J. Enzyme Inhibition, 2000, Vol. 15, pp. 471–486
Reprints available directly from the publisher
Photocopying permitted by license only

CARBONIC ANHYDRASE ACTIVATORS: SYNTHESIS OF HIGH AFFINITY ISOZYMES I, II AND IV ACTIVATORS, DERIVATIVES OF 4-(ARYLSULFONYLUREIDO-AMINO ACYL)ETHYL-1*H*-IMIDAZOLE*

CLAUDIU T. SUPURAN† and ANDREA SCOZZAFAVA

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

(Received 14 October 1999)

Based on the X-ray crystallographic structure of the adduct of human carbonic anhydrase II (hCA II) with the weak activator histamine (Briganti, F., Mangani, S., Orioli, P., Scozzafava, A., Vernaglione, G. and Supuran, C.T. (1997) *Biochemistry*, **36**, 10384–10392), a novel class of tight-binding CA activators was designed by using histamine (Hst) as lead molecule. Thus, *N*-1-tritylsulfenyl Hst was synthesized by reaction of Hst with tetrabromophthalic anhydride followed by protection of its imidazole moiety with tritylsulfenyl chloride. After hydrazinolysis, it afforded a key intermediate which was derivatized at the aliphatic amino group. Reaction of the key intermediate with 4-fluorophenylsulfonylureido amino acids (fpu-AA) or 2-toluenesulfonylureido amino acids (ots-AA) in the presence of carbodiimides, afforded after deprotection, a series of compounds with the general formula fpu/ots-AA-Hst (fpu = 4-$FC_6H_4SO_2NHCO$; ots = 2-$MeC_6H_4SO_2NHCO$). Some structurally related dipeptides with the general formula fpu/ots-AA1–AA2-Hst (AA, AA1 and AA2 represent amino acyl moieties), were also prepared, by a strategy similar to that used for the simple amino acyl compounds above. The new derivatives proved to be efficient *in vitro* activators of three CA isozymes. Best activity was shown against hCA I and bCA IV, for which some of the new compounds (such as the Lys, Arg, His or the dipeptide derivatives) showed affinities in the 2–12 nm range (h = human; b = bovine isozymes). hCA II was on the other hand somehow less prone to activation by the new derivatives, which possessed affinities around 30–60 nM for this isozyme. *Ex vivo* experiments showed some of the new activators to strongly enhance red cell CA activity (180–230%) after incubation with human erythrocytes. This new class of CA activators might lead to the development of drugs/diagnostic tools for the CA deficiency syndrome, a genetic disease of bone, brain and kidneys.

Keywords: Carbonic anhydrase; Histamine; Arylsulfonyl isocyanates; Ureas; Enzyme activators

* See Ref. [1].

† Corresponding author. Fax: +39-055-2757555. E-mail: cts@bio.chim.unifi.it.

INTRODUCTION

In previous contributions from this laboratory[1–3] it was shown that effective activators of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) can be designed by considering histamine (Hst) **1** as a lead molecule.[4] Indeed, the X-ray crystallographic structure for the adduct of human CA II (hCA II) with this weak activator (activation constant, $K_A = 125\ \mu M$) has been recently reported by our group.[4] As seen from the X-ray coordinates with which Figure 1 was generated, the activator molecule is bound at the entrance of the hCA II active site cavity, being anchored by hydrogen bonds to amino acid side chains and to water molecules.[4] Hst does not interact with the catalytically critical Zn(II) ion of the enzyme, in contrast to the inhibitors of this enzyme which generally directly coordinate to the zinc ion.[4–9]

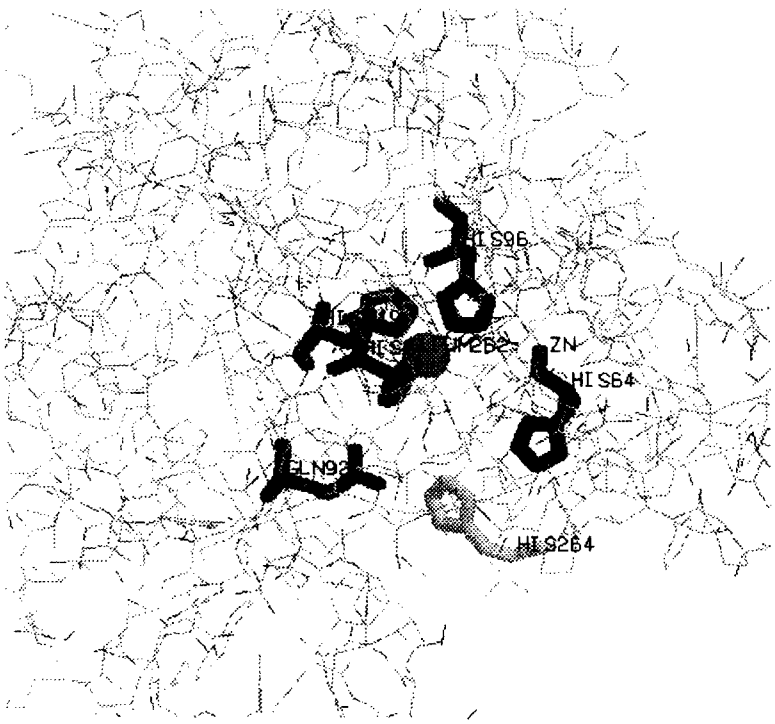


FIGURE 1 hCA II – histamine adduct: the Zn(II) ion (central sphere) and its three histidine ligands (His 94, His 96 and His 119) are shown at the center of the active site, whereas Hst (numbered as His 264) is situated at the entrance to it, between residues His 64 and Gln 92. The figure was generated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

The above-mentioned hydrogen bonds involve only the nitrogen atoms of the imidazole moiety, whereas the terminal aliphatic amino group does not experience any contact with the enzyme, but is extended away from the cavity into the solvent. The two imidazole atoms Nδ1 and Nε2 of the heterocyclic ring are engaged in hydrogen bonds with the side chains of Asn 62, His 64, Gln 92 and with Wat 152.[4] Positioned in such a favorable way, Hst facilitates the rate-limiting step of CA catalysis, i.e., the proton transfer processes between the active site and the environment.[1–4] Thus, one may consider the aliphatic NH_2 moiety of Hst as an interesting target for obtaining tighter-binding CA activators, in order to exploit the energy of binding of such derivatives with amino acid residues at the edge of the active site. This approach has in fact been successfully used both by Whiteside's[5,6] and our group[7–9] for the design of strong inhibitors of various CA isozymes, by attaching amino acyl/dipeptidyl or other types of "tails" to aromatic/heterocyclic sulfonamides.

The development of CA activators is of interest as a possible treatment of the CA deficiency syndrome, a genetic disease of bone, brain and kidney affecting a large enough number of patients, for which no medical treatment is known up to now.[10,11] 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.[10,11] But in many of the reported CA deficiency syndrome cases, only one CA isozyme is absent from the organism of the affected patient, the other isozymes generally being present in normal concentrations.[10,11] Thus, their activation by exogenous modulators of activity might lead to the restoration of the normal CA activity in the patients affected by this life-threatening genetic disorder.

Compounds interfering with the activity of these enzymes are widely used in clinical practice. Thus, CA 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,[7–9,12] epilepsy,[13] gastric and duodenal ulcers,[14] osteoporosis[15] or acid–base disequilibria[16] among others. In contrast to inhibitors, activators of this enzyme, for which at least 14 different isozymes have been isolated to date in higher vertebrates[17] have been 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 Hst[4] and phenylalanine (in this case a tertiary complex, in which azide is also bound to the Zn(II) ion)[18] have been reported by this group. Furthermore, few other QSAR[19] or synthetic chemistry studies[20–23] have been reported in the field of CA activators.

In this paper we report the synthesis of a series of arylsulfonylureido-amino acyl/dipeptidyl Hst derivatives possessing the general formula fpu/ots-AA-Hst and fpu/ots-AA1–AA2-Hst (AA, AA1, AA2 = amino acyl moieties; fpu = 4-fluorophenylsulfonylureido; ots = 2-toluenesulfonylureido), obtained by reaction of appropriately protected Hst with arylsulfonylureido amino acids/dipeptides, in the presence of carbodiimide derivatives. 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. *Ex vivo* experiments showed some of the new activators to strongly enhance red cell CA activity after incubation with human erythrocytes. SAR in this series of derivatives is also discussed.

MATERIALS AND METHODS

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 and ^{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 $\pm 0.4\%$ of the theoretical values. Preparative HPLC was done using C_{18} reversed-phase Bondapack or Dynamax-60A (25 × 250 mm) columns.

Compounds used in synthesis (Hst, natural and non-natural amino acids, tritylsulfenyl chloride, tetrabromophthalic anhydride, hydrazine, etc.) were commercially available compounds (from Sigma, Acros orAldrich). The arylsulfonylureido-amino acid/dipeptide derivatives were prepared as described previously[24] by the reaction of 4-fluorophenylsulfonyl isocyanate or *ortho*-tosylsulfonyl isocyanate (Aldrich) with 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 under anhydrous conditions.

Preparation of *N*-1-Tritylsulfenyl-Histamine 3

An amount of 5.55 g (50 mM) of Hst 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 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 **2** 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 precipitate in 200 mL of ethanol adding 15 mL of hydrazinium hydroxide, followed by stirring for 5 h at room temperature. The solvent was then evaporated, a small excess of 2 N HCl solution added and the precipitated tetrabromophthalhydrazide filtered and discarded. The solution containing **3** was brought to pH 7 with solid $NaHCO_3$, reduced 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 Hst, after the three steps described above). Tan crystals, m.p. 177–8°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). Found: C, 75.12%; H, 5.83%; N, 10.88%. $C_{24}H_{23}N_3S$ requires: C, 74.77%; H, 6.01%; N, 10.90%.

General Procedure for the Preparation of Arylsulfonylureido Amino Acids/Dipeptides fpu/ots-AA and fpu/ots-AA1–AA2

An amount of 20 mM of amino acid/dipeptide was suspended/dissolved in 50 mL of anhydrous acetone or acetonitrile, and the stoichiometric amount of 4-fluorophenylsulfonyl isocyanate or *ortho*-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 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 arylsulfonyl-ureido-amino acid/dipeptide contained variable amounts of unreacted amino acid and substituted-benzenesulfonamide). Conditions were: 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 under 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. The structure of the products has been 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 under standard conditions (data not shown).

General Procedure for the Preparation of Compounds A1–A24 and B1–B24

An amount of 10 mM *N*-1-tritylsulfenyl-Hst **3** was dissolved in 50 mL of anhydrous acetonitrile and then treated with a solution obtained from 10 mM of arylsulfonyl-ureido amino acid/dipeptide (10 mM) dissolved in 10 mL of the same solvent, followed by 10 mM of diisopropyl-carbodiimide (or EDCI · HCl + Et_3N) and 10 mM 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 removed *in vacuo*. In many cases the compounds of type **4** precipitated, were filtered, dried and deprotected at the N-1 imidazole moiety in the following way. 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 40°C for 6–8 h (TLC control). The solvent was then evaporated under reduced pressure, the residue taken up in 50 mL of a 5% solution of sodium bicarbonate and the tritylsulfenyl chloride formed during the deprotection step extracted in 2 × 50 mL of Et_2O. The extraction lasts for about 5–10 min, and this explains why the trityl sulfenyl chloride does not re-react with the imidazole moieties of the prepared derivatives. The water phase was evaporated *in vacuo* to a small volume when, generally, compounds **A1–A24**/**B1–B24** precipitated on 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-[3-(4-Fluorophenylsulfonylureido-Glycylamido)-Ethyl)]-1H-Imidazole

A1, as white crystals, m.p. 271–2°C (dec.). IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1289 (amide III), 1371 (SO_2^{as}), 1579 (amide II), 1710 (amide I), 3060 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.53 (t, 2H, $J = 7.0$, Hst CH_2), 2.96 (t, 2H, $J = 7.0$, Hst CONHCH_2), 3.65 (s, 2H, CH_2 of Gly), 7.34 (m, 1H, imidazole CH), 7.62 (d, $^3J_{HH} = 8.1$, 2H, H$_{ortho}$ of FC_6H_4), 7.94 (d, $^3J_{HH} = 8.1$,

2H, H*meta* of FC_6H_4), 8.25 (br s, 3H, CONH + NHCONH), 8.39 (s, 1H, imidazole CH), 8.82 (s, 1H, imidazole NH). ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.3 (s, CH_2 of Hst), 37.6 (s, CH_2 of Hst), 40.6 (s, CH_2 of Gly), 122.4 (s, C-4 of Hst), 130.5 (s, C*meta* of FC_6H_4), 132.9 (s, NHCONH), 134.8 (s, C-5 of Hst), 135.3 (s, C*ortho* of FC_6H_4), 137.6 (s, C-2 of Hst), 145.9 (s, C$_{ipso}$ of FC_6H_4), 148.4 (s, C*para* of FC_6H_4), 167.6 (CONH). Anal. ($C_{14}H_{16}FN_5O_4S$) C, H, N.

4-[β-(4-Fluorophenylsulfonylureido-Glycyl-Histidylamido)-Ethyl]-1H-Imidazole

A21: m.p. 213–4°C. IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1287 (amide III), 1375 (SO_2^{as}), 1594 (amide II), 1715 (amide I), 3065 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.55 (t, 2H, $J = 7.0$, Hst CH_2), 3.04 (t, 2H, $J = 7.0$, Hst CONHCH_2), 3.35–3.47 (m, 2H, CHCH_2 of His), 3.65 (s, 2H, CH_2 of Gly), 4.52–4.66 (m, 1H, CHCH_2 of His), 7.34 (s, 2H, CH-5 of His + Hst), 7.61 (d, $^3J_{HH} = 8.1$, 2H, H*ortho* of FC_6H_4), 7.95 (d, $^3J_{HH} = 8.1$, 2H, H*meta* of FC_6H_4), 8.30 (br s, 4H, 2CONH + NHCONH), 8.36 (s, 2H, CH-2 of His + Hst), 8.84 (s, 2H, imidazole NH from His and Hst). ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.1 (s, CH_2 of Hst), 37.9 (s, CH_2 of Hst), 40.8 (s, CH_2 of Gly), 59.6 (s, CHCH_2 of His), 122.4 (s, C-4 of His), 122.8 (s, C-4 of Hst), 130.4 (s, C*meta* of FC_6H_4), 132.0 (s, C-5 of His), 132.6 (s, NHCONH), 134.3 (s, C*ortho* of FC_6H_4), 134.9 (s, C-5 of Hst), 137.3 (s, C-2 of His), 137.8 (s, C-2 of Hst), 139.4 (s, C*para* of FC_6H_4), 145.1 (s, C$_{ipso}$ of FC_6H_4), 175.4 (s, CH_2CO of Gly), 176.6 (s, CONH of His). Anal. ($C_{20}H_{23}FN_8O_5S$) C, H, N.

4-[β-(2-Methylphenylsulfonylureido-Arginylamido)-Ethyl)]-1H-Imidazole

B11, as white crystals, m.p. 240–1°C (dec.). IR (KBr), cm^{-1}: 1154 (SO_2^{sym}), 1283 (amide III), 1375 (SO_2^{as}), 1585 (amide II), 1713 (amide I), 3066 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.71–2.04 (m, 2H, $CHCH_2CH_2$ of Arg), 2.49 (t, 2H, $J \doteq 7.0$ Hz, Hst CH_2), 2.51–2.65 (m, 2H, $CHCH_2CH_2$ of Arg), 2.69 (s, 3H, Me), 2.78 (t, $^3J_{HH} = 6.5$, 1H, $(CH_2)_2CH_2CO$ of Arg), 3.04 (t, 2H, $J = 7.0$ Hz, Hst CONHCH_2), 3.51–3.65 (m, 1H, CH_2CH(NH)CO of Arg), 7.36 (m, 1H, imidazole CH), 7.53–7.98 (m, 4H, MeC_6H_4), 8.27 (br s, 3H, CONH + NHCONH), 8.33 (s, 1H, imidazole CH), 8.81 (s, 1H, imidazole NH). ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.0 (s, Me of tosyl); 29.5 (s, $CH_2CH_2CH_2$ of Arg), 33.4 (s, CH_2 of Hst), 35.4 (s, $CHCH_2CH_2$ of Arg), 37.5 (s, CH_2 of Hst), 45.6 (s, CH_2CH_2NH of Arg), 59.8 (s, $CH_2CH(NH)CO_2H$ of Arg), 122.5 (s, C-4 of Hst), 130.4 (s, C*meta* of MeC_6H_4), 131.8 (s, NHCONH), 134.6 (s, C-5 of Hst), 135.2 (s, C*ortho* of MeC_6H_4), 137.4 (s, C-2 of Hst), 144.8 (s, C$_{ipso}$ of MeC_6H_4), 148.9 (s, C*para* of ClC_6H_4), 161.6 (s, NHC(=NH)NH_2 of Arg), 170.8 (CONH). Anal. ($C_{19}H_{28}N_8O_4S$) C, H, N.

4-[β-(2-Methylphenylsulfonylureido-Isonipecotylamido)-Ethyl)]-1H-Imidazole

B20: m.p. 244–5°C (dec.). IR (KBr), cm^{-1}: 1152 (SO_2^{sym}), 1279 (amide III), 1371 (SO_2^{as}), 1591 (amide II), 1715 (amide I), 3063 (NH). ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.86–2.30 (m, 8H, $2CH_2CH_2$ of Inp), 2.48 (t, 2H, $J = 7.0$, Hst CH_2), 2.61 (s, 3H, Me), 2.99 (t, 2H, $J = 7.0$, Hst CONHCH_2), 3.21–3.56 (m, 1H, CHCO of Inp), 7.33 (m, 1H, H-5 of Hst), 7.53–7.93 (m, 4H, MeC_6H_4), 8.24 (br s, 2H, CONH + Inp-NCONH), 8.35 (s, 1H, H-2 of Hst), 8.81 (s, 1H, imidazole NH). ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.3 (s, CH_2 of Inp), 26.0 (s, Me of tosyl), 33.3 (s, CH_2 of Hst), 37.5 (s, CH_2 of Hst), 47.3 (s, NCH_2 of Inp), 53.5 (s, CHCO of Inp), 122.3 (s, C-4 of Hst), 132.1 (s, NHCON), 132.6 (s, C_{meta} of MeC_6H_4), 134.9 (s, C-5 of Hst), 135.6 (s, C_{ortho} of MeC_6H_4), 137.6 (s, C-2 of Hst), 145.3 (s, C_{para} of MeC_6H_4), 148.4 (s, C_{ipso} of MeC_6H_4), 170.4 (s, Inp-CONH). Anal. ($C_{19}H_{25}N_5O_4S$) 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 Behravan *et al.*[25] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[26] and the enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[27] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49\,mM^{-1}\cdot cm^{-1}$ for CA I and $54\,mM^{-1}\cdot cm^{-1}$ for CA II, respectively, based on $M_r = 28.85$ kDa for CA I, and 29.30 kDa for CA II, respectively.[28] CA IV was isolated from bovine lung microsomes as described by Maren *et al.* and its concentration was determined by titration with ethoxzolamide.[29]

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.[30] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2\cdot10^{-2}$ and $1\cdot10^{-6}$ M, working at 25°C. A molar absorption coefficient ε of $18{,}400\,M^{-1}\cdot cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.40), as reported in the literature.[30] 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 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

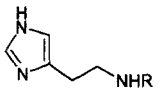
inhibitory/activatory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled–deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the enzyme–activator (E–A) complex. The activation constant K_A was determined as described by Briganti *et al.*[4] Enzyme concentrations were 3.2 nM for hCA II, 10 nM for hCA I and 33 nM for bCA IV (this isozyme has a decreased esterase activity[31] 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 was done at 37°C with gentle stirring, for periods of 30–60 min. After that time, the red cells were washed and then centrifuged for 10 min, the supernatant discarded, and the cells washed again three times with 5 mL of the buffer, in order to eliminate all unbound compound. The cells were then lysed in 5 mL of distilled water, centrifuged to eliminate membranes and other insoluble materials, and CA activity was determined as described above. Blank experiments were done in which no activator was added to the blood red cells treated as described above, and CA activity determined under such conditions was taken as 100%.[32]

RESULTS AND DISCUSSION

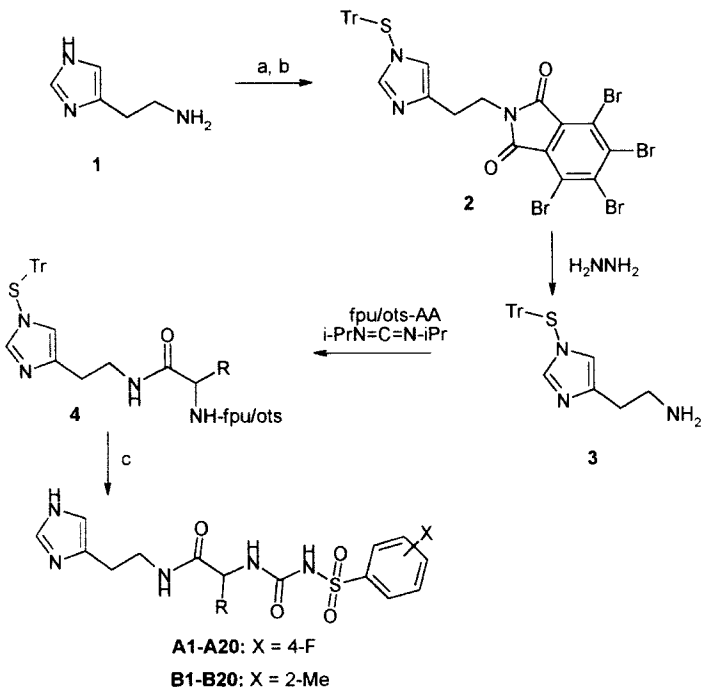
The study of CA activators has only recently made some progress, by the report of the first X-ray crystallographic data of adducts of Hst[4] and phenylalanine[18] with the rapid isozyme hCA II. Taking into account the binding mode of this compound to the enzyme, we designed tighter-binding activators, by derivatization of the aminoalkyl group of Hst **1** by means of alkyl/arylsulfonyl, carboxamido or ureido moieties among others. The compounds obtained by this approach, of types **1a–1c**,



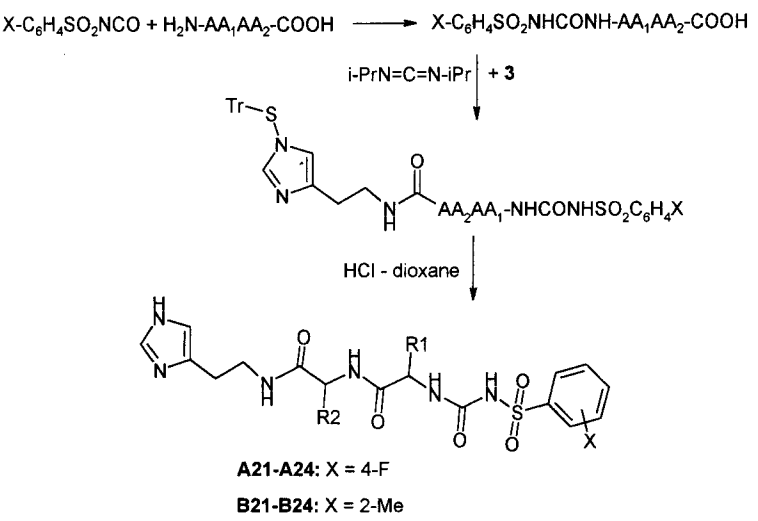
1: R = H
1a: R = $ArSO_2$
1b: R = ArCO
1c: R = ArNHCO

showed in some cases highly enhanced CA activatory properties as compared to the lead molecule.[1–3] Here we extend these studies[1–3] and report the synthesis and activation data for a new class of tight-binding activators, obtained by chemical modification of the aliphatic amino group of the lead compound, Hst **1**.

The key intermediate for obtaining the novel types of activators reported in this paper, *N*-1-tritylsulfenyl Hst **3** was obtained by standard procedures involving the initial protection of the primary amine moiety by means of phthalimide derivatives, followed by protection of the imidazole NH moiety with tritylsulfenyl chloride, and then hydrazinolysis of the phthalimido moiety under mild conditions (Scheme 1). The overall yield for the three steps was good (around 80%) and the purifications procedures quite simple. The approach shown here would thus predict that the tritylsulfenyl (TrS) moiety might be a good protecting group for the side chains of "difficult" amino acids such as histidine and arginine, for (solid phase) peptide synthesis.[2] Subsequent reaction of the key intermediate **3** with arylsulfonylureido-amino acid/dipeptide derivatives in the presence of carbodiimides afforded



SCHEME 1 Reagents: a – tetrabromophthalic anhydride; b – tritylsulfenyl chloride; c – 4 M HCl dioxane.



SCHEME 2

a series of *N*-tritylsulfenylated compounds **4**, which were deprotected under standard conditions (dioxane–HCl), leading to the desired derivatives **A1–A20** and **B1–B20**, respectively. Similarly were prepared some dipeptide derivatives of histamine, **A21–A24**, and **B21–B24**, as outlined in Scheme 2. 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 formulas).

The CA activation data of Table I show significant differences between the investigated isozymes in their behavior towards both "classical" activators, such as Hst **1**, and the new class of derivatives described in the present work. Thus, Hst **1** is a potent hCA I activator, and a relatively weak hCA II activator, whereas isozyme bCA IV possesses an intermediate behavior. The most interesting finding of the present study is represented by the high susceptibility of the cytosolic rapid isozyme, hCA II to be activated by some of the derivatives of Hst of types **A1–A24**/**B1–B24**, as compared to the lead molecule (compounds with activation constants in the 0.02–0.05 μM were frequently obtained). Furthermore, the highly abundant and most prone to activation (by Hst) 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 in 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 behavior towards the new class of activators, with activation constants in the 0.007–0.012 μM range for the

TABLE I CA isozymes I, II and IV activation by histamine **1** (Hst) and the new derivatives **A, B1–A, B24; A1–A20: fpu-AA-Hst, A21–A24: fpu-AA_1–AA_2-Hst; B1–B20: ots-AA-Hst, B21–B24: ots-AA_1–AA_2-Hst**

No.	*X*	*AA*; AA_1AA_2	K_A*(μM)			*Yield*
			hCA I[a]	*hCA II*[a]	*bCA IV*[b]	
1	Histamine	—	2	125	41	—
A1	—	Gly	0.21	11	1.7	67
A2	—	*L*-Ala	0.19	13	3.0	58
A3	—	*β*-Ala	0.20	16	1.9	71
A4	—	GABA	0.23	18	2.2	66
A5	—	GlyGly	0.15	10	2.1	54
A6	—	*L*-Val	0.16	9	1.4	62
A7	—	*L*-Leu	0.18	9	1.2	49
A8	—	*L*-Ile	0.20	8	2.0	76
A9	—	*L*-Asn	0.16	6	2.1	70
A10	—	*L*-Gln	0.13	5	2.0	75
A11	—	*L*-Arg	0.02	1.0	0.5	79
A12	—	*L*-Lys	0.03	1.1	0.7	80
A13	—	*L*-His	0.03	0.8	0.5	61
A14	—	*L*-Phg[c]	0.09	7	1.3	73
A15	—	*L*-Phe	0.08	7	1.1	85
A16	—	*L*-Trp	0.23	12	5.4	90
A17	—	*L*-Pro	0.16	10	3.0	64
A18	—	*L*-Pip[d]	0.15	9	3.1	79
A19	—	*D,L*-Nip[e]	0.16	8	3.0	70
A20	—	*D,L*-Inp[f]	0.15	8	3.0	77
A21		*L*-GlyHis	0.002	0.03	0.008	32
A22	—	*L*-*β*-AlaHis	0.001	0.03	0.007	51
A23		*L*-PhePro	0.004	0.04	0.009	33
A24		*L*-ProGly	0.006	0.05	0.011	30
B1	—	Gly	0.24	16	2.5	85
B2	—	*L*-Ala	0.25	17	3.0	81
B3	—	*β*-Ala	0.25	18	3.6	69
B4	—	GABA	0.24	15	2.2	62
B5	—	GlyGly	0.18	11	1.9	63
B6	—	*L*-Val	0.17	9	2.4	44
B7	—	*L*-Leu	0.20	11	3.4	56
B8	—	*L*-Ile	0.22	8	3.5	50
B9	—	*L*-Asn	0.17	7	3.3	67
B10	—	*L*-Gln	0.14	7	2.1	54
B11	—	*L*-Arg	0.03	1.2	0.6	75
B12	—	*L*-Lys	0.04	1.1	0.8	59
B13	—	*L*-His	0.04	0.9	0.6	71
B14	—	*L*-Phg[c]	0.10	9	2.0	77
B15	—	*L*-Phe	0.09	8	2.0	68
B16	—	*L*-Trp	0.26	15	5.8	93
B17	—	*L*-Pro	0.17	11	3.1	62
B18	—	*L*-Pip[d]	0.18	9	3.0	56
B19	—	*D,L*-Nip[e]	0.16	9	3.1	60
B20	—	*D,L*-Inp[f]	0.16	7	3.0	79
B21		*L*-GlyHis	0.003	0.03	0.009	56
B22	—	*L*-*β*-AlaHis	0.002	0.03	0.008	50
B23		*L*-PhePro	0.004	0.06	0.008	44
B24		*L*-ProGly	0.006	0.05	0.012	41

*Mean from at least three determinations by the esterase method.[30] Standard error was in the range of 5–10%. [a]Human cloned isozyme. [b]Purified from bovine lung microsomes. [c]Phg = phenylglycine. [d]Pip = pipecolic acid (piperidine-2-carboxylic acid). [e]Nip = nipecotic acid (piperidine-3-carboxylic acid). [f]Inp = isonipecotic acid (piperidine-4-carboxylic acid). fpu = 4-F–$C_6H_4SO_2NHCO$–; ots = 2-Me–$C_6H_4SO_2NHCO$–.

most active of such compounds. Efficient CA activators were as follows: (i) Derivatives of basic amino acids (Arg, Lys, His), such as **A11–13**, **B11–13**, as well as the phenylglycine and phenylalanine derivatives **A14**, **B14** and **A15**, **B15**. (ii) Slightly less active were the compounds derived from Pro, Pip, Nip, Inp, Asn, Gln as well as the hydrophobic amino acid derivatives (Val, Leu, Ile, Trp). (iii) The best activators in this series were those derived from dipeptides such as GlyHis (**A21**, **B21**), β-AlaHis (**A22**, **B22**), PhePro (**A23**, **B23**) or ProGly (**A24**, **B24**). These compounds possessed activation constants in the 2–12 nM range against hCA I and bCA IV, and in the 30–60 nM range against hCA II. Probably the many heteroatoms present in the arylsulfonylureido-dipeptidyl moieties confer "sticky" properties, i.e., they are able to participate in many interactions with amino acid residues at the active site, thus assuring the formation of very stable E–A adducts. The fluorophenylsulfonylureido derivatives were slightly more active than the corresponding *ortho*-toluenesulfonylureido compounds.

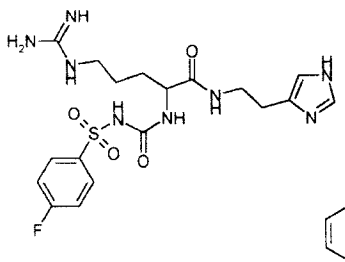
Similarly to all CA activators reported up to now, the compounds obtained in the present work presumably intervene in the catalytic cycle of the enzyme, leading to the formation of CA–activator complexes (similarly to the enzyme–inhibitor adducts, but without substitution of the metal bound solvent molecule) in which the activator bound within the active site facilitates the rate-limiting proton transfer steps (between the active site and the environment), as suggested by Lindskog's[33] and our works.[1–4] The driving force for this effect might be the fact that intramolecular reactions are more rapid than intermolecular ones.[1–4] Thus, in the presence of activators (symbolized as "A"), the rate-limiting step is described by Equation (1) below:

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

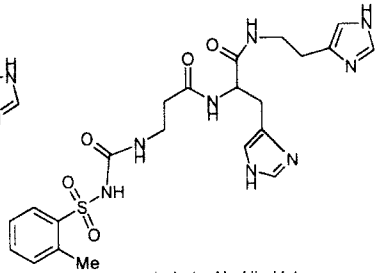
E–A complexes

Obviously, compounds of the types reported here possess the imidazole 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 the arylsulfonylureido amino acyl/dipeptidyl tails in their molecule they can bind more effectively to the enzyme, leading to more efficient activation processes as compared to **1**. Indeed, the active site edge of all three CA isozymes investigated by us contains a high proportion of polar amino acid residues which might interfere with polar groups such as $X-C_6H_4-SO_2NHCO-$amino acyl. 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.[1-4] 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)[4] which could easily participate in the formation of hydrogen bonds (as well as other types of interactions) with the Hst derivatives reported here. This might explain the greater efficiency of the compounds reported in the present work in activating this isozyme, as compared to Hst, which is a relatively weak hCA II activator.



fpu-ArgHst
A11



ots-beta-Ala-His-Hst
B22

A special mention should also be made regarding compounds such as **A11**, **B11**, **A12**, **B12**, **A13**, **B13**, **A21**, **B21**, **A22** or **B22**, which are very effective CA activators against all three isozymes investigated here due to the fact that they possess secondary moieties able to shuttle protons, in addition to the parent Hst one (the guanidino-, ε-amino- or imidazole ring of histidine, respectively).

Data of Table II show that after incubation of normal blood red cells (containing approximately 150 μM of hCA I and 20 μM of hCA II)[34] with

TABLE II *Ex vivo* CA activation data after 30 and 60 min of incubation of human erythrocytes with solutions containing 5 μM activators **1**, **A11**, **A22**, **B13** and **B22**

Activator	*% CA activity*[a]	
	30 min	60 min
1 (histamine)	121 ± 3	130 ± 5
A11	156 ± 8	184 ± 7
A22	207 ± 9	233 ± 12
B13	150 ± 4	178 ± 5
B22	198 ± 6	225 ± 11

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

micromolar concentrations of Hst **1** or Hst derivatives synthesized in the present work (such as **A11**, **A22**, **B13**, **B22**, etc.), total CA activity (measured by the esterase method, with 4-nitrophenyl acetate as substrate)[30] in the treated cells is enhanced as compared to that of cells treated in a blank experiment with buffer only. Thus, Hst produces only a weak activation of around 120% after half an hour's incubation, and of around 130% of the basal CA activity after 1 h incubation with the red cells. Some of the new Hst derivatives tested *ex vivo* (which showed strong *in vitro* CA activity enhancements) produced activations of 150–207% after half an hour's incubation, and of 178–233% after 1 h incubation. These are clear-cut experiments proving that some of the compounds reported in this paper might act as effective *in vivo* CA activators.

Acknowledgments

This research was financed by the EU grant ERB CIPDCT 940051. Thanks are addressed to Drs. M.A. Ilies, and M. Barboiu for expert technical assistance.

References

[1] This paper is part 25 of the series. Preceding part: Scozzafava, A. and Supuran, C.T. (2000) *Eur. J. Pharm. Sci.*, **10**, 29–41.
[2] F. Briganti, A. Scozzafava and C.T. Supuran (1999) *Bioorg. Med. Chem. Lett.*, **9**, 2043–2048.
[3] A. Scozzafava and C.T. Supuran (2000) *Eur. J. Med. Chem.*, **35**, 31–39.
[4] F. Briganti, S. Mangani, P. Orioli, A. Scozzafava, G. Vernaglione and C.T. Supuran (1997) *Biochemistry*, **36**, 10 384–10 392.
[5] L.Z. Avila, Y.H. Chu, E.C. Blossey and G.M. Whitesides (1993) *J. Med. Chem.*, **36**, 126–133.
[6] J.M. Gao, S. Qiao and G.M. Whitesides (1995) *J. Med. Chem.*, **38**, 2292–2301; Gao, J.M., Cheng, X.H., Chen, R.D., Sigal, G.B., Bruce, J.E., Schwartz, B.L., Hofstadler, S.A., Anderson, G.A., Smith, R.D. and Whitesides, G.M. (1996) *J. Med. Chem.*, **39**, 1949–1955.
[7] A. Scozzafava, L. Menabuoni, F. Mincione, F. Briganti, G. Mincione and C.T. Supuran (1999) *J. Med. Chem.*, **42**, 2641–2650.
[8] A. Scozzafava, F. Briganti, G. Mincione, L. Menabuoni, F. Mincione and C.T. Supuran (1999) *J. Med. Chem.*, **42**, 3690–3700.
[9] C.T. Supuran, A. Scozzafava, L. Menabuoni, F. Mincione, F. Briganti and G. Mincione (1999) *Eur. J. Pharm. Sci.*, **8**, 317–328.
[10] W.S. Sly (1991) Carbonic anhydrase II deficiency syndrome: Clinical delineation, interpretation and implications. In *The Carbonic Anhydrases* (Dodgson, S.J., Tashian, R.E., Gros, G. and Carter, N.D. (Eds.)), pp. 183–196. Plenum Press; New York and London.
[11] W.S. Sly and P.Y. Hu (1995) *Annu. Rev. Biochem.*, **64**, 375–401.
[12] T.H. Maren (1995) *J. Glaucoma*, **4**, 49–62.
[13] W.G. Reiss and K.S. Oles (1996) *Ann. Pharmacother.*, **30**, 514–519.
[14] C.T. Supuran (1994) Carbonic anhydrase inhibitors. In *Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism* (Puscas, I. (Ed.)), pp. 29–111. Helicon; Timisoara.

[15] L.G. Raisz, H.A. Simmons, W.J. Thompson, K.L. Shepard, P.S. Anderson and G.A. Rodan (1988) *Endocrinology*, **122**, 1083–1086.
[16] C.T. Supuran, C.W. Conroy and T.H. Maren (1996) *Eur. J. Med. Chem.*, **31**, 843–846.
[17] D. Hewett-Emmett (2000) Evolution and distribution of the carbonic anhydrase gene families. In *Carbonic Anhydrase – New Horizons* (Chegwidden, W.R. (Ed.)). Birkhauser; New York. (in press).
[18] F. Briganti, V. Iaconi, S. Mangani, P. Orioli, A. Scozzafava, G. Vernaglione and C.T. Supuran (1998) *Inorg. Chim. Acta*, **275–276**, 295–300.
[19] B.W. Clare and C.T. Supuran (1994) *J. Pharm. Sci.*, 1994, **83**, 768–779.
[20] C.T. Supuran, A.T. Balaban, P. Cabildo, R.M. Claramunt, J.L. Lavandera and J. Elguero (1993) *Biol. Pharm. Bull.*, **16**, 1236–1239.
[21] C.T. Supuran, R.M. Claramunt, J.L. Lavandera and J. Elguero (1996) *Biol. Pharm. Bull.*, **19**, 1417–1422.
[22] C.T. Supuran, M. Barboiu, C. Luca, E. Pop, M.E. Brewster and A. Dinculescu (1996) *Eur. J. Med. Chem.*, **31**, 597–606.
[23] (a) M.A. Ilies, M.D. Banciu, M. Ilies, F. Chiraleu, F. Briganti, A. Scozzafava and C.T. Supuran (1997) *Eur. J. Med. Chem.*, **32**, 911–918; (b) M.J. Tozer, E.A. Harper, S.B. Kalindjian, M.J. Pether, N.P. Shankley and G.F. Watt (1999) *Bioorg. Med. Chem. Lett.*, **9**, 1825–1830.
[24] A. Scozzafava and C.T. Supuran (1999) *J. Enz. Inhib.*, **14**, 343–363.
[25] G. Behravan, B.H. Jonsson and S. Lindskog (1990) *Eur. J. Biochem.*, **190**, 351–357.
[26] R.G. Khalifah, D.J. Strader, S.H. Bryant and S.M. Gibson (1977) *Biochemistry*, **16**, 2241–2247.
[27] S. Lindskog and J.E. Coleman (1964) *Proc. Natl. Acad. Sci. USA*, **70**, 2505–2508.
[28] H. Steiner, B.H. Jonsson and S. Lindskog (1975) *Eur. J. Biochem.*, **59**, 253–259.
[29] T.H. Maren, G.C. Wynns and P.J. Wistrand (1993) *Mol. Pharmacol.*, **44**, 901–906.
[30] Y. Pocker and J.T. Stone (1967) *Biochemistry*, **6**, 668–678.
[31] T.T. Baird, A. Waheed, T. Okuyama, W.S. Sly and C.A. Fierke (1997) *Biochemistry*, **36**, 2669–2678.
[32] C.T. Supuran, A. Scozzafava, M.A. Ilies, B. Iorga, T. Cristea, F. Briganti, F. Chiraleu and M.D. Banciu (1998) *Eur. J. Med. Chem.*, **33**, 577–595.
[33] S. Lindskog (1997) *Pharmacol. Ther.*, 1997, **74**, 1–20.
[34] P.J. Wistrand and A. Lindqvist (1991) Design of carbonic anhydrase inhibitors and the relationship between the pharmacodynamics and pharmacokinetics of acetazolamide. In *Carbonic Anhydrase – From Biochemistry and Genetics to Physiology and Clinical Medicine* (Botrè, F., Gros, G. and Storey, B.T. (Eds.)). pp. 352–378. VCH; New York, Weinheim.