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Carbonic Anhydrase Inhibitors: Synthesis of Water Soluble Sulfonamides Incorporating a 4-sulfamoylphenylmethylthiourea Scaffold, with Potent Intraocular Pressure Lowering Properties

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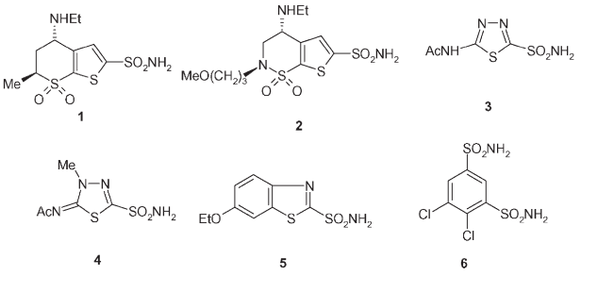
Reaction of thiophosgene with 4-aminomethyl-benzenesulfonamide afforded 4-isothiocyanatomethyl-benzenesulfonamide, which by reaction with amines, amino acids and oligopeptides, lead to a series of new sulfonamides incorporating a 4-sulfamoylphenylmethylthiourea scaffold. These new thioureas showed strong affinities towards isozymes I, II and IV of carbonic anhydrase (CA, EC 4.2.1.1). In vitro inhibitory potency was good (in the low nanomolar range) for the derivatives of: amino-benzoic acids, β-phenyl-serine, α-phenyl-glycine, for those incorporating hydroxy- and mercapto-amino acids (Ser, Thr, Cys and Met), hydrophobic amino acids (Val, Leu, Ile), aromatic amino acids (Phe, His, Trp, Tyr; DOPA); dicarboxylic amino acids as well as di-/tri-/tetrapeptides among others. Such CA inhibitors displayed very good water solubility (in the range of 2–3%) as sodium (carboxylate) salts, with pH values for the solutions obtained of 6.5–7.0. Furthermore, in normotensive rabbits, some of them showed an effective and prolonged intraocular pressure (IOP) lowering when administered topically, as 2% solutions.

Keywords: Carbonic anhydrase; Sulfonamide; Intraocular pressure; Glaucoma

INTRODUCTION

Sulfonamide inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) are extensively used in clinical medicine and as diagnostic tools, their main applications being in the treatment of glaucoma, macular edema, epilepsy and other neurological disorders.[1–3] Several such drugs are presently available, such as the recently introduced topical sulfonamides dorzolamide **1** and brinzolamide **2**, in addition to the classical, systemically acting inhibitors acetazolamide **3**, methazolamide **4**, ethoxzolamide **5** or dichlorophenamide **6**, which have been employed clinically for more than 45 years.[1–3]



Systemic inhibitors possess many undesired side effects due to inhibition of several CA isozymes (14 are presently known in higher vertebrates)[1] in tissues other than the target one, i.e., the eye (more precisely, the ciliary processes of the eye), and many agents such as **3**–**6** are presently used more in physiological studies or as diagnostic tools than as antiglaucoma drugs.[1] On the other hand, the two topically acting inhibitors **1** and **2**, show a much more diminished number of side effects, together with an efficient reduction of intraocular pressure

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(IOP) due to inhibition of the enzymes (mainly isozymes CA II and CA IV)[1–3] present in the ciliary processes, without appreciable inhibition of CAs from other tissues/organs.[1–4] Since sulfonamides **1** and **2** are both administered as hydrochloride salts, frequent stinging sensations, burning or reddening of the eye, blurred vision, pruritus, and other local irritations are common side effects. In order to reduce such inconveniences, many attempts were ultimately reported to design topical antiglaucoma sulfonamides devoid of the above-mentioned side effects.[5–8] Among them, one approach consisted in attaching water-solubilizing "tails" to the molecules of aromatic/heterocyclic sulfonamides possessing amino, imino or hydroxy moieties in their molecules.[6,7] Such tails included pyridine-carboximido, carboxypyridine-carboxamido, quinolinesulfonamido, picolinoyl, isonicotinoyl, as well as amino acyl groups among others, whereas ring systems derivatized by this procedure included: 2-, 3- or 4-aminobenzenesulfonamides, 4-(ω-aminoalkyl)-benzene-sulfonamides, 3-halogenosubstituted-sulfanilamides, 1,3-benzene-disulfonamides, 1,3,4-thiadiazole-2-sulfonamides, benzothiazole-2-sulfonamides as well as thienothiopyran-2-sulfonamides among others, and had as the main objective the possibility of formulating the ophthalmic solutions at pH values close to neutrality.[6–8]

Continuing our previous research in this field,[4–8] we report here an alternative approach for obtaining water soluble, potent CA inhibitors with applications as agents for the treatment of ocular hypertension, with potentially no side effects due to the acidic pH of the ophthalmic solution. Thus, 4-isothiocyanatomethyl-benzenesulfonamide (obtained from thiophosgene (CAUTION, this reagent is highly toxic and should be handled only under a well ventilated hood) and 4-aminomethyl-benzenesulfonamide) was reacted with amines, amino acids or oligopeptides, and the thioureas obtained in this way, showed excellent water solubilities either as sodium salts (for the amino acid/oligopeptide derivatives), or as hydrochlorides/triflates (in the case of the amine derivatives). The nucleophiles used in the above mentioned syntheses were chosen in such a way as to possess pK_a values in the "physiological" range. Most of these new sulfonamides showed excellent inhibitory properties against CA isozymes I, II and IV, and were effective IOP lowering agents when administered topically to normotensive rabbits.

MATERIALS AND METHODS

Chemistry

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss IR-80 instrument. ^{1}H-NMR spectra were recorded in DMSO-d_6 as solvent, with a Bruker CPX200 or Varian 300 instrument. Chemical shifts are reported as δ values, relative to Me_4Si as internal 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. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Analytical and preparative HPLC was performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Sulfanilamide, thiophosgene, and nucleophiles (amines, amino acids and oligopeptides) used in the synthesis were highest purity, commercially available compounds (from Sigma—Aldrich, Fluka, E. Merck or Acros). Acetonitrile, acetone (E. 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 4-isothiocyanatomethyl-benzenesulfonamide B

The method of McKee and Bost[9] has been modified as follows. An amount of 22.2 g (0.1 mol) of homosulfanilamide hydrochloride was dissolved in 200 mL of water and 10 mL of concentrated hydrochloric acid solution was added, together with 11.2 g (0.1 mol) of thiophosgene (CAUTION, this reagent is highly toxic and should be handled under a well ventilated hood). Stirring was begun immediately until all the red color of thiophosgene disappeared (around 3.5–4 h) and a white crystalline precipitate was formed. The product was filtered and recrystallized from acetone–water (1:1, v/v). The yield was of 95% (m.p. 179–81°C).

General Procedure for the Preparation of the Compounds B7–B47

An amount of 0.53 g (2.5 mMoles) of 4-isothiocyanatomethyl-benzenesulfonamide **A** and the stoichiometric amount of nucleophile **7–47** were suspended in 50–100 mL of dry acetone or acetonitrile and heated at reflux for 2–8 h (TLC control). The solvent was evaporated, and the crude product either recrystallized from ethanol or ethanol–water (for the greater majority of thioureas described here), or purified by preparative HPLC in the case when the reaction mixture contained appreciable amounts of impurities (as shown by TLC). This was particularly the case for the oligopeptidyl thioureas **B39–B47**. Conditions used for the purification were: C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) preparative columns; 90% acetonitrile/ 8% methanol/ 2% water; 30 mL/min.

1-(4-Sulfamoyl-phenylmethyl)-3-[(1H-imidazol)-4-yl-ethyl]-thiourea (Histamine Derivative) **B10**

White crystals, m.p. 173–4°C (acetone–water 1:1, v/v). IR (KBr), cm^{-1}: 1150 (SO_2^{sym}), 1245 (thioamide III), 1375 (SO_2^{as}), 1550 (thioamide II), 1680 (thioamide I), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.49 (t, 2H, J = 7.0, Hst CH_2), 2.99 (t, 2H, J = 7.0, Hst CSNHCH_2), 4.49 (d, 2H, $^3J_{HH} = 6.0$, $H_2NO_2SC_6H_4$-CH_2), 7.34 (m, 1H, imidazole CH), 7.52 (s, 2H, SO_2NH_2), 7.65 (d, $^3J_{HH} = 8.1$, 2H, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, 2H, $^3J_{HH} = 8.1$, H_{meta} of $H_2NO_2SC_6H_4$), 8.21 (br s, 2H, NHCSNH), 8.35 (s, 1H, imidazole CH), 8.80 (s, 1H, imidazole NH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.23 (s, CH_2 of Hst), 37.64 (s, CH_2 of Hst), 41.58 (CH_2), 123.70 (s, C-4 of Hst), 130.93 (s, C_{meta} of $H_2NO_2SC_6H_4$), 134.32 (s, C-5 of Hst), 135.13 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 137.30 (s, C-2 of Hst), 139.43 (s, NHCSNH), 144.68 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 148.14 (s, C_{para} of $H_2NO_2SC_6H_4$); Anal. Found: C, 45.94; H, 5.32; N, 20.36. $C_{13}H_{17}N_5O_2S_2$ requires C, 46.00; H, 5.05; N, 20.63%.

1-(4-Sulfamoyl-phenylmethyl)-3-(carboxy-methyl)-thiourea (Glycine Derivative) **B14**

White crystals, m.p. 168–9°C (acetone–water 1:2, v/v). IR (KBr), cm^{-1}: 1153 (SO_2^{sym}), 1240 (thioamide III), 1375 (SO_2^{as}), 1550 (thioamide II), 1682 (thioamide I), 1754 (COOH), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.67 (s, 2H, CH_2 of Gly), 4.47 (d, 2H, $^3J_{HH} = 6.0$, $H_2NO_2SC_6H_4$-CH_2), 7.54 (s, 2H, SO_2NH_2), 7.63 (d, 2H, $^3J_{HH} = 8.1$, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, 2H, $^3J_{HH} = 8.1$, H_{meta} of $H_2NO_2SC_6H_4$), 8.21 (br s, 2H, NHCSNH), 10.63 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 40.81 (s, CH_2 of Gly), 41.62 (CH_2), 131.53 (s, C_{meta} of $H_2NO_2SC_6H_4$), 135.49 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.76 (s, NHCSNH), 144.24 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.57 (s, C_{para} of $H_2NO_2SC_6H_4$), 179.83 (COOH); Anal. Found: C, 39.30; H, 4.68; N, 13.57. $C_{10}H_{13}N_3O_4S_2$ requires C, 39.55; H, 4.32; N, 13.85%.

1-(4-Sulfamoyl-phenylmethyl)-3-(1-carboxy-2-methyl-butyl)-thiourea (Isoleucine Derivative) **B26**

White crystals, m.p. 170–2°C (acetone–water 1:1, v/v). IR (KBr), cm^{-1}: 1151 (SO_2^{sym}), 1244 (thioamide III), 1376 (SO_2^{as}), 1557 (thioamide II), 1676 (thioamide I), 1750 (COOH), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.15 (d, 3H, $^3J_{HH} = 6.5$, CH_3 of Ile), 1.21 (t, 3H, $^3J_{HH} = 6.7$, CH_3 of Et moiety of Ile), 1.54 (m, 2H, CH_2 of Ile), 3.22 (m, 1H, EtCH(Me)- of Ile), 3.75 (m, 1H, NHCHCO of Ile), 4.48 (d, 2H, $^3J_{HH} = 6.0$, $H_2NO_2SC_6H_4$-CH_2), 7.51 (s, 2H, SO_2NH_2), 7.66 (d, 2H, $^3J_{HH} = 8.0$, H_{ortho} of $H_2NO_2SC_6H_4$), 7.92 (d, 2H, $^3J_{HH} = 8.0$, H_{meta} of $H_2NO_2SC_6H_4$), 8.20 (br s, 2H, NHCSNH), 10.42 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 21.78 (s, CHCH_3 of Ile), 22.57 (s, CH_3CH_2 of Ile), 31.42 (s, CH_2 of Ile), 34.58 (s, CH(CH_3)$_2$ of Leu), 41.57 (CH_2), 46.36 (s, EtCH(Me)- of Ile), 55.12 (s, NHCHCH$_2$ of Ile), 131.43 (s, C_{meta} of $H_2NO_2SC_6H_4$), 135.58 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.75 (s, NHCSNH), 144.28 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.64 (s, C_{para} of $H_2NO_2SC_6H_4$), 179.16 (COOH); Anal. Found: C, 46.90; H, 5.75; N, 11.50. $C_{14}H_{21}N_3O_4S_2$ requires C, 46.78; H, 5.89; N, 11.69%.

1-(4-Sulfamoyl-phenylmethyl)-3-(1-carboxy-2-phenyl-ethyl)-thiourea (Phenylalanine Derivative) **B33**

White crystals, m.p. 208–9°C (ethanol–water 1:1, v/v). IR (KBr), cm^{-1}: 1148 (SO_2^{sym}), 1243 (thioamide III), 1376 (SO_2^{as}), 1550 (thioamide II), 1684 (thioamide I), 1751 (COOH), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.10-3.55 (m, 2H, CH_2CH of Phe), 4.08 (dd, 1H, $^3J_{HH} = 5.0$, $^3J_{HH} = 7.8$, CH$_2$CH of Phe), 4.49 (d, 2H, $^3J_{HH} = 6.0$, $H_2NO_2SC_6H_4$-CH_2), 7.29–7.51 (m, 5H, H_{arom} of Phe), 7.57 (s, 2H, SO_2NH_2), 7.63 (d, 2H, $^3J_{HH} = 8.1$, H_{ortho} of $H_2NO_2SC_6H_4$), 7.96 (d, 2H, $^3J_{HH} = 8.1$, H_{meta} of $H_2NO_2SC_6H_4$), 8.20 (br s, 2H, NHCSNH), 10.71 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 41.23 (s, CH_2CH of Phe), 41.64 (CH_2), 59.30 (s, CH$_2$CH of Phe), 131.56 (s, C_{meta} of $H_2NO_2SC_6H_4$), 133.70 (s, C_{meta} of Phe), 134.24 (s, C_{ortho} of Phe), 135.50 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 139.61 (s, NHCSNH), 141.58 (s, C_{ipso} of Phe), 144.23 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 145.45 (s, C_{para} of Phe), 147.37 (s, C_{para} of $H_2NO_2SC_6H_4$), 179.25 (COOH); Anal. Found: C, 51.70; H, 4.67; N, 10.54. $C_{17}H_{19}N_3O_4S_2$ requires C, 51.89; H, 4.87; N, 10.68%.

4-Sulfamoylphenylmethyl-thioureido-β-alanyl-histidine **B40** (Carnosine Derivative)

White crystals, m.p. 155–7°C. IR (KBr), cm^{-1}: 1156 (SO_2^{sym}), 1240 (thioamide III),1287 (amide I), 1375 (SO_2^{as}), 1540 (amide I), 1550 (thioamide II), 1683 (thioamide I), 1720 (amide I), 1750 (COOH), 3065 (NH); ^{1}H-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), 4.45 (d, 2H, $^3J_{HH} = 6.0$, $H_2NO_2SC_6H_4$-CH_2), 4.57–4.63 (m, 1H, CHCH$_2$ of His), 7.33 (s, 1H, CH-5 of His), 7.54 (s, 2H, SO_2NH_2), 7.63 (d, 2H, $^3J_{HH} = 8.1$, H_{ortho} of $H_2NO_2SC_6H_4$), 7.94 (d, 2H, $^3J_{HH} = 8.1$, H_{meta} of $H_2NO_2SC_6H_4$), 8.21 (br s, 2H, NHCSNH), 8.29 (br s, 1H, CONH), 8.35 (s, 1H, CH-2 of His), 8.80 (s, 1H, imidazole NH from His), 10.63 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 33.27 (s, CH_2 of His), 37.50 (s, NHCH_2CH$_2$ of β-Ala), 40.69 (s, CH$_2$CH_2CO of β-Ala), 41.55 (CH_2), 59.48 (s, CHCH$_2$ of His), 122.21 (s, C-4 of His), 131.56 (s, C_{meta} of $H_2NO_2SC_6H_4$), 134.28 (s, C-5 of His), 135.09 (s, C_{ortho} of $H_2NO_2SC_6H_4$), 137.57 (s, C-2 of His), 139.33 (s, NHCSNH), 144.16 (s, C_{ipso} of $H_2NO_2SC_6H_4$), 147.24 (s, C_{para} of $H_2NO_2SC_6H_4$), 175.60 (s, CH$_2$CO of β-Ala), 180.09 (COOH); Anal. Found: C, 45.07;

H, 4.62; N, 18.30. $C_{17}H_{22}N_6O_5S_2$ requires C, 44.92; H, 4.88; N, 18.49%.

Carbonic Anhydrase Inhibition

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's group.[10] Cell growth conditions were those described in ref. 11, and enzymes were purified by affinity chromatography according to the method of Khalifah *et al.*[12] 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 29.3 kDa for CA II, respectively.[13,14] bCA IV was isolated from bovine lung microsomes as described by Maren *et al.*, and its concentration was determined by titration with ethoxzolamide.[15]

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC.[16] 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 $M^{-1} \cdot cm^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis under the conditions of the experiments (pH 7.40), as reported in the literature.[16] Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor 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 − I complex. The inhibition constant K_I was determined as described by Pocker and Stone.[16] Enzyme concentrations were 3.6 nM for CA II, 9.1 nM for CA I and 30 nM for CA IV (this isozyme has a decreased esterase activity[17] and higher concentrations had to be used for the measurements).

Determination of Water (Buffer) Solubility

A standard solution was prepared by dissolving a precisely weighted amount (generally 1 mg) of inhibitor in 10 mL of methanol. The UV absorption maximum of each compound was determined (with a Perkin Elmer Lambda 20 Bio spectrophotometer) at 25°C eventually diluting the solution (with MeOH) as necessary. A saturated solution of each compound was then prepared by stirring magnetically a small volume of 0.039 M phosphate buffer (pH 7.4) in the presence of excess inhibitor for 3 h. The obtained saturated solution was filtered to remove solid compound through a Millipore 0.45 μm filter and scanned by UV at the wavelength of the previously determined absorption maximum. Total solubility was determined by the relationship: $C' = A'C/A$, where C = concentration of standard solution (mg/mL); A = absorbance of standard solution; A' = absorbance of the saturated solution; C' = concentration of the saturated solution (mg/mL).[18]

Partition Coefficient Determinations

Chloroform–buffer partition coefficients were obtained by equilibrating for 90 min at room temperature the test compound, between chloroform and 0.1-ionic strength pH 7.4 phosphate buffer. The concentration in each phase was determined by UV spectrophotometry or HPLC.[18]

Measurement of Tonometric IOP

Adult male New Zealand albino rabbits weighing 3–3.5 kg were used in the experiments (three animals were used for each inhibitor studied). The experimental procedures conform to the Association for Research in Vision and Ophthalmology Resolution on the use of animals. The rabbits were kept in individual cages with food and water provided *ad libitum*, being maintained on a 12 h: 12 h light/dark cycle in a temperature controlled room, at 22–26°C. Solutions/suspensions of inhibitors (2%, by weight, as hydrochlorides or sodium carboxylates) were obtained in distilled deionized water. The pH of these solutions was in the range of 5.5–7.4.

IOP was measured using a Digilab 30R pneumatonometer (BioRad, Cambridge, MA, USA) as described by Maren's group.[19,20] The pressure readings were matched with two-point standard pressure measurements at least twice each day using a Digilab Calibration verifier. All IOP measurements were done by the same investigator with the same tonometer. One drop of 0.2% oxybuprocaine hydrochloride (novesine, Sandoz) diluted 1:1 with saline was instilled in each eye immediately before each set of pressure measurements. IOP was measured three times at each time interval, and the means reported. IOP was measured first immediately before drug administration, then at 30 min after the instillation of the pharmacological

agent, and then each 30 min for a period of 4–6 h. For all IOP experiments drug was administered to only one eye, leaving the contralateral eye as an untreated control. The ocular hypotensive activity is expressed as the average difference in IOP between the treated and control eye, in this way minimizing the diurnal, seasonal and interindividual variations commonly observed in the rabbit.[19,20] All data are expressed as mean ± SE.

Transcorneal Penetration of Drugs

The method of Maren *et al.*[15] with the modifications of Pierce's group[22,23] (for the HPLC assay of sulfonamides) have been used. Excised rabbit corneas with either intact or denuded epithelium were used in these experiments. The pH was 7.4 and the exposed area was 1.2 cm^2. Concentrations of drug, 40–2000 μM, were placed in the epithelial chamber and samples of fluid were collected from the endothelial chamber at different intervals, up to 4 h. Both chambers contained 6 mL. Drugs present in these fluids were assayed both by the HPLC method of Pierce *et al.*,[22,23] or enzymatically.[18] The results of the drug analyses were used to calculate the rate constant of transfer across the cornea (k_{in}). As described by Pierce,[22,23] this value was determined by using the formula:

$$k_{in}(\times 10^3\,hr^{-1}) = [drug]_{endo}/[drug]_{epi} \times 60/t \times 1000$$

where $[drug]_{endo}$ = concentration of drug on endothelial side; $[drug]_{epi}$ = concentration of drug on epithelial side; t = time (in min).

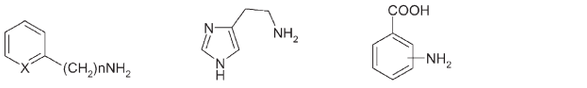
Drug Distribution in Ocular Fluids and Tissues

The general procedure of Maren's group was followed.[19,20] The animals were killed with an intracardiac injection. Aqueous humor (both posterior and anterior chamber fluids) were withdrawn. Then, the cornea and anterior uvea (iris plus attached ciliary body) were dissected, rinsed well with water, blotted, weighed and put into 1–2 mL of water. For isolation of the ciliary processes, intact anterior uvea rings were placed on a parafilm covered piece of polystyrene foam in a Petri dish. The tissue was wetted with normal saline and dissected under a microscope, when ciliary processes were liberated from their attachment to the iris, cut, weighed and put into 0.5 mL of distilled water. The tissue from 4 eyes (average weight of 8 mg/ eye) was pooled for drug analysis. Samples were boiled for 5 min (in order to denature CA, and free drug from the E − I complex), diluted and then incubated with a known amount of enzyme. The activity of the free enzyme and in the presence of the inhibitor were determined as described above. A calibration curve was used in order to determine the fractional inhibition in the different tissues, as described in ref 19,20.

RESULTS AND DISCUSSION

Chemistry

The synthesis of 4-isothiocyanato-benzenesulfonamide **A** was reported in 1946 by McKee and Bost,[9] in the search for more effective antibacterial sulfonamides. Still, this highly versatile compound has only recently been used for the preparation of sulfonamides possessing CA inhibitory properties.[8] A large number of derivatives has been prepared by reaction of 4-isothiocyanato-benzenesulfonamide **A** with amines **7**–**13**, amino acids **14**–**38** or oligopeptides **39**–**47**, and many such derivatives possessed effective IOP lowering properties when administerd topically to rabbits.[8] Thus, we decided to further explore this approach and here we report the synthesis of the structurally related isothiocyanate **B** as well as the corresponding thioureas **B7**–**B47**.



7: X=N; n = 1
8: X=N; n = 2
9: X=CH; n =2

10

11: 2-NH_2
12: 3-NH_2
13: 4-NH_2

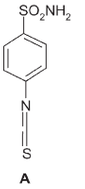
14: Gly; **15:** Ala; **16:** beta-Ala; **17:** GABA; **18:** alpha-Ph-Gly; **19:** Ser; **20:** beta-Ph-Ser

21: Thr; **22:** Cys; **23:** Met; **24:** Val; **25:** Leu; **26:** Ile; **27:** Asp; **28:** Asn; **29:** Glu; **30:** Gln

31: Pro; **32:** His; **33:** Phe; **34:** Tyr; **35:** DOPA; **36:** Trp; **37:** Lys; **38:** Arg; **39:** GlyGly

40: beta-AlaHis; **41:** HisGly; **42:** HisPhe; **43:** AlaPhe; **44:** LeuGly; **45:** AspAsp

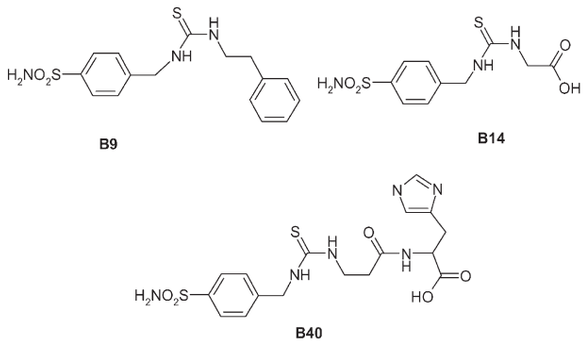
46: ProGlyGly; **47**: $(Asp)_4$



A

The newly obtained derivatives are numbered as **B7**–**B47**, denoting that the 4-H_2NO_2S-C_6H_4-CH_2-NHCSNH-group is attached to the corresponding moiety of the starting nucleophile **7**–**47**. For instance, **B9** is the thiourea obtained by the reaction of phenethylamine **9** with 4-isothiocyanatomethyl-benzenesulfonamide **B**; **B14** is the thiourea obtained by reaction of glycine **14** with 4-isothiocyanatomethyl-benzenesulfonamide **B**; **B40** is the thiourea obtained by reaction of carnosine

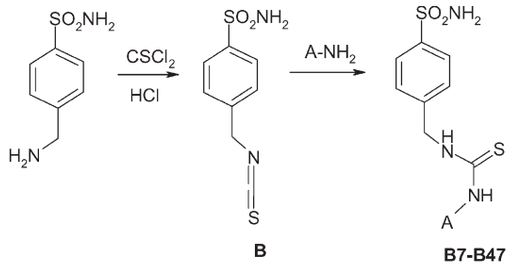
40 with 4-isothiocyanatomethyl-benzenesulfonamide **B**; etc.



Compound **B** has been obtained by adapting the reported literature procedure for the preparation of **A**,[9] from homosulfanilamide hydrochloride and thiophosgene, and was subsequently reacted with a large number of amines, amino acids and oligopeptides, of type **7**–**47**, leading to the new thioureas **B7**–**B47** (Scheme 1).[8,9,24,25]

The reaction generally proceeded in very good yields and without the formation of side-products mainly for amines and simple amino acids. Still, in the case of oligopeptides (such as **39**–**47**) used as nucleophiles in these syntheses, a lot of tar was formed during the reaction, and pure compounds **B39**–**B47** could only be obtained after repeated crystallizations.

The nucleophiles used for the preparation of the new compounds reported here (**7**–**47**) were chosen in such a manner as to contain water-solubilizing moieties in the presence of acids/bases, such as the pyridine or imidazole moieties in the case of **7**, **8** and



SCHEME 1 Synthesis of 4-isothiocyanatomethyl-benzenesulfonamide **B** and the thioureas **B7**–**B47**.

10 (hydrochlorides[6,7] or triflates[6] of **B7**, **B8** or **B10** would presumably lead to water soluble CA inhibitors, but the compound unable to form such a salt—**B9**—has also been prepared for comparison, and confirmed the hypothesis mentioned above). Thioureas obtained from other nucleophiles (**B11**–**B47**) on the other hand readily form water soluble sodium salts due to the presence of at least one carboxyl group in their molecules. It was previously noted in this laboratory[2,26] that sulfonamides incorporating carboxylate moieties in their molecules might interact with a histidine cluster at the entrance of the hCA II active site (this is the isozyme that plays the most important function in aqueous humor secretion).[1–3] This cluster (Figure 1) comprises the residues His 64 (at the center of the active site cavity), His 4 and His 3 (at the rim of it) as well as several residues on the external surface of the enzyme, near the entrance to the active site cavity (His 10, His 15 and His 17; hCA I numbering).[26] All imidazolic side chains of these residues may interact (better when in a charged state, as imidazolium ions) with negatively charged inhibitors, containing for

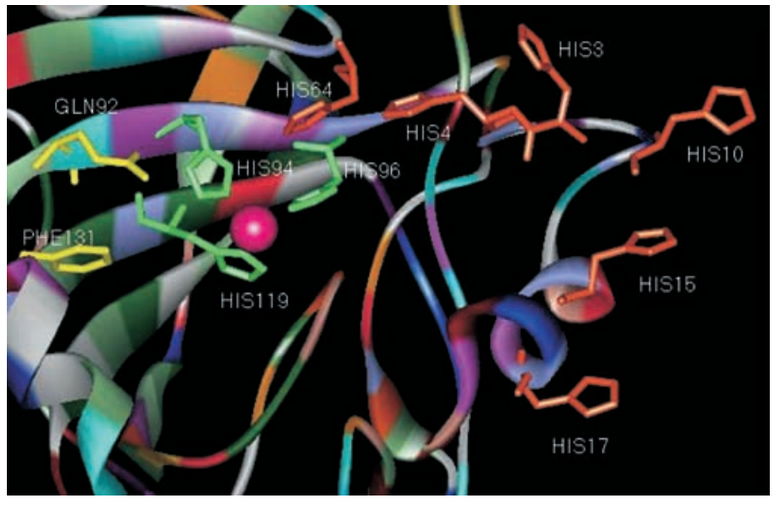


FIGURE 1 hCA II active site with the histidine cluster shown. This cluster comprises residues 64, 3 (not seen in this figure), 4, 17, 15 and 10, and assures a tight binding of inhibitors incorporating negatively-charged moieties. The zinc ion (central pink sphere) and its three histidine ligands (His 94, 96 and 119, in green) are also shown.

instance carboxylate anions, leading in this way to a supplementary stabilization of the enzyme-inhibitor adduct.[8] This is the reason why so many compounds of this type have been designed and prepared in this study.

The greatest majority of these new compounds contain one COO^- moiety (when in solution as sodium salts), but derivatives with two (**B45**) or four (**B47**) carboxylate moieties were also obtained. As will be discussed shortly, indeed, many such derivatives showed very good CA inhibitory properties.

In Vitro CA Inhibition

CA inhibition data against three isozymes, hCA I, hCA II and bCA IV (h = human; b = bovine isozyme) for the prepared compounds and standard inhibitors are shown in Table I, whereas some important physico-chemical properties for selected inhibitors are shown in Table II.

All the new compounds reported here showed better CA inhibitory properties when compared to the parent sulfonamide **B** from which they were obtained, this being a moderately weak inhibitor, similarly to many benzene sulfonamides reported in the literature.[1,2,27–30] Thus, the four derivatives of aromatic/heterocyclic amines **B7–B10** (which are the most ineffective CA inhibitors among the new compounds reported here) showed affinities of 30–43 nM against hCA II, 52–76 nM against bCA IV, and 88–126 nM against the slow isozyme hCA I. Increased inhibitory power was observed for the derivatives obtained from **B** and aminobenzoic acids **11–13** or amino acids **14–38**. Thus, the most active of such compounds were those incorporating β-phenyl-serine (**B20**), hydroxy-(Ser, Thr; **B19–B21**) and mercapto-(Cys, Met; **B22**, **B23**) amino acids, hydrophobic amino acids (Val, Leu, Ile; **B24–B26**), aromatic amino acids (His, Phe, Tyr, DOPA; **B32–B35**); or dicarboxylic (Asp, Glu, and their corresponding amides, Asn, Gln; **B27–B30**) amino acid moieties. Such compounds possessed inhibition constants in the range 1–10 nM against hCA II, 7–23 nM against bCA IV and 20–40 nM against hCA I (being more active than the classical inhibitors of type **1–6** mentioned above, which have been used as standards in these measurements, Table I). Slightly less active on the other hand were the derivatives of aminobenzoic acids (**B11–B13**), those of Gly, Ala, β-Ala and GABA (**B14–B17**), as well as those of Trp, Lys and Arg (**B36–B38**), with inhibition constants in the range 10–18 nM against hCA II, 23–56 nM against bCA IV and 38–103 nM against hCA I (but it must be mentioned that these compounds possess the same potency as acetazolamide or methazolamide, clinically used CA inhibitors). Among all the thioureas reported here, the most ineffective CA inhibitor was the proline derivative **B31**, probably due to its relatively bulky nature (this compound is anyhow a strong inhibitor, comparable with the clinically used dichlorophenamide **6**). Strong inhibitory properties were on the other hand observed for the oligopeptidyl derivatives **B39–B47**. Thus, except for the GlyGly (**B39**) and AspAsp (**B45**) derivatives, which possess the same activity as methazolamide **4** against hCA II, all the other oligopeptidyl thioureas prepared in the present study were very potent

TABLE I CA inhibition data with standard inhibitors **1–6**, 4-isothiocyanatobenzenesulfonamides **A**, **B**, and the new sulfonamides **B7–B47** reported in the present study

	K_I (nM)		
Inhibitor	hCA I[a]	hCA II[a]	bCA IV[b]
Dorzolamide **1**	50000	9	43
Acetazolamide **3**	900	12	220
Methazolamide **4**	780	14	240
Ethoxzolamide **5**	25	8	13
Dichlorophenamide **6**	1200	38	380
A	5000	185	300
B	4300	162	270
B7	124	43	74
B8	120	38	75
B9	126	39	76
B10	88	30	52
B11	54	11	23
B12	38	10	29
B13	47	9	21
B14	56	17	34
B15	48	10	29
B16	39	8	28
B17	40	10	27
B18	40	5	12
B19	23	2	9
B20	20	1	7
B21	24	2	13
B22	21	3	8
B23	19	3	10
B24	20	4	9
B25	17	3	11
B26	18	4	8
B27	27	7	15
B28	22	5	9
B29	39	10	23
B30	36	9	21
B31	89	21	55
B32	33	6	14
B33	27	5	14
B34	24	3	9
B35	30	6	14
B36	42	9	17
B37	73	10	34
B38	103	18	56
B39	50	13	26
B40	22	4	9
B41	12	1	5
B42	19	2	7
B43	21	3	10
B44	20	4	11
B45	37	12	25
B46	22	3	8
B47	51	9	24

[a]Human (cloned) isozymes; [b]From bovine lung microsomes, by the esterase method.

TABLE II Solubility, chloroform-buffer partition coefficients and in vitro corneal permeability of some classical and new sulfonamide CA inhibitors

Compound	Solubility[a] mM	Log P[b]	$k_{in} \times 10^3$ (hr^{-1})[c]	
			Cornea intact	No epithelium
Dorzolamide[d]	60	2.0[e]	3.0	5.2
Acetazolamide	3.2	0.001	0.37	7.0
Methazolamide	12	0.06	1.90	13
Ethoxzolamide	0.04	25	27	40
B11[e]	41	1.238	2.9	8.3
B12[e]	40	1.313	3.0	8.7
B13[e]	46	1.428	3.3	9.1
B18[e]	59	3.721	5.6	12.0
B19[e]	70	1.662	3.3	8.5
B20[e]	51	2.518	4.9	10.7
B22[e]	65	0.124	0.4	0.9
B40[e]	57	0.933	2.8	5.9

[a]Solubility in pH 7.40 buffer, at 25°C. [b]Chloroform-buffer partition coefficient. [c]Determined as described in ref. 28,29. [d]As hydrochloride, at pH 5.8. [e]As sodium salts, at pH 7.0.

inhibitors, with inhibition constants in the range 1–9 nM against hCA II, 5–25 nM against bCA IV and 12–51 nM against hCA I. Very good inhibitory properties were correlated with the presence of His moieties in such oligopeptidyl thioureas (e.g. **B40–B42**). It is also interesting to note the influence of the number of aspartyl residues in some of the thioureas prepared in this study, on the CA inhibitory properties of the obtained compounds. Thus, among the Asp, Asp–Asp and $(Asp)_4$ derivatives, best activity was observed for the simple monoderivative (**B27**), whereas the most ineffective inhibitor was the dipeptidyl derivative (**B45**; with its K_I against hCA II almost doubled as compared to **B27**). On the other hand, the tetrapeptide derivative **B47** regained much of the hCA II inhibitory activity, but showed a much lower affinity for bCA IV and hCA I. It is difficult to explain at the present moment this intricate behavior. From all these data, the main SAR conclusion is that the best inhibitors are obtained when the molecule of the thiourea is prolonged in the direction of the axis passing through the Zn(II) ion of the enzyme, the sulfonamide sulfur atom as well as the thiourea proximal nitrogen atom of the inhibitor (with three exceptions), as was in fact explained theoretically by QSAR studies from our group for other series of sulfonamide CA inhibitors[27,28] (the exceptions from this rule being the aspartyl derivatives mentioned above, **B27**, **B45** and **B47**). Among the three CA isozymes investigated here, the most susceptible to inhibition was hCA II, followed by bCA IV and then hCA I.

IOP Lowering in Normotensive Rabbits

In vivo IOP lowering experiments were done in normotensive rabbits, with many of the new compounds reported here, due to their strong CA II and CA IV inhibitory properties, such as **B7**, **B10–B13**, **B15**, **B18–B22**, **B25**, **B27**, **B28**, **B33**, **B37**, and **B40** (Table III).

The following may be noted regarding the data of Table III: (i) some of the new derivatives, such as **B19**, **B20** and **B28**, provoked a very strong and rapid decrease of IOP when applied topically, with IOP lowering of 6.3–8.6 mm Hg at 30 minutes after administration (compared with 2.2 mm Hg IOP lowering after dorzolamide), which at 1 hour amounted to 9.1–10.3 mm Hg (versus 4.1 mm Hg of dorzolamide) and this potent effect was maintained at 1.5 hours (7.4 −9.4 mm Hg). Furthermore, IOP returned to baseline values after 5–6 hours from administration (see Figure 2). Thus, such new

TABLE III Fall of IOP of normotensive rabbits (18.0 ± 2.5 mm Hg), after treatment with one drop (50 μL) 2% solution of CA inhibitor (as hydrochloride salt in the case of dorzolamide, **B7** and **B10**, and as sodium salts for the other derivatives, with the pH value shown below) directly into the eye, at 30, 60 and 90 minutes after administration

Inhibitor	pH	Δ IOP ± SE[a] (mm Hg)		
		30 min	60 min	90 min
Dorzolamide	5.5	2.2 ± 0.15	4.1 ± 0.15	2.7 ± 0.10
B7	6.5	1.2 ± 0.10	3.5 ± 0.20	0.9 ± 0.25
B10	6.5	3.2 ± 0.20	0.7 ± 0.15	0.0
B11	7.0	4.2 ± 0.15	8.0 ± 0.25	4.3 ± 0.10
B12	7.0	4.0 ± 0.10	8.1 ± 0.20	9.0 ± 0.30
B13	7.0	1.2 ± 0.15	7.0 ± 0.15	10.3 ± 0.20
B15	7.0	3.3 ± 0.10	5.2 ± 0.15	5.0 ± 0.20
B18	7.5	6.4 ± 0.30	2.5 ± 0.15	2.0 ± 0.10
B19	7.0	6.7 ± 0.15	9.1 ± 0.25	8.3 ± 0.30
B20	7.0	8.6 ± 0.20	10.3 ± 0.25	9.4 ± 0.15
B22	7.0	0.0	0.0	0.0
B25	7.0	5.4 ± 0.15	6.0 ± 0.20	6.8 ± 0.30
B27	7.0	1.7 ± 0.10	2.5 ± 0.20	2.3 ± 0.20
B28	7.0	6.3 ± 0.15	9.1 ± 0.30	7.4 ± 0.20
B33	7.0	4.3 ± 0.15	4.0 ± 0.30	2.9 ± 0.25
B37	7.0	0.0	0.0	0.0
B40	7.0	1.5 ± 0.15	3.0 ± 0.30	4.6 ± 0.20

[a]Δ IOP = $IOP_{control\ eye} - IOP_{treated\ eye}$ (n = 3).

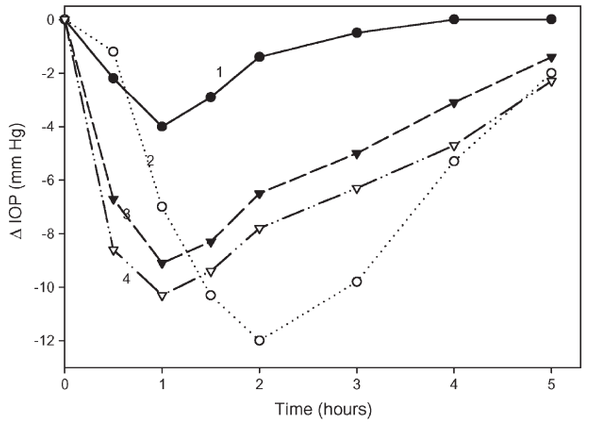


FIGURE 2 Effect of topically administered sulfonamide inhibitors (2% water solutions) on the IOP of normotensive albino rabbits. Curve 1: dorzolamide **1** (hydrochloride salt, pH 5.5); curve 2: compound **B13** (sodium salt, pH 7.0); curve 3: compound **B19** (sodium salt, pH 7.0);); curve 4: compound **B20** (sodium salt, pH 7.0).

inhibitors are much more potent hypotensor agents, and their effect is much longer, than that of the standard drug dorzolamide. It is interesting to note that two of these very effective IOP lowering agents mentioned above are the Ser and β-Ph-Ser derivatives, whereas the third one is the Asn derivative. More surprisingly, the very similar Asp derivative (**B27**) is a modest IOP hypotensor agent. This fact demonstrates that very small structural modifications (replacement of the $CONH_2$ moiety by COOH) leads to a completely different pharmacological profile for the two derivatives containing them, although their CA inhibitory properties are rather similar; (ii) compounds such as **B11**, **B12**, **B15**, **B18**, **B25** and **B33** showed an IOP lowering of 3.3–6.4 mm Hg at 30 minutes after administration (being more effective than dorzolamide), whereas at one hour their effect was in the range of 2.5–8.1 mm Hg, and at 90 minutes 2.0–9.0 mm Hg. Thus, although more effective than the standard drug at 30 minutes after administration, some of these agents showed a diminished effect at later times, probably due to their being washed away from the ciliary processes. This may be due to an improper balance between lipo- and hydrophilicity for some of these compounds, as will be discussed shortly; (iii) derivatives such as **B7, B10, B22, B27, B37** and **B40** showed a modest effect in the decrease of IOP after administration, with IOP lowering of 0.0–3.2 mm Hg after 30 minutes, 0.0–3.5 mm Hg after 60 minutes, and 0.0–4.6 mm Hg after 90 minutes; (iv) compound such **B13** showed a modest reduction of IOP after 30 minutes, but was very effective at one hour and longer times post administration (Table III and Figure 2).

At this point it is important to return to the correlation between the physico-chemical properties of the new CA inhibitors reported here (Table II), and their in vivo activity as topical hypotensor agents. One of the most interesting observations in this work is that some of the newly obtained compounds, although acting as very potent hCA II (and bCA IV) inhibitors, showed an unexpectedly small IOP lowering effect via the topical route, whereas structurally very similar derivatives acted as strong hypotensors (compare **B19** and **B22**, which differ minimally, by the presence of an OH moiety and an SH moiety, respectively; or the example mentioned above, the Asp and Asn derivatives, (**B27** and **B28**). Maren[31] has already noted that the most restrictive conditions needed for a sulfonamide to act as an effective IOP lowering agent is to possess a modest (but not insignificant) lipid solubility (attributable to its unionized form), accompanied by a good water solubility (eventually conferred by the presence of ionizable groups of appropriate pK_a). As seen from the data of Table II, the very active compounds reported here to act as effective topical hypotensors, such as **B13**, **B19**, **B20**, possessed just this type of balanced physico-chemical properties, which lead to good accession rates across the cornea, followed by sustained inhibition of the ciliary processes enzyme. In contrast, the Cys (**B22**) derivative mentioned above, although an effective CA inhibitor, possessing a very good water solubility, exhibits a too low hydrophobicity (due to the presence of the ionizable SH moiety) and in consequence an impaired penetration through the cornea (observe the low k_{in} values of Table II). The same seems to be true for other derivatives investigated here, such as for instance the Lys or oligopeptidyl derivatives (**B37** or **B40**), which again showed relatively modest IOP lowering, although they were extremely efficient in vitro CA inhibitors. It must also be mentioned that a very hydrophobic inhibitor (such as ethoxzolamide **6**) is also ineffective topically, although it possesses very high accession rates through the cornea.[31] Still, this property also allows its rapid diffusibility into red blood cells, and in consequence its washing away from the ciliary processes due to the blood circulation.[31] This might explain why some of the compounds reported here (such as the α-Ph-Gly derivative **B18**) initially showed a potent IOP lowering (at 30 minutes after administration), whereas this effect rapidly diminished at later periods after administration. It may be observed that **B18** was the most hydrophobic among the inhibitors investigated in some detail in this study (Table II), and this probably leads not only to very good accession rates across the cornea but also to its rapid clearing from the ciliary processes, similarly to ethoxzolamide **6** mentioned above.

TABLE IV Ocular tissue concentrations (μM) after one and two hours, following corneal application of one drop (50 μL) of 2% solution of inhibitors **B13** and **B20** in albino rabbits (with dorzolamide hydrochloride **1** as standard)

		Drug concentration (μM)*	
Time (h)	Cornea	Aqueous humor	Ciliary process
1 (HCl)			
1 h	105 ± 5	32 ± 3	15 ± 3
2 h	39 ± 4	21 ± 2	6 ± 1
B13			
1 h	123 ± 9	204 ± 13	50 ± 7
2 h	76 ± 5	62 ± 4	23 ± 2
B20			
1 h	133 ± 10	205 ± 12	49 ± 5
2 h	70 ± 9	53 ± 5	15 ± 1

*Mean ± standard deviation (n = 3).

Thus, this series of CA inhibitors proves in a clear-cut manner that in order to obtain hypotensors with prolonged duration of action via the topical route, the physico-chemical and enzyme inhibitory properties must be fine-tuned in a very precise manner, and that such compounds should possess the right balance between lipo- and hydrophobicity, in addition to acting as potent hCA II inhibitors and possessing an acceptable water solubility.

Drug Distribution in Ocular Fluids and Tissues

In Table IV the drug distribution in ocular fluids and tissues is shown, after the topical administration of compounds **B13** and **B20**. It is seen that one and two hours after topical administration of the drug, high levels of inhibitors were found in the cornea, aqueous humor and ciliary processes. This is due to the good accession rates of these sulfonamides through the cornea, as measured both for the intact eye (cornea) as well as for the eyes with no epithelium, as described in the literature.[23] Based on the inhibition constant of these compounds, the fractional inhibition estimated in these tissues/fluids is of 99.5–99.9%,[2] proving the fact that the IOP decrease is indeed due to CA inhibition. Furthermore, as seen from the data of Table IV, the new compounds reported here, tend to concentrate in the aqueous humor (concentrations of around 204 −205 μM were detected one hour after administration), whereas dorzolamide reaches much lower concentrations (32 μM after one hour). High concentrations of the inhibitor were also maintained at 2 hours after administration. Thus, the strong and long lasting IOP lowering properties of the new compounds reported here are probably due to this concentrating effect reached mainly in the aqueous humor, but which is also present in the cornea and ciliary processes.

Conclusions

We report here a novel class of sulfonamides, obtained by reaction of 4-isothiocyanatobenzenesulfonamide with amines, amino acids and oligopeptides. Many of the newly reported derivatives showed very good water solubility, at nearly neutral pH values, whereas their lipid solubility, hydrophobicity (Log P) as well as accession rates across the cornea were those appropriate for acting as efficient topical IOP lowering agents. Some of these new CA inhibitors possessed affinities in the nanomolar range for isozymes hCA II and bCA IV, acting as effective enzyme inhibitors in vitro. Several of the most active inhibitors strongly lowered IOP pressure in normotensive and glaucomatous rabbits, showing a prolonged duration of action as compared to dorzolamide. The new compounds reported here might lead to the development of more efficient antiglaucoma drugs from the class of the sulfonamide CA inhibitors.

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