Carbonic Anhydrase Activators: High Affinity Isozymes I, II, and IV Activators, Incorporating a β-Alanyl-histidine Scaffold

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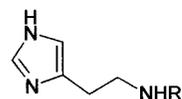
A novel class of tight binding carbonic anhydrase (CA) activators was designed by using histamine and histidine as lead molecules. Carnosine (β-Ala-His) derivatives were synthesized by reaction of appropriately derivatized β-alanines with imidazole/carboxy-protected histidine in the presence of carbodiimides, followed by removal of the various protecting groups. The derivatized β-alanines mentioned above were in turn obtained by coupling of 4-fluorophenyl-sulfonylureido amino acids (fpu-AA) or 2-toluenesulfonylureido amino acids (ots-AA) with β-Ala. Some structurally related dipeptides with the general formula fpu/ots-AA1-AA2 (AA, AA1, and AA2 represent amino acyl moieties) were also prepared by a similar strategy and used thereafter for obtaining CA activators incorporating a modified tetrapeptide scaffold. Many of the new tri-/tetrapeptide derivatives reported here proved to be efficient in vitro activators of three CA isozymes. Very good activity was detected against hCA I and bCA IV, for which some of the new compounds showed affinities in the 1–20 nM range (h = human; b = bovine isozymes), whereas against hCA II, their affinities were in the range of 10–40 nM. Ex vivo experiments showed some of the new activators to strongly enhance cytosolic red cell CA activity after incubation with human erythrocytes. This new class of CA activators might lead to the development of drugs/diagnostic tools for the management of CA deficiency syndromes, as well as for the pharmacological enhancement of synaptic efficacy, spatial learning, and memory. This may constitute a new approach for the treatment of Alzheimer's disease and other conditions in need of achieving memory therapy.

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) inhibition with sulfonamides, discovered by Mann and Keilin,[1] and its activation by different classes of compounds, reported by Leiner,[2] although simultaneous, had completely different consequences for research of these enzymes and their modulators of activity. Whereas CA inhibitors (CAIs) were extensively studied, leading to a detailed understanding of the catalytic and inhibition mechanisms and also to several valuable drugs,[3] CA activators (CAAs) constituted a controversial issue immediately after they were first described.[4] Thus, activation of crude human red cell enzyme (a mixture of isozymes CA I and CA II) by different compounds, such as histamine, amino acids, and some purine derivatives, has been reported and retracted several times by the above-mentioned and other authors,[5] without arriving at a clear-cut answer regarding the mere existence of such a class of CA activity modulators. This topic, then, received little attention from the scientific community for at least two reasons: (i) the statement by Clark and Perrin that activators of CA do not exist[6] and (ii) the idea that the reported activation is not a phenomenon per se but an artifact generally due to restoration of CA activity possibly lost in the presence of adventitious metal ions or other impurities (or due to enzyme adsorption at interfaces, or even due to enzyme denaturation followed by renaturation in the presence of activators).[7] Leiner,[2] the researcher whose role in

discovering this important class of modulators of CA activity should be completely reevaluated, observed among others that the activation was readily detected when working with highly purified enzyme preparations, and this may explain the large discrepancies between the different early studies describing this phenomenon. Only recently our group reported the X-ray crystallographic structures of adducts of the human isozyme hCA II with different activators, proving undoubtedly the existence of this class of modulators of enzyme activity as well as elucidating their mechanism of action at the molecular level.[8,9]

The very recent report[10] that some CAAs (such as phenylalanine and imidazole) administered to experimental animals may produce an important pharmacological enhancement of synaptic efficacy, spatial learning, and memory proves that this class of relatively unexplored enzyme modulators may have pharmacological applications in conditions in which learning and memory are impaired, such as for example Alzheimer's disease or aging. One must also mention that it was previously reported that the levels of CA are significantly diminished in the brain of patients affected by Alzheimer's disease,[11] and these facts strongly support

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1a: R = ArSO$_2$
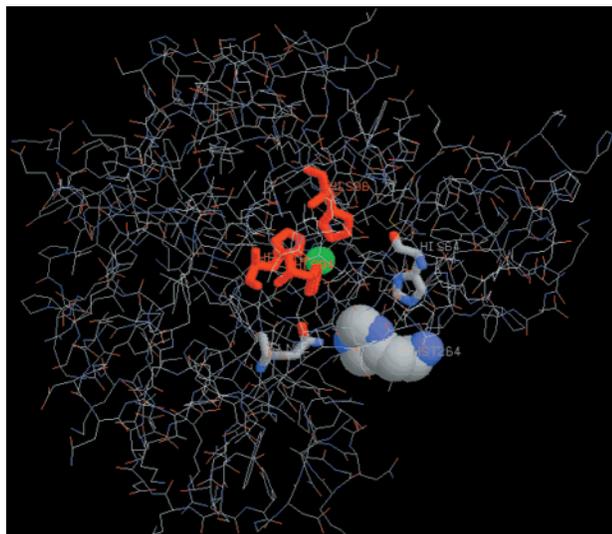**1b:** R = ArCO
1c: R = ArNHCO

Figure 1. hCA II−histamine adduct: the Zn(II) ion (central green sphere) and its three histidine ligands (in red, His 94, His 96, and His 119) are shown at the center of the active site, whereas histamine (in spacefill, Hst 264) is situated at the entrance in it, between residues His 64 (at the right) and Gln 92 (at the left). The coordinates of this structure are available in the Brookhaven Protein Database (PDB entry 4TST).[8]

the involvement of different CA isozymes in cognitive functions.[10,11]

In previous contributions from this laboratory[12−14] it was shown that effective CAAs of the type **1a**−**c** can be designed by considering histamine as a lead molecule.[8] Indeed, the X-ray crystallographic structure of the adduct of human CA II (hCA II) with histamine, a weak activator (activation constant, $K_A = 125 \mu$M), has recently been reported by our group,[8] showing (Figure 1) that the activator molecule is bound at the entrance of the active site cavity, anchored by hydrogen bonds to three amino acid side chains and to a water molecule. These hydrogen bonds involve only the nitrogen atoms of the imidazole moiety of histamine (the Nδ1 and Nϵ2 are engaged in hydrogen bonds with the side chains of Asn 62, His 64, and Gln 92 and with Wat 152), whereas the aliphatic amino group is not experiencing any contact with the enzyme but is extending away from the cavity into the solvent.[8] Positioned in such a favorable way, histamine facilitates the rate-limiting step of CA catalysis, i.e., the proton-transfer processes between the active site and the environment,[5] and also allows its easy derivatization (at the aliphatic amino group), in order to obtain stronger activators, as the compounds of type **1a**−**c** previously reported.[12−14]

The same activator-binding site as the one in the hCA II−histamine adduct was then shown to be occupied by phenylalanine, the second activator for which the X-ray structure has been reported (as a tertiary complex, hCA II−Phe−azide).[9] Correlated with the fact that, similar to phenylalanine, several other aromatic amino acids (for example, histidine) do show good CA activating properties, we decided to use this last amino acid (His) as a lead molecule for obtaining new types of tight-binding CAAs. The histidine dipeptide carnosine (β-Ala-His) is an even stronger CAA than histidine, and the new compounds described here were obtained starting from this compound. In this paper we report the

Scheme 1[a]

A1-A20: X = 4-F
B1-B20: X = 2-Me
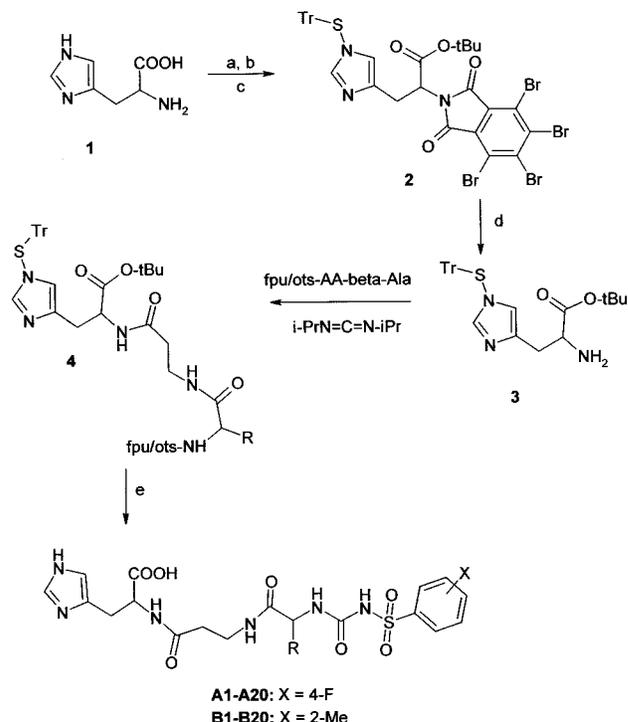
[a] Reagents: (a) tetrabromophthalic anhydride; (b) tritylsulfenyl chloride; (c) SOCl$_2$, then t-BuONa; (d) hydrazine hydrate; (e) 4 M HCl−dioxane.

Scheme 2

A21-A24: X = 4-F
B21-B24: X = 2-Me
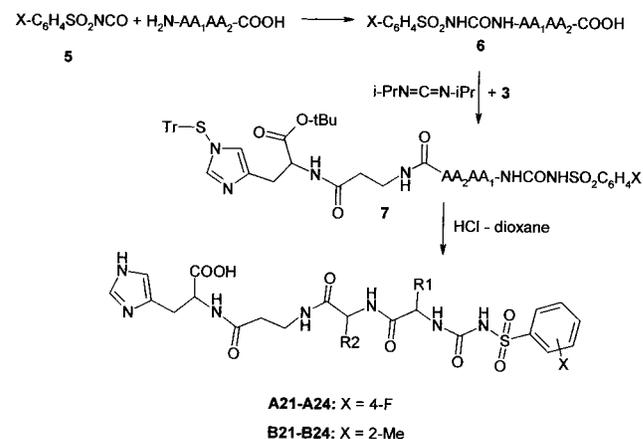
synthesis of a series of arylsulfonylureido-amino acyl/dipeptidyl carnosine derivatives possessing the general formula fpu/ots-AA-β-Ala-His and fpu/ots-AA1-AA2-β-Ala-His (AA, AA1, AA2 = amino acyl moieties; fpu = 4-fluorophenyl-sulfonylureido; ots = 2-toluenesulfonyl-ureido), which show excellent CA activatory properties against several physiologically relevant isozymes such as CA I, CA II, and CA IV. Ex vivo experiments and SAR for this new class of potent CAAs are also discussed.

Results

Synthesis. Preparation of compounds **A1**−**A24** and **B1**−**B24** is shown in Schemes 1 and 2. Routine synthetic procedures for the derivatization of amino acid/dipeptide derivatives, previously reported for the preparation of histamine-based CAAs, have been employed.[12−14]

Table 1. CA Isozymes I, II, and IV Activation with Histamine, Histidine 1, Carnosine, and the New Derivatives (**A**,**B**)**1**−**24**

A1-A20 :fpu-AA-β-AlaHis   A21 − A24: fpu-AA₁-AA₂-β-AlaHis
B1-B20 :ots-AA-β-AlaHis   B21 − B24: ots-AA₁-AA₂-β-AlaHis

no.	AA/AA₁AA₂	$K_A{}^a$ (μM) hCA I[b]	hCA II[b]	bCA IV[c]	yield
histamine		2	125	41	
histidine **1**		4	113	39	
carnosine (β-AlaHis)		1.3	35	18	
A1	Gly	0.20	15	2.1	71
A2	L-Ala	0.21	13	2.0	74
A3	β-Ala	0.20	13	3.9	63
A4	GABA	0.19	12	3.2	79
A5	GlyGly	0.16	10	1.3	38
A6	L-Val	0.13	10	1.2	66
A7	L-Leu	0.12	9	1.2	51
A8	L-Ile	0.15	8	1.0	70
A9	L-Asn	0.14	7	2.0	62
A10	L-Gln	0.12	5	2.3	65
A11	L-Arg	0.01	1.2	0.2	36
A12	L-Lys	0.02	1.3	0.3	40
A13	L-His	0.03	0.5	0.1	41
A14	L-Phg[d]	0.06	7	1.0	79
A15	L-Phe	0.07	6	1.1	80
A16	L-Trp	0.19	13	3.2	86
A17	L-Pro	0.18	11	3.6	50
A18	L-Pip[e]	0.13	6	2.1	73
A19	D,L-Nip[f]	0.15	6	2.4	75
A20	D,L-Inp[g]	0.12	7	2.2	79
A21	L-GlyHis	0.004	0.03	0.006	28
A22	L-β-AlaHis	0.002	0.01	0.005	45
A23	L-PhePro	0.005	0.02	0.008	43
A24	L-ProGly	0.005	0.03	0.010	36
B1	Gly	0.23	16	2.2	80
B2	L-Ala	0.24	13	2.3	89
B3	β-Ala	0.23	14	2.6	71
B4	GABA	0.22	13	2.4	60
B5	GlyGly	0.16	10	1.5	67
B6	L-Val	0.18	9	2.4	53
B7	L-Leu	0.20	12	3.0	58
B8	L-Ile	0.20	8	3.1	59
B9	L-Asn	0.15	8	3.3	34
B10	L-Gln	0.14	6	3.1	39
B11	L-Arg	0.05	1.4	0.7	27
B12	L-Lys	0.04	1.5	0.8	41
B13	L-His	0.03	0.7	0.5	53
B14	L-Phg[d]	0.10	9	1.6	79
B15	L-Phe	0.08	10	1.9	77
B16	L-Trp	0.21	14	3.7	85
B17	L-Pro	0.14	12	3.8	54
B18	L-Pip[e]	0.15	9	2.9	59
B19	D,L-Nip[f]	0.16	9	3.0	67
B20	D,L-Inp[g]	0.13	9	3.1	72
B21	L-GlyHis	0.005	0.03	0.010	33
B22	L-β-AlaHis	0.004	0.02	0.008	31
B23	L-PhePro	0.005	0.04	0.008	40
B24	L-ProGly	0.006	0.03	0.010	28

[a] Mean from at least three determinations by the esterase method.[25] Standard error was in the range of 5−10%. [b] Human cloned isozyme. [c] Purified from bovine lung microsomes. fpu = 4-F-$C_6H_4SO_2NHCO$-; ots = 2-Me-$C_6H_4SO_2NHCO$-. [d] Phg = phenylglycine. [e] Pip = pipecolic acid (piperidine-2-carboxylic acid). [f] Nip = nipecotic acid (piperidine-3-carboxylic acid). [g] Inp = isonipecotic acid (piperidine-4-carboxylic acid).

Carbonic Anhydrase Activation. In vitro activation data of isozymes hCA I, hCA II, and bCA IV with the new derivatives **A1**−**A24** and **B1**−**B24** as well as several standard activators, are presented in Table 1.

Ex Vivo Activation. Data with some of the best in vitro activators against human red cell isozymes (hCA I + hCA II), after incubation of red cells with the

Table 2. Ex Vivo CA Activation Data after 30 and 60 min of Incubation of Human Erythrocytes with Solutions Containing 5 μM Activators: Histamine (as standard), **A11**, **A22**, **B13**, and **B22**

activator	% CA activity[a] 30 min	60 min
histamine	121 ± 3	130 ± 5
A11	186 ± 5	225 ± 10
A22	254 ± 8	283 ± 12
B13	180 ± 7	211 ± 9
B22	236 ± 5	267 ± 11

[a] Mean ± standard error ($n = 3$); erythrocyte CA activity (hCA I + hCA II) in the absence of activator is taken as 100%.

activator solution for different periods of time, are presented in Table 2.

Discussion

Chemistry. The study of CAAs has only recently registered some progress since the report of the first X-ray crystallographic data of adducts of histamine[8] and phenylalanine[9] with isozyme hCA II. Histidine **1** (formally a histamine derivative) also acts as a CAA, possessing an activity quite similar to that of histamine and phenylalanine, against isozymes hCA I and hCA II (Table 1).[5] Taking into account the binding mode of these last two compounds to the enzyme, as well as the fact that the best histamine-based tighter-binding activators previously reported were those obtained by derivatization of the aminoalkyl group of histamine by means of alkyl/arylsulfonyl, carboxamido, or ureido moieties (in compounds of types **1a**−**c**),[12−18] we decided to use the same type of approach for designing histidine-based CAAs. Rather than derivatizing histidine itself, we used one of its dipeptide derivatives, carnosine (β-Ala-His), as starting point in the synthesis, since this compound proves to be a much stronger CAA as compared to histidine (Table 1). The fact that an activator with a longer molecule would be more effective than a shorter one has in fact been explained in a QSAR study on a series of amine and amino acid CAAs.[15] It must also be mentioned that carnosine is a naturally occurring dipeptide present in high concentrations (up to 20 mM) in many inervated tissues (such as muscle and brain).[16−18] Its physiological role is rather uncertain, but it seems that carnosine possesses protective functions as an antioxidant, free radical scavenger, aldehyde scavenger, and heavy metal ion complexing agent.[16−18] The clinical use of this compound as a protective, antiaging pharmacological agent has also been proposed.[16−18] Furthermore, in contrast to histidine (or phenylalanine), carnosine is not readily metabolized in vivo,[16−18] and this may be an important factor for a putative pharmacological agents of this class.

The key intermediate for obtaining the novel activators reported here, *N*-1-tritylsulfenyl-*tert*-butyl histidine **3**, was obtained from histidine **1** by protection of the primary amine moiety by means of phthalimide derivatives, followed by protection of the imidazolic NH moiety with tritylsulfenyl chloride,[12−14] and conversion of the carboxylic acid group to the *tert*-butyl ester (with thionyl chloride, followed by reaction with sodium *tert*-butoxyde), leading thus to the tri-protected intermediate **2** (Scheme 1). Hydrazinolysis of the phthalimido moiety of **2** in mild conditions led to the imidazole- and carboxy-
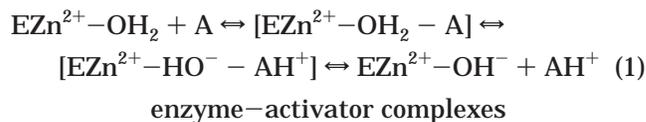
protected intermediate **3**, as the tritylsulfenyl and *tert*-butoxy moieties are resistant to these deprotection conditions (Scheme 1). The overall yield of these steps was good (around 65%), and the purifications procedures were rather simple. The approach used here showed that the tritylsulfenyl (TrS) moiety is a good protecting group for the side chains of "difficult" amino acids such as histidine (this protecting group has also successfully been used for the imidazolic moiety, in the synthesis of some histamine derivatives possessing CA activatory properties).[12–14] Subsequent reaction of the key intermediate **3** with arylsulfonylureido-amino acyl-β-alanine derivatives[13] in the presence of carbodiimides afforded a series of *N*-tritylsulfenylated compounds **4**, which were deprotected in standard conditions (dioxane–HCl), leading to the desired derivatives **A1–A20** and **B1–B20,** respectively. In these acidic conditions, both the imidazole as well as the carboxy moieties of **4** were deprotected (Scheme 1). The fpu/ots-aminoacyl-β-Ala derivatives used in these synthesis were obtained as described previously,[12,13,19,20] whereas the side chains of amino acids (His, Arg, Ser, etc.) incorporated in some of these derivatives were protected by standard protecting groups resistant to the coupling reaction conditions, which were removed in the final hydrolytic step (step e of Scheme 1) together with the other protecting groups of derivatives **4**.

Similarly were prepared some dipeptide derivatives of carnosine, of types **A21–A24** and **B21–B24**, as outlined in Scheme 2. Derivatization of dipeptides with arylsulfonyl isocyanates **5** afforded the arylsulfonylureido dipeptides **6**,[13,19,20] which were coupled with the key intermediate **3** in the presence of carbodiimides, as outlined above. Deprotection has been achieved as for the previously mentioned derivatives **A1–A20** and **B1–B20** (Scheme 2). One must mention that the two tails used here for the derivatization of the amino acids/dipeptides, i.e., 4-fluorosulfonylureido (fpu) and 2-methylphenylsulfonylureido (ots), were previously shown to induce tight-binding within the CA active site for the histamine-based activators,[14b] and this is the reason we used them to obtain histidine/carnosine-based activators. All the new compounds reported here have been characterized by IR, ^{1}H, and ^{13}C NMR spectroscopy, as well as elemental analysis ($\pm$0.4% of the theoretical data, calculated for the proposed formulas).

In Vitro CA Activation. The CA activation data of Table 1 show significant differences between the investigated isozymes in their behavior toward both "classical" activators, such as histamine, and histidine **1**, as well as the new class of derivatives described in the present work. Thus, histidine is a good hCA I activator (with an affinity of 4 μM) and a relatively weak hCA II activator (affinity of 113 μM), whereas isozyme bCA IV possesses an intermediate behavior (affinity of 39 μM). Thus, histidine has a behavior with histamine rather similar to that of CAA (Table 1). Carnosine, on the other hand, is already a stronger CAA, with affinities of 1.3–35 μM against the three investigated isozymes. A very strong susceptibility to activation is shown by the cytosolic rapid isozyme, hCA II, with some of the new derivatives (**A,B)1–24**, reported here. Thus, many of these tetrapeptide derivatives (such as, for example, **A21–A24** or **B21–B24**) showed activation constants in

the 0.01–0.04 μM range against this isozyme. The highly abundant and most prone to activation isozyme hCA I was also very susceptible to activation by this type of derivative (with activation constants in the low nanomolar range, i.e., 2–6 nM). bCA IV, on the other hand, had again an intermediate behavior toward the new class of activators, with activation constants in the 5–10 nM range for the most active such compounds (**A21–A24** and **B21–B24**). Efficient CA activators were also the derivatives incorporating basic amino acids (Arg, Lys, His), such as (**A,B)11–13**, as well as the phenylglycine and phenylalanine derivatives (**A,B)14** and (**A,B)15**. Slightly less active were the compounds derived from Pro, Pip, Nip, Inp, Asn, and Gln as well as the hydrophobic amino acid derivatives (Val, Leu, Ile, Trp). The GlyGly (**A,B)5** and GABA (**A,B)4** derivatives were generally more active than the β-Ala derivative (**A,B)3**, which in turn were more active than the Ala or Gly derivatives (**A,B)2** and (**A,B)1**. Undoubtedly, the best activators in this series were those derived from dipeptides such as Gly-His, β-Ala-His (carnosine), Phe-Pro, or Pro-Gly mentioned above. These compounds possessed activation constants in the 2–10 nM range against hCA I and bCA IV and in the 10–40 nM range against hCA II, respectively. These compounds, but probably also the other quite active derivatives, such as (**A,B)11**, (**A,B)12**, (**A,B)13**, (**A,B)21**, or (**A,B)22**, incorporate additional moieties able to shuttle protons (except for the imidazole of the carnosine moiety), such as the guanidino-, ϵ-amino-, or imidazole groups of Arg, Lys, and His moieties present in their molecules, respectively. Such compounds behave as effective CA activators against all three isozymes investigated here, probably due to the various moieties able to act as proton shuttles in the rate-determining step of the catalytic cycle (see later in the text). The fluorophenylsulfonylureido derivatives of type **A1–A24** were generally slightly more active than the corresponding *o*-toluenesulfonylureido compounds of type **B1–B24**.

Similarly to all CA activators reported up to now,[5] the compounds obtained in the present study exert their enzyme modulation properties by intervening in the catalytic cycle of CA, leading to the formation of enzyme–activator complexes (similar to the enzyme–inhibitor adducts, but without substitution of the metal bound solvent molecule), in which the activator bound within the active site facilitates the rate-limiting proton-transfer steps between the active site and the environment.[5,21–23] The driving force of this effect is probably the fact that intramolecular reactions are more rapid than intermolecular ones.[5] Thus, in the presence of activators (symbolized as "A"), the rate-limiting step in the CA catalytic cycle is described by eq 1.[5]

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

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, but due to the presence of the arylsulfonylureido amino acyl/dipeptidyl tails, they bind much more effectively to the enzyme, allowing thus for

more efficient activation processes as compared to the original lead molecule. Indeed, the active site edge of all three CA isozymes investigated here contains a high proportion of polar amino acid residues which might interfere with polar groups such as the $X-C_6H_4-SO_2-NHCO$-amino acyl moieties present in these new CA activators. This explains the much greater efficiency of the compounds reported in the present work in activating these CA isozymes, as compared to histamine or histidine, which behaves as a relatively weak activator.

Ex Vivo CA Activation. After incubation of normal blood red cells (containing approximately 150 μM of hCA I and 20 μM of hCA II)[24] with micromolar concentrations of histamine or new activators synthesized in the present work (such as **A11**, **A22**, **B13**, **B22**, etc.), the total CA activity (measured by the esterase method, with 4-nitrophenyl acetate as substrate)[25] in homogenates of treated cells is enhanced as compared to that of cells treated in a blank experiment only with buffer (Table 2). Thus, histamine produces only a weak activation of around 120% after 0.5 h incubation, and of around 130% of the basal CA activity after 1 h incubation with red cells. Some of the new derivatives tested ex vivo (which showed strong in vitro CA activity enhancements) produced activations of 180−250% after 0.5 h incubation, and of 210−280% after 1 h incubation (Table 2). These are clear-cut experiments proving that some of the compounds reported here might act as effective in vivo CA activators and might thus constitute interesting candidates for animal studies regarding their involvement in cognitive processes. Furthermore, a syndrome of CA deficiency has been described some time ago by Sly's group,[26] who showed that CA II (or CA IV) may be absent in some patients affected by rare genetic disorders. Such patients generally show normal levels of CA I in their tissues, but this is a catalytically less effective isozyme, and this might explain the acid−base disequilibria and other pathologies associated with this syndrome.[26] Improving the function of CA I by activators of the type described here might constitute a pharmacological approach for the treatment of the CA deficiency syndrome, a condition which has no pharmacological cure for the moment.

Conclusions

We report here a novel class of tight binding CA activators synthesized by an original approach, using histamine, histidine, and carnosine as lead molecules. These new derivatives possessing the general formulas fpu/ots-AA-β-Ala-His or fpu/ots-AA1-AA2-β-Ala-His (AA, AA1, and AA2 represent amino acyl moieties; fpu = 4-fluorophenylsulfonylureido moiety; ots = 2-toluenesulfonylureido group) were obtained by reaction of appropriately derivatized β-alanines with imidazole/carboxy-protected histidine in the presence of carbodiimides, followed by removal of the various protecting groups. Best activity was observed for compounds incorporating basic amino acid residues (Arg, Lys, etc.) as well as those incorporating a tetrapeptide scaffold, which showed nanomolar affinities for all the investigated CA isozymes, i.e., hCA I, hCA II, and bCA IV. This new class of CA activators might lead to the development of drugs/diagnostic tools for the management of CA deficiency syndromes as well as for the

pharmacological enhancement of synaptic efficacy, spatial learning, and memory, constituting thus a radically new approach for the treatment of Alzheimer's disease and other conditions in need of achieving memory therapy.

Experimental Section

General. 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 were obtained 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 (histidine, natural and nonnatural amino acids, tritylsulfenyl chloride, tetrabromophthalic anhydride, hydrazine, sodium *tert*-butoxide, thionyl chloride, etc.) were commercially available compounds (from Sigma, Acros, or Aldrich). The arylsulfonylureido-amino acid/dipeptide derivatives were prepared as described previously[13,19,20] by the reaction of 4-fluorophenylsulfonyl isocyanate or *o*-tosylsulfonyl isocyanate (Aldrich) with (protected) amino acids/dipeptides (from Sigma or Aldrich). Acetonitrile, acetone, 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.

Preparation of *N*-1-Tritylsulfenyl-histidine *tert*-Butyl Ester 3. An amount of 7.75 g (50 mmol) of histidine and 23.15 g (50 mmol) of tetrabromophthalic anhydride were suspended in 300 mL of dry toluene and refluxed under Dean−Stark conditions until water was separated (generally 3−4 h). The solvent was evaporated in vacuo, and the crude product was dissolved in 150 mL of anhydrous acetonitrile and treated with 15.5 g (50 mmol) of tritylsulfenyl chloride and 6.95 mL (50 mmol) 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 next step, which consisted of treatment with thionyl chloride (5.5 mL, 75 mmol) in 100 mL of dry benzene. The mixture was refluxed till cessation of HCl evolution (about 3 h), the solvent and excess $SOCl_2$ were distilled in vacuo, and the crude product was treated with the stoichiometric amount of sodium *tert*-butoxide (4.8 g, 50 mmol) in 100 mL of anhydrous acetonitrile. After the mixture was stirred at room temperature for 2 h, the precipitated NaCl was filtered, the acetonitrile evaporated, and the crude **2** used for the deprotection step. Hydrazinolysis was effected by dissolving the above-mentioned compound in 200 mL of ethanol and addition of 15 mL of hydrazinium hydroxide, followed by stirring for 5 h at room temperature. The solvent was evaporated, a small excess of 2 N HCl solution was added, and the precipitated tetrabromophthalylhydrazide was filtered and discarded. The solution containing **3** was brought to pH 7 with solid $KHCO_3$ and brought to a small volume by in vacuo evaporation of the solvent, and the precipitated **3** was recrystallized from ethanol (yield of 74%, based on histidine). Tan crystals, mp 154−5 °C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm) 1.80 (s, 9H, *t*-Bu), 3.32−3.46 (m, 2H, CHCH_2 of His), 4.01−4.08 (m, 1H, CHCH$_2$ of His), 4.23 (m, 2H, NH$_2$), 7.46 (s, 1H, CH-5 of His), 7.11−7.35 (m, 15H, trityl), 8.50 (s, 1H, CH-2 of His). Anal. found: C, 71.53; H, 6.61; N, 8.57%. $C_{29}H_{31}N_3O_2S$ requires: C, 71.72; H, 6.43; N, 8.65%.

General Procedure for the Preparation of Arylsulfonylureido Amino Acids/Dipeptides fpu/ots-AA and fpu/ots-AA1-AA2. An amount of 20 mmol of (protected) amino acid/dipeptide was suspended/dissolved in 50 mL of anhydrous acetone or acetonitrile, and the stoichiometric amount of 4-fluorophenylsulfonyl isocyanate or *o*-tosylsulfonyl isocyanate

was added in one portion, with energetic stirring and eventual cooling of the reaction mixture. The mixture was then stirred for 1−2 h at 4 °C, the solvent was evaporated in vacuo, and the product was purified either by recrystallization from water−ethanol (1:1, v/v) or by preparative HPLC (in the case of fpu-GlyGly, ots-His, fpu-Val, fpu-Trp, and ots-Phe, when the arylsulfonylureido-amino acid/dipeptide contained variable amounts of unreacted amino acid and substituted benzenesulfonamide). Conditions were as follows: C_{18} reversed-phase Bondapack or Dynamax-60A (25 × 250 mm) columns; 90% acetonitrile/8% ethanol/2% water, 30 mL/min. Remarkably, the reaction of L-Lys monohydrochloride or L-Arg monohydrochloride with the two arylsulfonyl isocyanates in the conditions mentioned above led to the formation of only one very pure product, i.e., the α-derivatized compound, without derivatization of the ε-amino moiety in the case of Lys or the guanidino one in the case of Arg. This is probably due to the fact that H^+ acts in this case as a very good side chain protecting group for these two amino acids. This has been further confirmed by the synthesis of α-fpu-Lys and α-fpu-Arg from the appropriately protected amino acid derivatives (*N*-ε-acetyl-L-Lys and ω-*N*-tritylsulfenyl-L-Arg) and 4-fluorophenylsulfonyl isocyanate, followed by deprotection of the side chain in standard conditions (data not shown).

General Procedure for the Preparation of Compounds A1−A24 and B1−B24. An amount of 10 mmol of **3** was dissolved in 50 mL of anhydrous acetonitrile and treated with a solution obtained from 10 mmol of arylsulfonyl-ureido amino acid/dipeptide (10 mmol) dissolved in 10 mL of the same solvent, followed by 10 mmol of diisopropyl-carbodiimide (or EDCI·HCl + Et₃N) and 10 mmol of 1-hydroxybenzotriazole in anhydrous acetonitrile as solvent. The reaction mixture was stirred at 4 °C for 3−9 h (TLC control). The solvent was then 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 was removed in vacuo. In many cases the compounds of type **4** or **7** were precipitated, filtered, dried, and deprotected at the N-1 imidazolic moiety in the following way. The crude **4/7** was dissolved in 20 mL of dioxane and treated with 25 mL of a 4 M HCl solution in dioxane, followed by heating at 40 °C for 6−8 h (TLC control). The solvent was then evaporated under reduced pressure, the residue was taken up in 50 mL of a 5% solution of sodium bicarbonate, and the trityl sulfenyl chloride formed during the deprotection step was extracted in 2 × 50 mL of Et₂O. The water phase was evaporated in vacuo to a small volume, when generally compounds (**A**,**B**)**1−24** 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 title derivatives.

4-Fluorophenylsulfonylureido-glycyl-β-alanyl-histidine A1: tan crystals, mp 202−4 °C (dec); IR (KBr, cm⁻¹) 1147 (SO_2^{sym}), 1285 (amide III), 1362 (SO_2^{as}), 1580 (amide II), 1715 (amide I), 3060 (NH); ¹H NMR (DMSO-d_6, δ, ppm) 2.79−2.88 (m, 2H, CH_2 of β-Ala), 3.11−3.26 (m, 2H, CH_2 of β-Ala), 3.34−3.45 (m, 2H, CHCH_2 of His), 3.65 (s, 2H, CH_2 of Gly), 4.57−4.63 (m, 1H, CHCH₂ of His), 7.32 (s, 1H, CH-5 of His), 7.62 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of FC₆H₄), 7.91 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of FC₆H₄), 8.28 (br s, 4H, 2CONH + NHCONH), 8.35 (s, 1H, CH-2 of His), 8.84 (s, 1H, imidazole NH), 10.23 (br s, 1H, COOH); ¹³C NMR (DMSO-d_6, δ, ppm) 33.3 (s, CH_2 of His), 37.4 (s, NHCH_2CH₂ of β-Ala), 40.6 (s, CH_2 of Gly); 40.8 (s, CH₂CH_2CO of β-Ala), 59.6 (s, CHCH₂ of His), 122.2 (s, C-4 of His), 130.5 (s, C_{meta} of FC₆H₄), 132.9 (s, NHCONH), 134.2 (s, C-5 of His), 135.3 (s, C_{ortho} of FC₆H₄), 137.2 (s, C-2 of His), 145.9 (s, C_{ipso} of FC₆H₄), 148.4 (s, C_{para} of FC₆H₄), 167.6 (CONH of Gly), 175.6 (s, CH₂CO of β-Ala), 180.4 (s, CO_2H of His). Anal. ($C_{18}H_{21}$-FN₆O₇S) C, H, N.

4-Fluorophenylsulfonylureido-glycyl-histidyl-β-alanyl-histidine A21: mp 239−40 °C; IR (KBr, cm⁻¹) 1151 (SO_2^{sym}), 1283 (amide III), 1369 (SO_2^{as}), 1596 (amide II), 1715 (amide I), 3063 (NH); ¹H NMR (DMSO-d_6, δ, ppm) 2.77−2.89 (m, 2H, CH_2 of β-Ala), 3.06−3.23 (m, 2H, CH_2 of β-Ala), 3.35−3.47 (m, 4H, CHCH_2 of 2 His), 3.61 (s, 2H, CH_2 of Gly), 4.52−4.66 (m, 1H, CHCH₂ of 2 His), 7.33 (s, 2H, CH-5 of 2 His), 7.60 (d, $^3J_{HH}$ = 8.1, 2H, H_{ortho} of FC₆H₄), 7.93 (d, $^3J_{HH}$ = 8.1, 2H, H_{meta} of FC₆H₄), 8.39 (br s, 5H, 3CONH + NHCONH), 8.47 (s, 2H, CH-2 of 2 His), 8.84 (s, 2H, imidazole NH from 2 His), 10.23 (br s, 1H, COOH); ¹³C NMR (DMSO-d_6, δ, ppm) 33.5 (s, CH_2 of His), 37.8 (s, NHCH_2CH₂ of β-Ala), 40.9 (s, CH_2 of Gly), 41.3 (s, CH₂CH_2CO of β-Ala), 59.6 (s, CHCH₂ of His), 122.7 (s, C-4 of His), 130.1 (s, C_{meta} of FC₆H₄), 132.3 (s, C-5 of His), 133.5 (s, NHCONH), 134.7 (s, C_{ortho} of FC₆H₄), 137.3 (s, C-2 of His), 139.9 (s, C_{para} of FC₆H₄), 145.0 (s, C_{ipso} of FC₆H₄), 175.4 (s, CH₂CO of Gly), 175.6 (s, CONH of His-β-Ala), 175.9 (s, CH₂CO of β-Ala), 180.9 (s, CO_2H of carboxyterminal His). Anal. ($C_{24}H_{28}$-FN₉O₈S) C, H, N.

2-Methylphenylsulfonylureido-arginyl-β-alanyl-histidine B11: white crystals, mp 237−8 °C (dec); IR (KBr, cm⁻¹) 1157 (SO_2^{sym}), 1284 (amide III), 1370 (SO_2^{as}), 1585 (amide II), 1718 (amide I), 3060 (NH); ¹H NMR (DMSO-d_6, δ, ppm) 1.71−2.04 (m, 2H, CHCH₂CH_2 of Arg), 2.51−2.65 (m, 2H, CHCH_2-CH₂ of Arg), 2.72 (s, 3H, Me), 2.78 (t, $^3J_{HH}$ = 6.5, 1H, (CH₂)₂CH_2CO of Arg), 2.79−2.87 (m, 2H, CH_2 of β-Ala), 3.11−3.26 (m, 2H, CH_2 of β-Ala), 3.30−3.46 (m, 2H, CH₂CH_2NH of Arg), 3.36−3.48 (m, 2H, CHCH_2 of His), 3.51−3.65 (m, 1H, CH₂CH(NH)CO of Arg), 4.57−4.68 (m, 1H, CHCH₂ of His), 7.34 (s, 1H, CH-5 of His), 7.50−7.98 (m, 4H, 2-MeC₆H₄), 8.27 (br s, 4H, 2CONH + NHCONH), 8.38 (s, 1H, CH-2 of His), 8.81 (s, 1H, imidazole NH), 10.12 (br s, 1H, COOH); ¹³C NMR (DMSO-d_6, δ, ppm) 26.0 (s, Me of tosyl), 29.5 (s, CH₂CH_2CH₂ of Arg), 33.3 (s, CH_2 of His), 35.4 (s, CHCH₂CH_2 of Arg), 37.4 (s, NHCH_2CH₂ of β-Ala), 40.8 (s, CH₂CH_2CO of β-Ala), 45.6 (s, CH_2CH₂NH of Arg), 59.5 (s, CHCH₂ of His), 59.8 (s, CH₂CH-(NH)CO₂H of Arg), 122.7 (s, C-4 of His), 130.4 (s, C_{meta} of MeC₆H₄), 132.0 (s, NHCONH), 134.3 (s, C-5 of His), 135.2 (s, C_{ortho} of MeC₆H₄), 137.5 (s, C-2 of His), 144.8 (s, C_{ipso} of MeC₆H₄), 148.9 (s, C_{para} of ClC₆H₄), 161.6 (s, NHC$(=NH)$NH₂ of Arg), 170.8 (CONH from Arg), 175.9 (s, CONH of β-Ala), 180.3 (s, CO_2H of carboxyterminal His). Anal. ($C_{23}H_{33}N_9O_7S$) C, H, N.

2-Methylphenylsulfonylureido-isonipecotyl-β-alanyl-histidine B20: mp 253−5 °C (dec); IR (KBr, cm⁻¹) 1158 (SO_2^{sym}), 1284 (amide III), 1361 (SO_2^{as}), 1584 (amide II), 1720 (amide I), 3065 (NH); ¹H NMR (DMSO-d_6, δ, ppm) 1.86−2.30 (m, 8H, 2 CH₂CH₂ of Inp), 2.61 (s, 3H, Me), 2.79−2.88 (m, 2H, CH_2 of β-Ala), 3.11−3.20 (m, 2H, CH_2 of β-Ala), 3.24−3.59 (m, 3H, CHCO of Inp + CHCH_2 of His), 4.57−4.63 (m, 1H, CHCH₂ of His), 7.32 (s, 1H, CH-5 of His), 7.56−7.99 (m, 4H, MeC₆H₄), 8.27 (br s, 3H, 2CONH + Inp-NCONH), 8.35 (s, 1H, CH-2 of His), 8.81 (s, 1H, imidazole NH), 10.10 (br s, 1H, COOH); ¹³C NMR (DMSO-d_6, δ, ppm) 21.2 (s, CH_2 of Inp), 26.4 (s, Me of tosyl), 33.0 (s, CH_2 of His), 37.3 (s, NHCH_2CH₂ of β-Ala), 40.5 (s, CH₂CH_2CO of β-Ala), 47.3 (s, NCH₂ of Inp), 53.6 (s, CHCO of Inp), 59.8 (s, CHCH₂ of His), 122.5 (s, C-4 of His), 132.3 (s, NHCON), 132.8 (s, C_{meta} of MeC₆H₄), 134.0 (s, C-5 of His), 135.6 (s, C_{ortho} of MeC₆H₄), 137.6 (s, C-2 of His), 145.3 (s, C_{para} of MeC₆H₄), 148.4 (s, C_{ipso} of MeC₆H₄), 173.8 (s, CONH of Inp), 175.0 (s, CONH of β-Ala), 179.7 (s, CO_2H of His). Anal. ($C_{23}H_{30}N_6O_7S$) C, H, N.

Enzyme Preparations. Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog et al.[27] (The two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this 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 CA I and 54 mM⁻¹ cm⁻¹ for CA II, respectively, based on M_r = 28.85 kDa for CA I and 29.30 kDa for CA II, respectively.[30] CA IV was isolated from bovine lung mi-

crosomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[31]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[25] 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.[25] Nonenzymatic 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 mean of such results. Stock solutions of activator (1 mM) were prepared in distilled−deionized water with 10−15% (v/v) DMSO (which is not inhibitory/activatory at these concentrations), and dilutions up to 0.1 nM were done thereafter with distilled−deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, to allow for the formation of the E−A complex. The activation constant K_A was determined as described by Briganti et al.[8] Enzyme concentrations were 3.1 nM for hCA II, 9.5 nM for hCA I, and 39 nM for bCA IV (this isozyme has a decreased esterase activity,[32] and higher concentrations had to be used for the measurements).

Ex Vivo CA Activation. An amount of 2 mL of freshly isolated human blood was thoroughly washed several times with 5 mL of Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min. The obtained erythrocytes were then treated with 2 mL of a 5 μM solution of CA activator. Incubation has been done at 37 °C with gentle stirring, for periods of 30−60 min. After that time, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 5 mL of the above-mentioned buffer, in order to eliminate all unbound compound. The cells were then lysed in 5 mL of distilled water and centrifuged for eliminating membranes and other insoluble materials, and CA activity has been assayed as described above. Blank experiments were done in which no activator has been added to the blood red cells treated as described above, and CA activity determined in such conditions has been taken as 100%.[33]

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