] or synthetic chemistry [10–13] studies were reported in the field of CA activators, although some of these compounds might be used in the treatment of the CA deficiency syndrome, a genetic disease of bone, brain and kidney affecting a large enough number of patients [14]. In this condition, a certain CA isozyme gene (generally CA II, I or IV) is either not expressed, or its protein product is unstable due to deleterious mutations, and the corresponding CA isozyme is absent in the blood, kidney or lung of such patients. No pharmacologically specific treatment for this condition is available up to now. CA activators are also important for understanding the CA catalytic and inhibition mechanisms [7–13].

The lead molecule considered by us for obtaining tighter binding CA activators was histamine **1** itself. As seen from the X-ray co-ordinates with which *figure 1* was generated, the activator molecule is bound at the entrance of the hCA II active site cavity, where it is anchored by hydrogen bonds to amino acid side-chains and to water molecules. Such hydrogen bonds involve only the nitrogen atoms of the imidazole moiety, whereas the terminal aliphatic amino group is not experiencing any contact with the enzyme, but is extending away from the cavity into the solvent. On the other hand, the Nδ1 and Nε2

[#]See Ref. [1]

*Correspondence and reprints: cts@bio.polosci.unifi.it

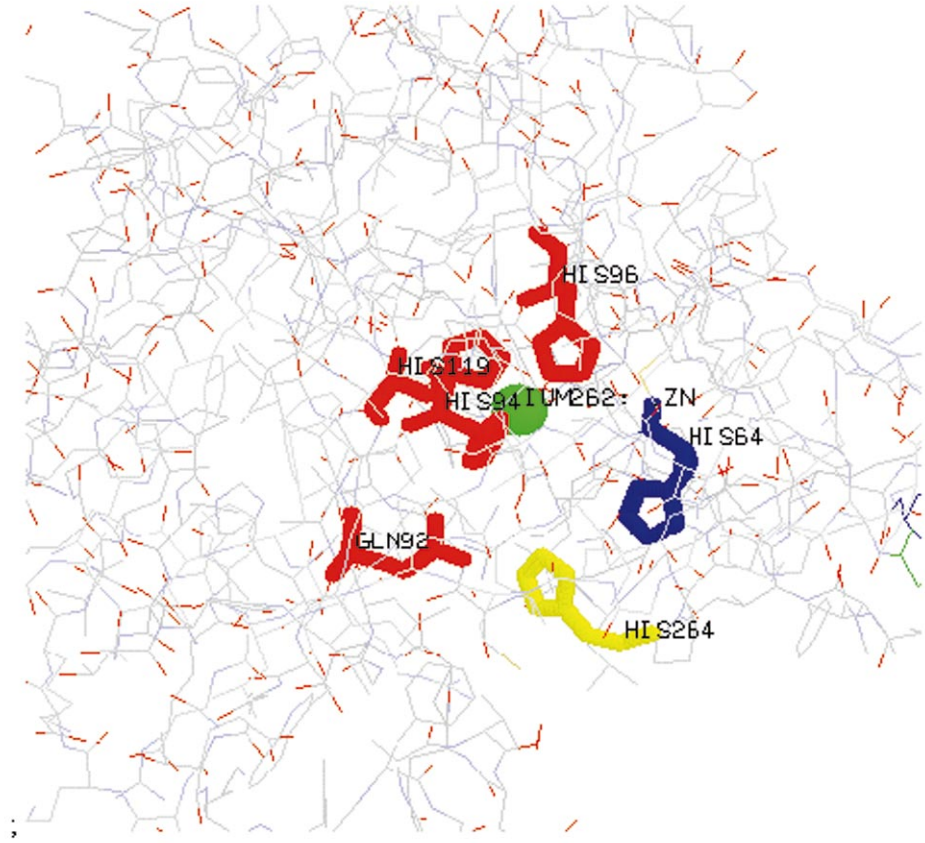


Figure 1. hCA II–histamine adduct: the Zn(II) ion (central sphere) and its three histidine ligands (His 94, His 96 and His 119) are shown at the centre of the active site, whereas histamine (numbered as His 264) is situated at the entrance to it, between residues His 64 and Gln 92. The figure was generated from the X-ray coordinates of the hCA II–histamine adduct reported by this group [7] with the program RasMol for Windows 2.6. The coordinates of this structure are depositated in the Brookhaven Protein Database (PDB entry 4TST).

atoms of the histidine imidazole ring are engaged in hydrogen bonds with the side-chains of Asn 62, His 64, Gln 92 and with Wat 152 [7]. Thus, it appeared of interest to derivatize the lead at its aliphatic NH_2 moiety, just in order to exploit the energy of binding of such modified groups with amino acid residues at the edge of the active site. This approach has been successfully used both by Whiteside's [15] and our group [7, 16, 17] for the design of tight-binding, isozyme-specific sulfonamide CA inhibitors. Moreover, recently we have reported some sulfonylated derivatives of histamine (at the aliphatic amino group) possessing high affinities for the three CA isozymes mentioned above [1]. Thus, it appeared of interest to explore other types of moieties that might be attached at the aliphatic end of the molecule, and carboxamido, ureido and thioureido derivatives appeared of interest due to the possible interactions of these highly polarized groups with amino acid residues at the edge of the active site, which presumably would lead to increased stabilities of the enzyme–activator adducts.

In this paper we report the synthesis of a series of carboxamido, ureido, thioureido and guanidino histamine derivatives obtained by reaction of appropriately protected [1, 18] histamine with carboxylic acids, acyl chlorides, carboxylic anhydrides, isocyanates, isothiocyanates or cyanamide. The new compounds were assayed as activators of three CA isozymes, hCA I, hCA II and bCA IV (h = human, b = bovine isozyme) and generally showed very good activities. SAR in these series of derivatives are also discussed.

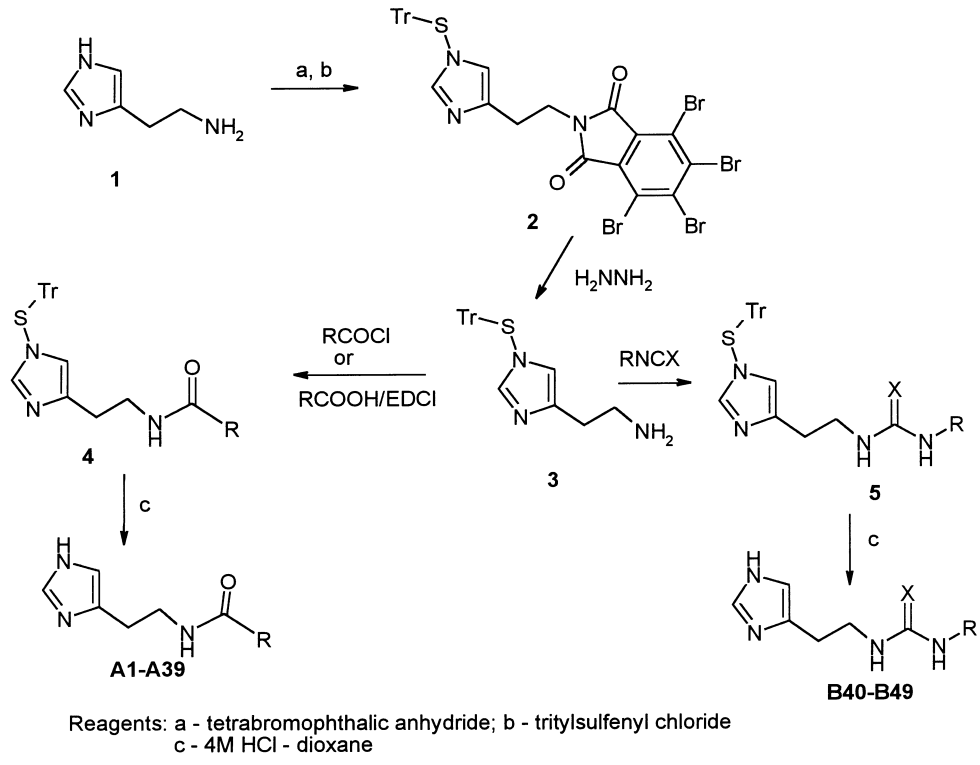


Figure 2. Synthesis of CA activators **A1**–**A39**.

2. Chemistry

Reaction of histamine **1** with tetrabromophthalic anhydride afforded the corresponding phthalimide, which was further protected at the imidazole NH moiety with triphenylmethanesulfenyl chloride (tritylsulfenyl chloride), leading to **2**. The key intermediate (N-1-tritylsulfenyl histamine, **3**) was easily obtained by room temperature hydrazinolysis of **2**, whereas its treatment with carboxylic acid anhydrides, acyl halides in the presence of triethylamine or carboxylic acids in the presence of carbodiimide derivatives, afforded derivatives of type **4** in high yields. These last compounds were then deprotected with 4 M HCl-dioxane, giving the target compounds **A1–A39**. Alternatively, another series of compounds was obtained by treating N-1-tritylsulfenyl histamine **3** with aryl/alkyl isocyanates/isothiocyanates, cyanamide or dicyandiamide, followed by deprotection of the imidazolic moiety in a similar manner as that described above, leading to derivatives of type **B40–B49** (*figure 2*). The first procedure mentioned above is relatively similar to that reported recently by Wolin et al. [18] for the syntheses of some sulfonylated homologues of histamine possessing H_3 antagonistic properties, except that different protecting groups were used by us, making it possible to work in much milder reaction conditions.

Another two compounds, **C1** and **C2**, were obtained by the reaction of EDTA dianhydride or DTPA dianhydride with histamine in molar ratios of 1:2 (*figure 3*).

3. Pharmacology

The new activators reported here were assayed against three CA isozymes (*table I*) due to the fact that hCA I, hCA II and bCA IV are the most abundant among the eight CA's isolated so far in higher vertebrates [6] on one hand, and on the other one, the high activity forms hCA II and bCA IV are considered to play the key physiological function in many tissues in which CA activity is present [2, 3, 6]. Although hCA I is the second most abundant protein in blood (after serum albumin), its physiological role is unknown [2, 4, 6]. Its activation process has never been considered from the physiological point of view up to now.

Thus, it was recently proven by this group that a concentration as low as 10^{-7} M of histamine produces a 150% activation of hCA I [7]. Although hCA II, one of

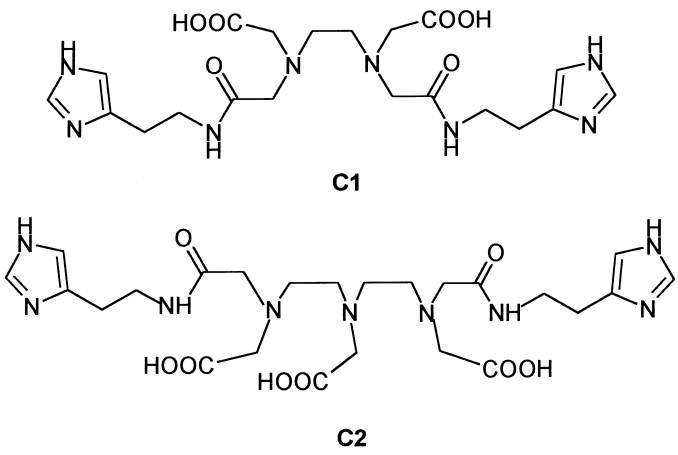


Figure 3. Structures of the potent CA activators **C1** and **C2**.

the most powerful catalysts ever evolved in nature [4], is not so abundant as hCA I [4, 6], this high activity isozyme is present in virtually every cell [2, 4, 6] where it catalyses the hydration of CO_2 generated in metabolic processes. In many tissues (such as ciliary processes within the eye, gastric or pancreatic mucosa, kidneys, etc.) it also participates in the processes of electrolyte secretion, generating H^+ or HCO_3^- ions (the products of CO_2 hydration) [2, 4]. Isozyme IV is the only membrane-bound one, being very abundant in the lung, kidney, gastro-intestinal tract and hepatocytes of vertebrates among others [2, 4, 14]. Like hCA II, it is a high activity form [4]. It is thus of considerable interest to search activators (and obviously, also inhibitors) that would react predominantly with one or the other of these physiologically important enzymes. This should constitute an interesting means for assessing the physiological function of many of the presently known CA isozymes, as well as for the development of agents able to activate CA isozymes in patients affected by CA deficiency syndromes [14].

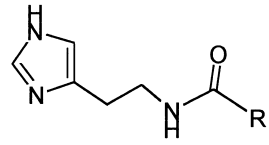
4. Results and discussion

Silverman's laboratory [19] reported activation of hCA II by histidine, and then considered that the activation initially observed was due to chelation of trace Cu(II) ions (which inhibit CA) eventually present in the enzyme preparation, by EDTA added in the buffer [20]. We explained in a previous publication the problems caused by the above mentioned experimental protocols [21]; and only recently, with the report of the first X-ray crystallographic data [7, 8] of adducts of histamine and phenylalanine with hCA II, has this topic received again its deserved importance, and some other types of high affinity activators investigated mainly by this [7–13] and Chegwidden's [22] group.

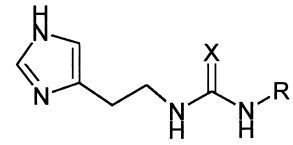
The key intermediate for obtaining novel types of activators reported in this paper, N-1-tritylsulfenyl histamine **3**, was obtained by well-known procedures involving the initial protection of the primary amine moiety by means of phthalimide derivatives, followed by protection of the imidazolic NH moiety with tritylsulfenyl chloride, and hydrazinolysis of the phthalimido moiety in mild conditions. The overall yield of the three steps was good (around 80%) and the purification procedures quite simple. Subsequent reaction of the key intermediate **3** with carboxylic acids or their derivatives [23, 24], or with isocyanates/isothiocyanates/cyanamides [25], afforded two series of N-tritylsulfenylated compounds, **4** and **5**, which were deprotected in standard conditions, leading thus to the desired derivatives **A1**–**A39** and **B40**–**B49**. Alternatively, reaction of EDTA dianhydride or DTPA dianhydride with histamine at molar ratios of 1:2 afforded the bis-functionalized derivatives **C1** and **C2**. All the new compounds reported here have been characterized by IR, ^{1}H- and ^{13}C-NMR spectroscopy, as well as elemental analysis (± 0.4% of the theoretical data, calculated for the proposed formulae). Spectral data for one representative example of each series are shown in the Experimental protocols.

The data of *table I* show significant differences between the investigated isozymes in their behaviour towards both 'classical' activators, such as histamine **1**, as well as the new class of activators synthesized in the present work. Thus, histamine **1** is a potent hCA I activator and a relatively weak hCA II activator, whereas isozyme bCA IV possesses an intermediate behaviour. The most interesting finding of the present study is represented by the high susceptibility of the cytosolic isozyme, hCA II, to be activated by some of the derivatives of histamines of types **A–C**, as compared to the lead molecule (compounds with activation constants in the 0.10–0.15 μM range were frequently obtained). Moreover, the highly abundant and most prone to activation (by histamine) isozyme hCA I, was also susceptible to activation by the new derivatives reported here (with constants in the nanomolar range for the most active derivatives), but differences of activity are not so pronounced as compared to the situation for the rapid isozyme hCA II. bCA IV, on the other hand, had an intermediate behaviour towards the new class of activators, with activation constants in the 0.01–0.10 μM range for the most active of such compounds.

Table I. CA isozymes I, II and IV activation with histamine **1** and its derivatives of types **A1–A39**, **B40–B49** and **C1**, **C2**, together with their synthetic methods.



A1-A39



B40-B49

No.	X	R	K_A* (μM)			Synth. method
			hCA I[a]	hCA II[a]	bCA IV[b]	
1	– histamine	–	2	125	41	–
A1	–	CF_3	0.016	1.0	2.5	A
A2	–	CCl_3	0.020	1.3	3.6	B
A3	–	$CHCl_2$	0.18	4.9	5.5	B
A4	–	CH_2Cl	0.67	5.5	6.3	C
A5	–	$CH_3(CH_2)_{10}$	0.21	70	21	B
A6	–	$HOOCCH_2CH_2$	0.18	84	19	A
A7	–	$HOOC(CH_2)_4$	0.20	86	16	C
A8	–	*cis*-HOOCCH=CH	0.21	62	14	A
A9	–	$HOOCCH_2OCH_2$	0.12	39	13	A
A10	–	4-F-C_6H_4	0.11	12	2.9	B
A11	–	2-Cl-C_6H_4	0.10	13	2.7	B
A12	–	3-Cl-C_6H_4	0.10	13	2.5	B
A13	–	2,4-$Cl_2C_6H_3$	0.07	9	3.0	B
A14	–	4-I-C_6H_4	0.08	10	3.2	C
A15	–	3-I-C_6H_4	0.05	8	4.0	C
A16	–	2-I-C_6H_4	0.06	7	4.4	C
A17[c]	–	4-tsHN-C_6H_4	0.03	1.3	0.15	C
A18[c]	–	3-tsHN-C_6H_4	0.06	0.8	0.10	C
A19[c]	–	2-tsHN-C_6H_4	0.05	0.5	0.65	C
A20	–	C_6F_5	0.02	0.10	0.02	B
A21	–	1-naphthyl	0.14	10	18	C
A22	–	2-naphthyl	0.12	9	15	C
A23	–	2-thienyl	0.13	8	10	B
A24	–	2-pyridyl	0.009	0.15	1.4	C
A25	–	3-pyridyl	0.008	0.20	1.5	B
A26	–	4-pyridyl	0.006	0.18	1.0	B
A27[d]	–	NTA	0.005	3.1	0.9	C
A28[e]	–	EDTA	0.004	0.7	0.9	C
A29[f]	–	DTPA	0.005	0.5	0.8	C
A30	–	2-HOOC-C_6Br_4	0.05	14	5.1	A
A31	–	2-HOOC-C_6H_4	0.26	25	6.6	A
A32	–	2-HOOC-C_6Hr_{11}	0.27	29	7.5	A
A33[c]	–	ts-Nipecotinoyl	0.08	9	3.2	C
A34[c]	–	ts-Isonipecotinoyl	0.09	5	3.1	C
A35	–	Ph_2N	0.15	13	5.8	B
A36	–	4-Cl-3-H_2NO_2S-C_6H_3	strong CA inhibitor[g]			C
A37	–	4-*n*-Pr_2NSO_2-C_6H_4	0.04	1.9	4.2	C
A38	–	4-H_2NO_2S-C_6H_4	strong CA inhibitor[h]			C
A39	–	H_2N	0.21	45	24	D
B40	O	4-Cl-C_6H_4	0.005	0.19	0.05	D
B41	O	3-Cl-C_6H_4	0.006	0.15	0.03	D
B42	O	2,4-$F_2C_6H_3$	0.005	0.08	0.02	D
B43	O	2,4-$Cl_2C_6H_3$	0.006	0.12	0.07	D
B44	O	1-naphthyl	0.015	0.40	0.10	D
B45	S	CH_2=$CHCH_2$	0.020	3.5	0.25	E
B46	S	C_6H_5CONH	0.004	0.12	0.03	E
B47	S	H	0.036	16	12	E
B48	NH	H	0.015	1.50	0.3	F
B49	NH	$H_2NC(=NH)$-	0.010	0.25	0.5	G
C1	–	–	0.006	0.12	0.03	–
C2	–	–	0.003	0.08	0.02	–

* Mean from at least two determinations by the esterase method [22]. Standard error was in the range of 5–10%; [a] Human cloned isozyme; [b] Purified from bovine lung microsomes [21]; [c] ts = 4-Me-$C_6H_4SO_2NHCO$-; [d] NTA = nitrilotriacetyl, $(HOOC)_2N$-CO-; [e] EDTA = ethylenediaminotetraacetyl, $(HOOCCH_2)_2N$-$CH_2CH_2N(CH_2COOH)CH_2CO$-; [f] DTPA = diethylenetriaminopetaacetyl, $(HOOCCH_2)_2N$-CH_2CH_2N $(CH_2COOH)CH_2CH_2N(CH_2COOH)CH_2CO$-; [g] K_I of: 32 nM (hCA I); 3.4 nM (hCA II) and 12 nM (bCA IV), respectively; [h] K_I of: 35 nM (hCA I); 4.1 nM (hCA II) and 13 nM (bCA IV), respectively.
Synthetic methods: **A**: N-1-tritylsulfenyl-histamine (**3**) + $(RCO)_2O$, followed by deprotection; **B**: N-1-tritylsulfenyl-histamine (**3**) + RCOCl/Et_3N, followed by deprotection; **C**: N-1-tritylsulfenyl-histamine (**3**) + RCOOH/carbodiimide/1-HO-benzotriazole, followed by deprotection; **D**: N-1-tritylsulfenyl-histamine (**3**) + isocyanate, followed by deprotection; **E**: N-1-tritylsulfenyl-histamine (**3**) + isothiocyanate, followed by deprotection; **F**: N-1-tritylsulfenyl-histamine (**3**) + cyanamide, followed by deprotection; **G**: N-1-tritylsulfenyl-histamine (**3**) + dicyandiamide, followed by deprotection.

Substitution patterns leading to efficient CA activators were: (i) perfluoroalkyl and perfluoroaryl, such as in derivatives **A1**, **A2**, or **A20**, which were among the most active compounds in the whole series of derivatives **A**, against all three isozymes; (ii) For the substituted-aryl derivatives, good CA activation was detected for compounds possessing 4-halogenophenyl; 4-, 3- or 2-iodophenyl, 4-, 3- or 2-toluenesulfonylureido-phenyl as well as 2-, 3- or 4-pyridyl- moieties in their molecule; (iii) very efficient activators were also those derived from polyamino-polycarboxylic acids, such as nitrilotriacetic (NTA); EDTA (ethylenediaminotetraacetic acid) or DTPA (diethylenetriaminopentaacetic acid), both in their mono- as well as bis-derivatives, of types **A27**–**A29** or **C1** and **C2**. The many heteroatoms present in these moieties probably contribute to the obtained compounds 'sticky' properties, i.e., they are able to participate in many interactions with amino acid residues from the active site, thus assuring the formation of very stable E–A (enzyme-activator) adducts; (iv) An interesting case is represented by the two compounds containing free sulfamoyl moieties in their molecule, **A36** and **A38**, which behave as very strong inhibitors of all the investigated isozymes. These two compounds, and the structurally related probenecid derivative **A37** were designed in such a way as to contain a moiety able to interact with the inhibitor binding site of the enzyme (i.e., the Zn(II) ion, where the sulfonamide anion coordinates) and with the activator binding site, where the histamine molecule binds (the edge of the entrance to the cavity, between amino acid residues Asn 62, His 64, and Gln 92 (*figure 1*). It was important to understand which of the binding forces would be stronger, and it is obvious from the data of *table I* that the inhibitory one predominates. It is also probable that the very strong inhibition observed with these two compounds (**A36** and **A38**) might be due to a binding in which the activator-binding site also participates in the interaction, further stabilizing the E–I adduct. The probenecid derivative **A37** on the other hand behaves as a relatively potent CA activator, since its sulfonamido moiety, being substituted, is unable to interact with the metal ion; (v) Slightly less active compounds were those containing long alkyl chains, carboxy-alkyl and carboxy-alkenyl ones, such as in compounds **A5**–**A9**; (vi) The ureido/thioureido derivatives of type **B40–B49** included some of the best CA activators reported here, with affinities in the 3–6 nM range for hCA I, 80–150 nM for hCA II and 10–30 nM for bCA IV (*table I*). The halogeno-containing derivatives **B40**–**B43** were slightly more active than the 1-naphthyl- or allyl derivatives **B44** and **B45**, but for the moment this subseries of compounds is too small for having a detailed SAR. The simple ureido and thioureido compounds were much less active, whereas the guanidino/bis-guanide ones were more active against all three investigated isozymes. Generally, no great differences in activity were evidenced between the isocyanates, the isothiocyanates or the guanidines of this type, although the sub-series is relatively small for the moment.

5. Conclusions

Similarly to all CA activators reported up to now, the compounds obtained in the present work presumably intervene in the catalytic cycle, leading to the formation of an enzyme–activator complex (similarly to the enzyme–inhibitor adducts, but without substitution of the metal bound solvent molecule), in which the activator bound within the active site facilitates proton transfer processes (which represent the rate-limiting step in catalysis) [7–13]. The driving force of this effect might be the fact that intramolecular reactions are more rapid than intermolecular ones. Thus, in the presence of activators (symbolized as 'A'), the rate-limiting step is described by equation 1 below [7–13]:

$$EZn^{2+}-OH_2 + A \Leftrightarrow [EZn^{2+}-OH_2-A] \Leftrightarrow [EZn^{2+}-HO^- - AH^+] \Leftrightarrow EZn^{2+}-OH^- + AH^+ \quad (1)$$

enzyme–activator complexes

Obviously, compounds of the types reported here possess the imidazolic moiety which can participate in the proton transfer processes between the active site and the environment (similarly to histamine **1**), but due to the presence of alkyl/arylcarboxamido (ureido) tails in their molecule they can bind more effectively to the enzyme, thus allowing for more efficient activation processes as compared to **1**. Indeed, the active site edge of all three CA isozymes investigated by us contain a high proportion of polar amino acid residues which might interfere with polar groups such as RCONH or RNHCXNH (X = O, S, NH). In fact, such amino acid residues might explain the different catalytic properties of the diverse isozymes, as well as their diverse susceptibility to be inhibited/activated by modulators of activity [7, 8]. For instance, the entrance of the active site of isozyme hCA II contains a cluster of six histidine residues (His 3, His 4, His 10, His 15, His 17 and His 64), some of which possess different conformations (as shown by X-ray crystallography) [7, 8] which could easily participate in hydrogen bond formation (as well as other types of interactions) with the histamine derivatives reported here. This might explain, in fact, the greater efficiency of the compounds

reported in the present work in activating this isozyme, as compared to histamine, which is a relatively weak hCA II activator.

6. Experimental protocols

6.1. Chemistry

Melting points were determined with a heating plate microscope and are not corrected; IR spectra were obtained in KBr pellets with a Perkin-Elmer 16PC FTIR spectrometer, whereas ^{1}H-NMR spectra with a Varian 300CXP apparatus in solvents specified in each case. Chemical shifts are expressed as δ values relative to Me_4Si as standard. Elemental analyses were done by combustion for C, H, N with an automated Carlo Erba analyzer, and were ± 0.4% of the theoretical values. Preparative HPLC was done (C_{18} reversed-phase Bondapack or Dynamax-60A (25 × 250 mm) columns.

Compounds used in synthesis (histamine, carboxylic acids, carboxylic acid anhydrides, acyl chlorides, alkyl/aryl isocyanates/isothiocyanates, cyanamide, dicyandiamide, tritylsulfenyl chloride, tetrabromophthalic anhydride, hydrazine, EDTA dianhydride, DTPA dianhydride, etc.) were commercially available compounds (from Sigma, Acros or Aldrich). The tosylureido-amino acid derivatives were prepared as described previously [23] by the reaction of tosylisocyanate with amino acids. Acetonitrile, dioxane (Merck) or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

6.1.1. Preparation of N-1-tritylsulfenyl-histamine **3**

An amount of 5.55 g (50 mM) of histamine and 23.15 g (50 mM) of tetrabromophthalic anhydride were suspended in 300 mL of dry toluene and refluxed under Dean-Stark conditions until water was separated (generally 2–3 h). The solvent was evaporated in vacuo, the crude product dissolved in 150 mL of anhydrous acetonitrile and was treated with 15.5 g (50 mM) of tritylsulfenyl chloride and 6.95 mL (50 mM) of triethylamine. The mixture was stirred at room temperature for 3 h (TLC control), then the solvent was evaporated and the crude product stirred with 100 mL of water and ice. The tan precipitate obtained was filtered, dried and used directly in the deprotection step. Hydrazinolysis was effected by dissolving the above mentioned precipitate in 200 mL of ethanol and addition of 15 mL of hydrazinium hydroxide, followed by stirring for 5 h at room temperature. Then the solvent was evaporated, a small excess of 2 N HCl solution was added, the precipitated tetrabromophthalhydrazide was filtered and discarded, whereas the solution containing **3** was brought to pH 7 with solid $NaHCO_3$, brought to a small volume by in vacuo evaporation of the solvent, and the precipitated **3** was then recrystallized from ethanol (yield of 80%, based on histamine, after the three steps described above). Tan crystals, m.p. 177–178 °C, ^{1}H-NMR (300 MHz, DMSO-d_6), δ ppm: 2.47 (t, 2H, J = 7.0 Hz, CH_2); 2.96 (q, 2H, J = 6.2, 12.5 Hz, H_2NCH_2); 4.23 (m, 2H, NH_2); 7.10–7.30 (m, 15H, trityl); 7.34 (m, 1H, imidazole CH); 8.35 (s, 1H, imidazole CH); Anal. ($C_{24}H_{23}N_3S$) C, H, N, S.

6.1.2. General procedure for the preparation of compounds **A1–A39** *and* **B40–B49**

Methods A–C: an amount of 10 mM N-1-tritylsulfenyl-histamine **3** was dissolved in 50 mL of anhydrous acetonitrile and then treated with a solution obtained from 10 mM of carboxylic acid anhydride (method A); acyl chloride (10 mM) and Et_3N (10 mM) (method B) or carboxylic acid (10 mM), diisopropyl-carbodiimide (or EDCI) (10 mM) and 1-hydroxybenzotriazole (10 mM) (method C) in the same solvent. The reaction mixture was stirred at 4 °C for 3–9 h (TLC control). The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (50 mL), poured into a 5% solution of sodium bicarbonate (50 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. In many cases the compounds of type **4** precipitated, were filtered, dried and deprotected at the N-1 imidazolic moiety in the following way. The crude **4** was dissolved in 20 mL of dioxane and treated with 25 mL of a 4 M HCl solution in dioxane, followed by heating at reflux for 2 h. The solvent was evaporated, the residue taken up in 50 mL of a 5% solution of sodium bicarbonate and the trityl sulfenyl chloride formed during the deprotection step extracted in 2 × 50 mL of Et_2O. The water phase was evaporated in vacuo to a small volume, at which point, generally, compounds **A1**–**A39** precipitated by letting the mixture stand at 4 °C overnight. The pure compounds were obtained after recrystallization from ethanol/water (1:1, v/v). In some cases, preparative HPLC was done (C_{18} reversed-phase Bondapack or Dynamax-60A (25 × 250 mm) columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min) in order to obtain the pure compounds of type **A1**–**A39**.

Methods D and E: an amount of 10 mM N-1-tritylsulfenyl-histamine **3** was dissolved in 50 mL of anhydrous acetonitrile and then treated with a solution obtained from 10 mM of isocyanate (method D) or isothiocyanate (method E) dissolved in the same solvent. The mixture was refluxed for 2–4 h, then the work-up and

the deprotection was effected as described above in methods A–C.

Methods F and G: an amount of 10 mM N-1-tritylsulfenyl-histamine **3** was dissolved in 50 mL of ethanol and then treated with a solution obtained from 12 mM of cyanamide (method F) or dicyandiamide (method G), and 5 mL of concentrated aqueous HCl. The reaction mixture was refluxed for 2 h, then the solvent was evaporated in vacuo, the tritylsulfenyl chloride extracted with Et_2O, and the crude guanidines **B48** and **B49** recrystallized from iso-propanol.

6.1.3. Preparation of compounds **C1** *and* **C2**

The procedure of Aimé et al. [26] (developed for DTPA-based NMR contrast agents) has been followed, since it was observed that no N-1 acylated derivatives were formed, and protection of the imidazolic NH group was thus not necessary. An amount of 222 mg (2 mM) of histamine was dissolved in 20 mL of anhydrous acetonitrile and treated with 1 mM of EDTA dianhydride or DTPA dianhydride dissolved in the same solvent. The mixture was stirred at room temperature for 6 h, the solvent was evaporated and the crude residue recrystallized from acetone/ethanol (2:1). Yields in **C1** and **C2** were around 95%.

6.1.4. 4-[(-(Pyridine-2-carboxamidoethyl)]-1H-imidazole **A24**

White crystals, m.p. > 300 °C; IR (KBr), cm^{-1}: 1 295 (amide III), 1 565 (amide II), 1 690 (amide I), 3 060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.47 (t, 2H, J = 7.0 Hz, CH_2); 2.93 (t, 2H, J = 7.0 Hz, CONHCH_2); 7.34 (m, 1H, imidazole CH); 7.62 (t, 1H, J = 7.9 Hz, H^4 of pyridine); 7.70–7.93 (m, 3H, H^3, H^5 and H^6 of pyridine); 8.15 (s, 1H, CONH); 8.35 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3; 37.9; 121.5; 125.9; 137.2; 157.3; 164.4; 167.1; 176.9 (CONH); Anal. ($C_{11}H_{12}N_4O$) C, H, N.

6.1.5. 4-[(-(4-Chlorophenylureido)-ethyl)]-1H-imidazole **B40**

White crystals, m.p. > 294 °C (dec.); IR (KBr), cm^{-1}: 1 287 (amide III), 1 579 (amide II), 1 710 (amide I), 3 060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.49 (t, 2H, J = 7.0 Hz, CH_2); 2.99 (t, 2H, J = 7.0 Hz, CONHCH_2); 7.34 (m, 1H, imidazole CH); 7.44 (d, 2H, J = 7.8 Hz, p-Cl-C_6H_4); 7.79 (d, 2H, J = 7.8 Hz, p-Cl-C_6H_4); 8.21 (br s, 2H, NHCONH); 8.35 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3; 37.9; 130.5; 134.5; 135.3; 145.8; 154.9 (NHCONH); 157.3; 164.4; 167.1; Anal. ($C_{12}H_{13}ClN_4O$) C, H, N.

6.1.6. 14-(1H-Imidazole-4-yl)-3-[2-[[2-(1H-Imidazole-4-yl)ethyl]amino]-2-oxoethyl]-6,9-bis(carboxymethyl)-11-oxo-3,6,9,12-tetraazatetradecanoic acid **C2**

White crystals, m.p. 173–175 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.45 (t, 2H, J = 7.0 Hz, histamine CH_2); 2.94 (t, 2H, J = 7.0 Hz, histamine CONHCH_2); 3.19 (t, 4H, ethylenic CH_2 near lateral nitrogens); 3.35 (s, 4H, CH_2 of the lateral acetates); 3.47 (t, 4H, ethylenic CH_2 near central nitrogen); 3.56 (s, 4H, CH_2 of the acetamido groups); 3.89 (s, 2H, CH_2 of the central acetate); 7.34 (m, 1H, imidazole CH); 8.35 (s, 1H, imidazole CH); 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3; 37.9; 51.6; 54.4; 56.0; 59.8; 60.7; 135.3; 145.8; 154.9; 171.6 (central COOH); 173.6 (CONH); 180.1 (lateral COOH); Anal. ($C_{24}H_{37}N_9O_8$) C, H, N.

6.2. Pharmacology

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 Forsman et al. [27] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group [28], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [29]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 $mM^{-1}.cm^{-1}$ for hCA I and 54 $mM^{-1}.cm^{-1}$ for hCA II, respectively, based on M_r = 28.85 kDa for hCA I, and 29.30 kDa for hCA II, respectively [30, 31]. bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration was determined by titration with ethoxzolamide [32].

Initial rates of 4-nitrophenyl acetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [33]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1.10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18 400 $M^{-1}.cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature [34]. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each activator concentration and the values reported throughout the paper are the means of such results. Stock solutions of activator (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Activator and enzyme

solutions were preincubated together for 10 min at room temperature prior to assay in order to allow for the formation of the E–A complex. The activation constant K_A was determined as described in ref. [7]. Enzyme concentrations were 3.2 nM for hCA II, 9 nM for hCA I and 16 nM for bCA IV (this isozyme has a decreased esterase activity [34] and higher concentrations had to be used for the measurements).

Acknowledgements

This research was financed by the EU grant ERB CIPDCT 940051.

References

[1] Briganti F., Scozzafava A., Supuran C.T., Bioorg. Med. Chem. Lett. 9 (1999) 2043–2048. Part 20 of the series.

[2] Supuran C.T., Scozzafava A., Ilies M.A., Iorga B., Cristea T., Briganti F., Chiraleu F., Banciu M.D., Eur. J. Med. Chem. 33 (1998) 577–595.

[3] Reiss W.G., Oles K.S., Ann. Pharmacother. 30 (1996) 514–519.

[4] Supuran C.T., in: Puscas I. (Ed.), Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism, Helicon, Timisoara, 1994, pp. 29–112.

[5] Supuran C.T., Conroy C.W., Maren T.H., Eur. J. Med. Chem. 31 (1996) 843–846.

[6] Hewett-Emmett D., Tashian R.E., Mol. Phylogenet. Evol. 5 (1996) 50–77.

[7] Briganti F., Mangani S., Orioli P., Scozzafava A., Vernaglione G., Supuran C.T., Biochemistry 36 (1997) 10384–10392.

[8] Briganti F., Iaconi V., Mangani S., Orioli P., Scozzafava A., Vernaglione G., Supuran C.T., Inorg. Chim. Acta 275-276 (1998) 295–300.

[9] Clare B.W., Supuran C.T., J. Pharm. Sci. 83 (1994) 768–779.

[10] Supuran C.T., Balaban A.T., Cabildo P., Claramunt R.M., Lavandera J.L., Elguero J., Biol. Pharm. Bull. 16 (1993) 1236–1239.

[11] Supuran C.T., Claramunt R.M., Lavandera J.L., Elguero J., Biol. Pharm. Bull. 19 (1996) 1417–1422.

[12] Supuran C.T., Barboiu M., Luca C., Pop E., Brewster M.E., Dinculescu A., Eur. J. Med. Chem. 31 (1996) 597–606.

[13] Ilies M.A., Banciu M.D., Ilies M., Chiraleu F., Briganti F., Scozzafava A., Supuran C.T., Eur. J. Med. Chem. 32 (1997) 911–918.

[14] Sly W.S., in: Dodgson S.J., Tashian R.E., Gros G., Carter N.D. (Eds.), The Carbonic Anhydrases, Plenum Press, New York and London, 1991, pp. 183–196.

[15] Jain A., Whitesides G.M., Alexander R.S., Christianson D.W., J. Med. Chem. 37 (1994) 2100–2105.

[16] Supuran C.T., Ilies M.A., Scozzafava A., Eur. J. Med. Chem. 33 (1998) 739–751.

[17] Scozzafava A., Menabuoni L., Mincione F., Briganti F., Mincione G., Supuran C.T., J. Med. Chem. 42 (1999) 2641–2650.

[18] Wolin R., Connolly M., Afonso A., Hey J.A., She H., Rivelli M.A., Williams S.M., West R.E., Bioorg. Med. Chem. Lett. 8 (1998) 2157–2162.

[19] Silverman D.N., Tu C., Wynns G.C., J. Biol. Chem. 253 (1978) 2563–2567.

[20] Tu C., Wynns G.C., Silverman D.N., J. Biol. Chem. 256 (1981) 9466–9471.

[21] Supuran C.T., Puscas I., in: Puscas I. (Ed.), Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism, Helicon, Timisoara, 1994, pp. 113–146.

[22] Shelton J.B., Chegwidden W.R., Comp. Biochem. Physiol. 114A (1996) 283–289.

[23] Scozzafava A., Supuran C.T., J. Enzyme Inhib. 14 (1999) 343–363.

[24] Kurzer F., Douraghi-Zadeh K., Chem. Rev. 67 (1967) 107–152.

[25] Supuran C.T., Scozzafava A., Jurca B.C., Ilies M.A., Eur. J. Med. Chem. 33 (1998) 83–93.

[26] Aimè S., Fasano M., Paoletti S., Terreno E., Gazz. Chim. Ital. 125 (1995) 125–131.

[27] Forsman C., Behravan G., Osterman A., Jonsson B.H., Acta Chem. Scand. B42 (1988) 314–318.

[28] Behravan G., Jonasson P., Jonsson B.H., Lindskog S., Eur. J. Biochem. 198 (1991) 589–592.

[29] Khalifah R.G., Strader D.J., Bryant S.H., Gibson S.M., Biochemistry 16 (1977) 2241–2247.

[30] Nyman P.O., Lindskog S., Biochim. Biophys. Acta 85 (1964) 141–151.

[31] Henderson L.E., Henriksson D., Nyman P.O., J. Biol. Chem. 251 (1976) 5457–5463.

[32] Maren T.H., Wynns G.C., Wistrand P.J., Mol. Pharmacol. 44 (1993) 901–906.

[33] Pocker Y., Stone J.T., Biochemistry 6 (1967) 668–678.

[34] Baird T.T., Waheed A., Okuyama T., Sly W.S., Fierke C.A., Biochemistry 36 (1997) 2669–2678.