Carbonic Anhydrase Inhibitors: Synthesis and Topical Intraocular Pressure Lowering Effects of Fluorine-Containing Inhibitors Devoid of Enhanced Reactivity

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Polyfluorinated carbonic anhydrase inhibitors (CAIs) show very good inhibitory properties against carbonic anhydrase (CA) and excellent in vivo antiglaucoma properties after topical administration in rabbits. Still, the pentafluorinated compounds reported previously by this group (Scozzafava et al. *J. Med. Chem.* **2000**, *43*, 4542−4551) showed high reactivity with thiol groups of cysteine, glutathione, and presumably other proteins containing such moieties, which may lead to severe ocular side effects. Here, we report an approach for obtaining fluorinated CA inhibitors without the undesired enhanced reactivity. Thus, new compounds have been obtained by attaching moieties with reduced reactivity toward aromatic substitution reactions to the molecules of aromatic/heterocyclic sulfonamides possessing derivatizable amino moieties. The employed tails of the 2,3,5,6-tetrafluorobenzoyl, 2,3,5,6-tetrafluophenylsulfonyl, and pentafluorophenylureido types induced excellent CA inhibitory properties in the new reported sulfonamides, mainly against the isozymes involved in aqueous humor secretion, CA II and CA IV, whereas affinity for CA I was lower. Several low-nanomolar CA II inhibitors were detected, which did not react with cysteine or glutathione, in contrast to the corresponding perfluorinated compounds previously reported. These derivatives also showed a potent reduction of the intraocular pressure (IOP) in hypertensive rabbits, amounting to 13−21 mmHg at 1 h postadministration (compared to 5 mmHg obtained with dorzolamide, a clinically used drug), and the decreased IOP was maintained for 4−5 h after the administration. These compounds constitute valuable candidates for obtaining topically acting antiglaucoma CA inhibitors of a new generation, with enhanced efficacy, prolonged duration of action, and reduced side effects.

Introduction

Although the treatment of glaucoma with inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) is very effective in reducing elevated intraocular pressure (IOP), the systemic administration of drugs such as acetazolamide AAZ, methazolamide MZA, ethoxzolamide EZA, and dichlorophenamide DCP leads to a wide range of unpleasant side effects due to inhibition of the enzyme present in tissues (kidneys, red cells, stomach, etc.) other than the eye.[1−4] The possibility of the topical administration of the classical drugs from this class mentioned above (acetazolamide, methazolamide, ethoxzolamide, or dichlorophenamide) was extensively investigated by several researchers, but negative results have been constantly obtained, and for more than 40 years it was considered that CA inhibitors (CAIs) could only be given systemically.[1] Important advances in this field have then been achieved by the Merck group, which discovered the first clinically used, topically effective antiglaucoma sulfonamide, dorzolamide DZA.[5] The approach for arriving at this compound (and in fact also for brinzolamide BRZ, the second such clinically used pharmacological agent)[6] is known as the "ring approach" because it involved the exploration of a wide range of ring systems to which sulfamoyl moieties were incorporated.[2−4] We have explored an alternative approach for the design of topically acting antiglaucoma sulfonamides that consists of attaching tails that will induce the desired physicochemical properties (such as water solubility, enhanced penetrability through the cornea, etc.) to scaffolds of aromatic/heterocyclic sulfonamides also incorporating derivatizable moieties of the amino/hydroxy type.[2−4,7−10]

Many tails have been used for the design of topically acting sulfonamide CAI antiglaucoma agents, such as pyridinecarboxamido, quinolinesulfonylamido, polyaminopolycarboxamido, perfluoroalkyl/arylcarboxamido/sulfonamido, aminoacyl, or oligopeptidyl among others,[2−4,7−10] but those leading to the most impressive biological activity were the fluorine-containing ones.[10] The constant search of novel topically acting antiglau-

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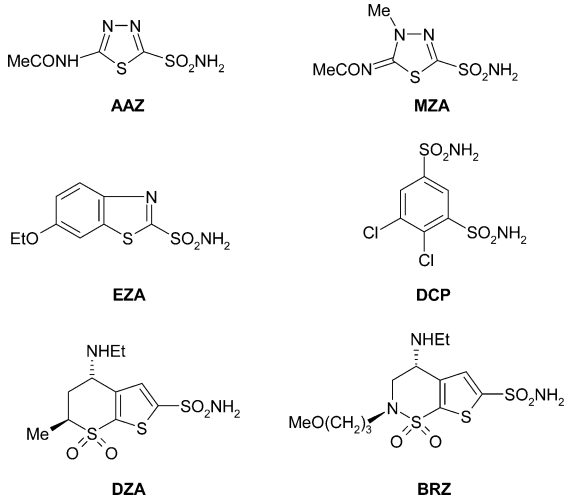


coma sulfonamides is motivated by the rather wide range of side effects associated with the two clinically used drugs, dorzolamide and brinzolamide, which include local irritation and eye reddening, pruritus, blurred vision, nephrolithiasis, anorexia, dementia, corneal decompensation, etc.[11]

In a previous contribution from our laboratory,[10] it was shown that by attaching perfluoroalkyl/arylcarboxamido/sulfonamido tails, such as perfluorobutylsulfonyl, perfluorooctylcarboxamido, perfluorophenylcarboxamido, or perfluorophenylsulfonyl, to the aromatic/heterocyclic sulfonamides also incorporating derivatizable amino moieties, of types **1**−**15**, very effective CAIs can be obtained, which also showed good water solubility and efficacy as topically acting antiglaucoma agents in an animal model of the disease. The best CAIs and antiglaucoma agents in that series of derivatives were those incorporating the perfluorinated aromatic moieties (such as C_6F_5CO and $C_6F_5SO_2$), a fact that was then explained after the report of the high-resolution X-ray crystal structure of isozyme CA II with one of these agents, the perfluorobenzoylated analogue of methazolamide, PFMZ (Figure 1).[12]

PFMZ is almost 10 times more effective a CA II inhibitor (K_I = 1.5 nM) compared to methazolamide (K_I = 14 nM). Its binding to the enzyme active site was shown to be similar to that of other sulfonamide inhibitors,[13,14] considering the interactions of the sulfonamide zinc anchoring group and thiadiazoline ring contacts, but differs considerably when the perfluorobenzoylimino fragment of the molecule has been analyzed. Thus, several unprecedented strong hydrogen bonds involving the imino nitrogen, carbonyl oxygen, a meta fluorine atom belonging to the inhibitor, and two water molecules, as well as Gln 92 of the enzyme active site, were evidenced. A stacking interaction of the perfluorophenyl ring of the inhibitor and the aromatic ring of Phe 131 was also observed for the first time in a CA−sulfonamide adduct (Figure 1).[12] All these findings proved that the pentafluorophenyl tail of such CAIs is indeed quite beneficial for obtaining very potent, topically effective CAIs, but an undesired property of such compounds was then discovered, related to the very electrophilic character of the carbon atom bearing the fluorine atom in the position para to the carboxamido/sulfonamido moiety.[15] Indeed, Medina et al.[16,17] reported a series of perfluorophenylsulfonamide derivatives of types **16**−**21** (among which is T138067, compound **21** in phase II clinical trials for the treatment of human cancers with MDR+ phenotype), which strongly inhibit the growth of a variety of tumors, including multidrug-

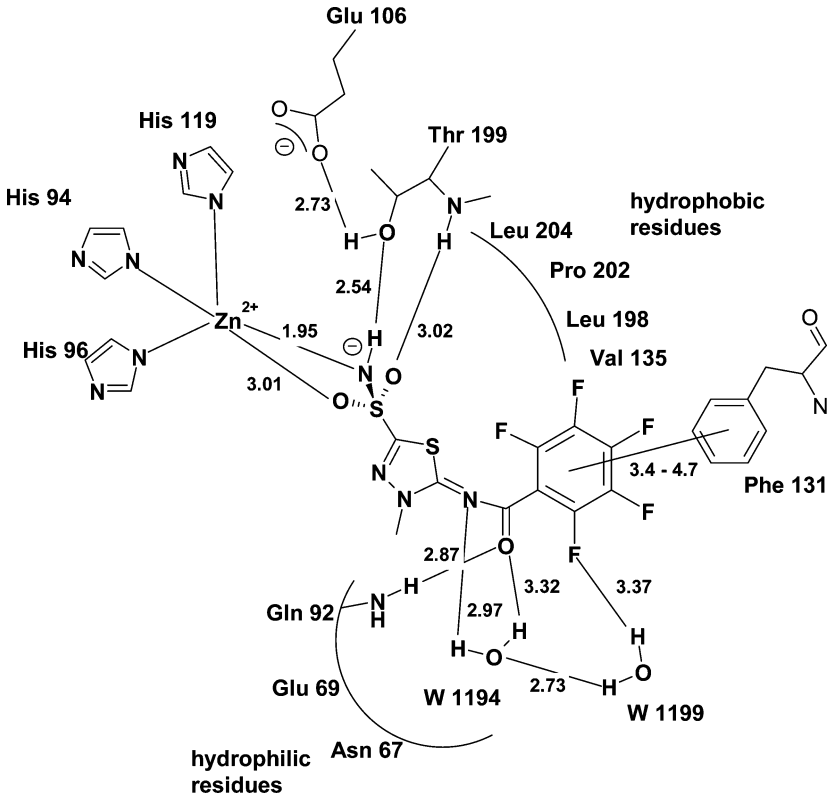
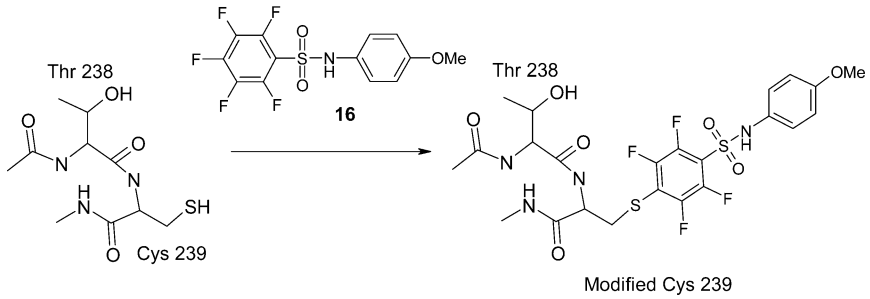
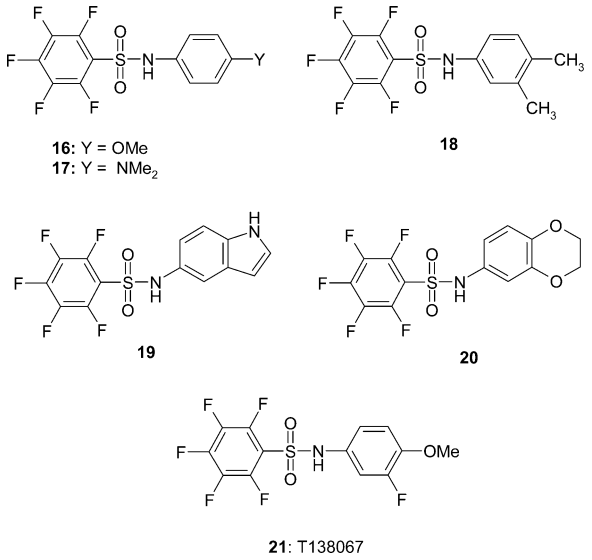


Figure 1. Schematic representation of PFMZ binding to the hCA II active site (distances in Å). Reprinted with permission from ref 12. Copyright 2003 Taylor and Francis. see also http://www.tandf.co.uk.

Scheme 1



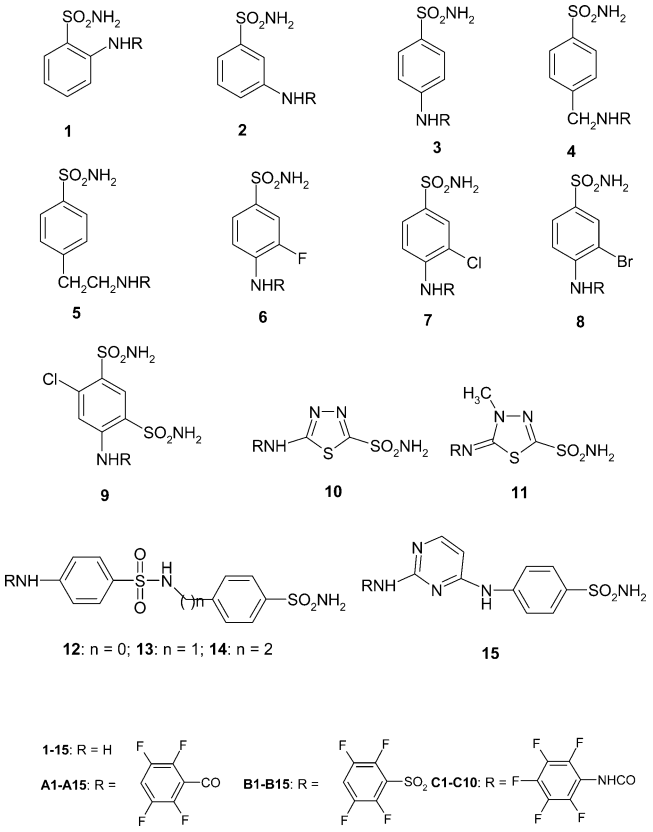
resistant ones. Furthermore, the corresponding derivatives possessing other halogens instead of the five fluorine atoms of **16**−**21**, together with the corresponding isomeric trifluoro- and tetrafluorosulfonamides, were much less active than the lead compound **16**.[16,17] This



was subsequently shown to be due to the mechanism of action of these compounds (Scheme 1), which covalently bind to Cys 239 of β-tubulin, by means of a nucleophilic aromatic substitution reaction in which the most labile fluorine atom of the antimitotic agent (i.e., the fluorine in the position para to the sulfonamide moiety) reacts with the thiol moiety of the Cys 239 residue of β-tubulin, leading to modification of the protein.[18]

The enhanced lability of the *p*-fluorine group is desired in antitumor agents of type **16**−**21** mentioned above[19,20] but is detrimental for topically acting CAIs because of the fact that the eye fluids are very rich in glutathione.[21] In fact, many topically acting sulfonamide CAIs could not be developed clinically just because of the fact that their sulfonamido group was easily displaced by reduced glutathione with formation of a glutathione conjugate,[21] which proved to be a potent allergen, leading to eye irritations in experimental animals.[4,22] To avoid such problems, we disclose a new class of potent, topically acting antiglaucoma CAIs which, although possessing the favorable properties associated with the perfluorophenyl-containing compounds previously described,[10] are devoid of enhanced sensitivity to nucleophilic substitution and thus potential side effects due to reaction with glutathione or cysteine-containing proteins.

Chart 1



Results

Synthesis. The new derivatives **A1**−**A8**, **A10**−**A14**, **B3**−**B7**, **B10**, **B15**, and **C1**−**C10** described here were prepared as previously described for related derivatives, from the aminosulfonamides **1**−**15** and 2,3,5,6-tetrafluorobenzoyl chloride, 2,3,5,6-tetrafluorobenzenesulfonyl chloride, or pentafluorophenyl isocyanate, respectively (Chart 1).[10,23−25]

Carbonic Anhydrase Inhibitory Activity. The new sulfonamides reported here were assayed for inhibition of three CA isozymes, two of them known to play a critical role in aqueous humor formation (CA II and CA IV), whereas the other one, CA I, is known to be important for the possible systemic side effects of such drugs (Table 1).[4]

Reaction of Fluorinated Sulfonamides with Thiol Reagents. Reactivity of some of the newly synthesized CAIs as well as other types of fluorinated sulfonamides with thiol reagents (cysteine and glutathione) is shown in Table 2.

IOP Measurements. In vivo IOP lowering data with some of the most active CAIs reported here, in hyper-

Table 1. CA Inhibition Data with Standard Inhibitors and the New Sulfonamides Reported in the Present Study

	K_I [a] (nM)		
inhibitor	hCA I[b]	hCA II[b]	bCA IV[c]
acetazolamide	900 ± 42	12 ± 0.7	220 ± 16
methazolamide	780 ± 30	14 ± 1.5	240 ± 13
ethoxzolamide	25 ± 1.8	8 ± 0.3	13 ± 0.7
dichlorophenamide	1200 ± 63	38 ± 3	380 ± 24
dorzolamide	50000 ± 100	9 ± 0.2	43 ± 3
A1	1500 ± 39	38 ± 3	690 ± 40
A2	1700 ± 62	21 ± 1.5	430 ± 13
A3	975 ± 18	16 ± 0.5	400 ± 27
A4	980 ± 54	13 ± 0.6	120 ± 5
A5	900 ± 68	12 ± 0.7	94 ± 7
A6	760 ± 30	1.5 ± 0.1	31 ± 2
A7	1080 ± 47	76 ± 5	42 ± 3
A8	1870 ± 80	84 ± 6	50 ± 4
A10	250 ± 13	2.1 ± 0.2	24 ± 3
A11	270 ± 15	1.4 ± 0.1	28 ± 2
A12	6300 ± 36	19 ± 2	63 ± 4
A13	6100 ± 33	15 ± 1	54 ± 4
A14	6000 ± 38	13 ± 1	49 ± 5
B3	1300 ± 65	25 ± 2	330 ± 16
B4	860 ± 47	12 ± 1	135 ± 8
B5	750 ± 40	9 ± 0.4	76 ± 6
B6	580 ± 39	3.8 ± 0.3	42 ± 3
B7	930 ± 62	61 ± 5	49 ± 2
B10	13 ± 1	0.7 ± 0.1	15 ± 1
B15	320 ± 18	13 ± 1	21 ± 1
C1	1750 ± 90	44 ± 3	485 ± 26
C2	1800 ± 74	30 ± 2	430 ± 35
C3	950 ± 53	8 ± 0.4	21 ± 2
C4	1000 ± 76	15 ± 0.5	50 ± 3
C5	940 ± 41	15 ± 1	41 ± 4
C6	760 ± 33	18 ± 2	63 ± 5
C7	1300 ± 76	80 ± 5	245 ± 13
C8	1960 ± 150	95 ± 6	360 ± 22
C9	740 ± 40	16 ± 1	58 ± 4
C10	270 ± 18	3.6 ± 0.4	45 ± 2

[a] Mean ± standard error (from three different assays). [b] Human (cloned) isozymes [c] Bovine lung isozyme, by the esterase method.[27]

Table 2. Reactivity against Thiol Reagents (Cysteine (Cys) and Glutathione (Glt)) of Fluorinated Sulfonamide Derivatives[a]

	% SH modification[b]	
compd	Cys	Glt
A6	0	0
B10	0	0
C3	0	0
27	55	49
28	76	68
16	98	96

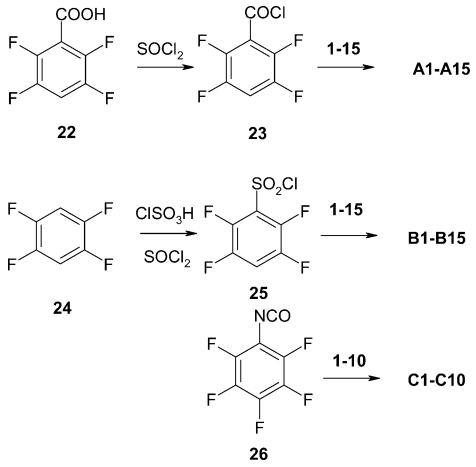
[a] The percent in the table means the amount of cysteine or glutathione that reacted with the fluorinated sulfonamide investigated in the paper. [b] After 24 h of incubation in a 1:1 molar ratio of the reagents in phosphate buffer (see Experimental Protocols). Errors were in the range 2−5% of the reported values, from three different determinations.[28]

tensive rabbits (a widely used animal model of glaucoma)[26] after topical administration of the drug, are shown in Figure 1.

Discussion

Chemistry. To prepare fluorine-containing CAIs devoid of enhanced sensitivity to nucleophilic substitution, two approaches have been used: (i) design of derivatives lacking the *p*-fluorine reactive group, with two types of such derivatives being prepared (the 2,3,5,6-tetrafluorophenylcarboxamides **A1**−**A8** and **A10**−**A14**; the 2,3,5,6-tetrafluorophenylsulfonamides **B3**−**B7**, **B10**, and **B15**); (ii) replacement of the carboxamido/sulfonamido linker between the perfluorinated aryl tail and the aromatic/heterocyclic ring bearing the sulfamoyl zinc anchoring group[10] by a new linker that should activate less the fluorine atom in the para position. We opted for the ureido linker because of the ease of preparing such compounds and because of the fact that we have previously shown that these types of CAI show high potency against the isozymes of interest for the secretion of aqueous humor within the eye[25] (Scheme 2). Furthermore, the ureido moiety is much less activating for aromatic nucleophilic substitution reactions compared to the carboxamido or sulfonamido moieties.[15]

Scheme 2

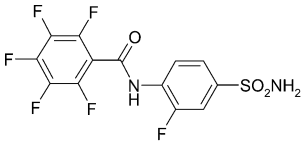


The carboxamides **A1**−**A8** and **A10**−**A14** were easily prepared from the commercially available 2,3,5,6-tetrafluorobenzoic acid **22**, which has been converted to the corresponding acyl chloride **23** by treatment with thionyl chloride, followed by reaction with the aminosulfonamides **1**−**8** and **10**−**14**, in Schotten−Baumann conditions, as previously reported.[10,23] For the preparation of the sulfonamides **B3**−**B7**, **B10**, and **B15**, 1,2,4,5-tetrafluorobenzene **24** was transformed to the monosulfonyl chloride by treatment with chlorosulfonic acid and thionyl chloride at 150 °C, followed by reaction with the aminosulfonamides **3**−**7**, **10**, and **15**, in Schotten−Baumann conditions as previously reported.[10,23,24] The pentafluorophenyl ureas **C1**−**C10** were easily obtained from the commercially available isocyanate **26** and the aminosulfonamides **1**−**10**, as previously reported.[25]

In Vitro CA Inhibition. CA inhibition data against the 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 1. All the new compounds reported here act as better CAIs than the parent sulfonamides from which they were obtained (data not shown), showing a biological activity quite similar to that of the corresponding perfluorinated derivatives bearing the same carboxamido/sulfonamido substitution pattern, as previously reported[10] (data not shown). Thus, the following SAR can be drawn from the data of Table 1. (i) Considering the three tails **A** (tetrafluorobenzoyl), **B** (tetrafluorophenylsulfonyl), and **C** (pentafluorophenylureido), generally derivatives of type **B** were more inhibitory than the corresponding derivatives of type **A**, which in turn were more inhibitory than the corresponding ureas of type **C** (but several exceptions were detected such as the carboxamide **A6**,

which is more inhibitory than the corresponding sulfonamide **B6** against isozymes I and II, among others). (ii) Considering the parent sulfonamides that were derivatized by the three tails mentioned above, the heterocyclic derivatives (such as the thiadiazoles and thiadiazolines **10**, **11**) led to more potent inhibitors compared to the benzenic derivatives. Among these last benzenic derivatives, the best inhibitors were the sulfonylated sulfanilamide/homosulfanilamide/*p*-aminoethylbenzenesulfonamide of types **12**−**14** (because of their long molecules, a fact explained in QSAR[24] and X-ray[14b] crystallographic studies) and the fluorosulfanilamide derivatives **A6** and **B6**, whereas lower activity was associated with the presence of the orthanilamide, metanilamide, or chlorinated/brominated sulfanilamides **1**, **2**, **7**, and **8** (the derivatives of these sulfonamides appreciably inhibited these enzymes, with potencies frequently comparable to that of dichlorophenamide, a clinically used drug). (iii) Many of the new CAIs reported here, such as **A6**, **A10**, **A11**, **A14**, **B5**, **B6**, **B10**, **B15**, **C3**, and **C10** among others, showed much better CA inhibitory properties compared to the clinically used drugs acetazolamide, methazolamide, dichlorophenamide, or dorzolamide. (iv) Isozyme hCA II was the most susceptible to inhibition by the new compounds reported here, generally followed by isozyme IV, whereas hCA I was the less susceptible. This is a general behavior of sulfonamides toward these three isozymes and is frequently observed for other types of investigated sulfonamides[2−4] (Table 1).

Reaction of Fluorinated Sulfonamides with Thiol Reagents. To validate the approach we designed for obtaining CAIs devoid of undesired nucleophilicity, the reactions of several new derivatives, the corresponding pentafluorophenylsulfonamides previously reported **27** and **28**,[10] and the standard compound **16**,[16] with thiol reagents (cysteine and glutathione) were investigated (Table 2).[28]



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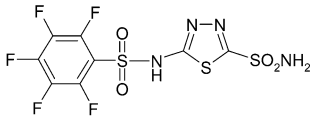

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As seen from the data of Table 2, the three new compounds reported here and investigated for their reactivity with thiol reagents, **A6**, **B10**, and **C3**, were totally unreactive, whereas the corresponding perfluorinated CAIs **27** and **28** appreciably reacted with cysteine as well as with glutathione after 24 h of incubation. In the same conditions, the standard drug, the antimitotic sulfonamide **16**, completely reacted with the two reagents. This clearly demonstrates that the approach we propose here is a valid one for obtaining fluorinated CAIs devoid of enhanced sensitivity toward nucleophilic substitution.

IOP Measurements. Some of the best in vitro CAIs detected here, such as **A6**, **B10**, and **C3**, as well as dorzolamide as standard drug, have been investigated in vivo in hypertensive rabbits, a widely used animal model of glaucoma, for their effects on intraocular pressure (IOP) after topical administration as 2% water solutions (Figure 2).[10,26]

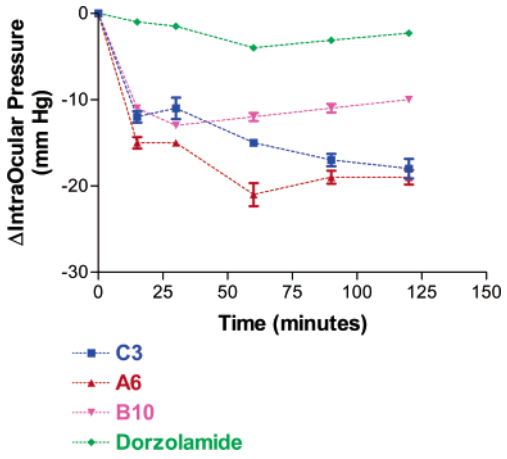


Figure 2. IOP lowering in hypertensive rabbits (initial pressure in the range of 34 ± 1 mmHg) after topical treatment with one drop (50 μL) of 2% solution of the CAIs dorzolamide, **A6**, **B10**, and **C3** (mean ± standard error, from three different determinations).

As seen from the data of Figure 2, the three new CAIs investigated in detail showed a potent and prolonged reduction of IOP in hypertensive rabbits. Thus, dorzolamide (as hydrochloride salt), the standard drug, induces a modest decrease of IOP after topical administration as a 2% water solution (pH 5.5), with the peak at 1 h postadministration of around 5 mmHg. The effect of this drug vanishes rapidly as the pressure returns to basal levels after 2−2.5 h. The fluorinated sulfonamides investigated here induced a very sharp decrease of IOP, with reductions of 12−15 mmHg after 15 min postadministration, with the maximal decrease of IOP generally also reaching the peak after 1 h postadministration (the pH of these solutions were in the range 6.5−7.0, being much less irritant than the dorzolamide hydrochloride solution). The maximal IOP reduction was much larger compared to that of the standard drug (dorzolamide), amounting to 21 mmHg for the best compound (**A6**) and 13−15 mmHg for the other two derivatives (**B10** and **C3**) after 1 h. It is interesting to note that the urea **C3** showed its maximal activity not at 1 h but at 2 h postadministration, when it reached a decrease of IOP of 17.5 mmHg, maintaining reduced IOP for another 2 h (data not shown). In fact, all these CAIs showed a much more prolonged duration of action compared to dorzolamide, of at least 4−5 h (data not shown), similar to the pentafluorophenyl derivatives previously reported.[10] No signs of eye irritations have been observed, even after prolonged administration of the eye drops containing the new derivatives mentioned above to the hypertensive rabbits used in these experiments. Thus, this significant reduction of IOP, correlating with the good water solubility (in neutral form, leading to nonirritating solutions with the pH in the neutral range), and the lack of enhanced reactivity toward thiol groups make these derivatives valuable candidates for the development of the next generation of topically acting antiglaucoma sulfonamides with enhanced activity and reduced side effects.

Conclusions

Polyfluorinated CAIs have been obtained by attaching fluorinated moieties with reduced sensitivity toward nucleophilic substitution reactions to the molecules of

aromatic/heterocyclic sulfonamides possessing derivatizable amino moieties. The employed tails of the 2,3,5,6-tetrafluorobenzoyl, 2,3,5,6-tetrafluophenylsulfonyl, and pentafluorophenylureido types induced excellent CA inhibitory properties in the new sulfonamides reported in the paper, mainly against the isozymes involved in aqueous humor secretion, CA II, and CA IV, whereas the affinity for CA I was lower. Several water-soluble, low-nanomolar CA II inhibitors were detected, which did not react with cysteine or glutathione, in contrast to the corresponding perfluorinated compounds previously reported. These derivatives also showed a potent reduction of IOP in hypertensive rabbits, amounting to 13−21 mmHg at 1 h postadministration (compared to 5 mmHg obtained with dorzolamide), and the decreased IOP was maintained for 4−5 h without signs of eye irritation. These compounds constitute valuable candidates for obtaining topically acting antiglaucoma CAIs of a new generation, with enhanced efficacy, prolonged duration of action, and reduced side effects.

Experimental Protocols

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 Hewlett-Packard instrument. Sulfonamides **1**−**15** used in the synthesis were commercially available compounds (from Sigma-Aldrich or Acros) or were prepared as described previously: halogenosulfanilamides **6**−**8** by halogenation of sulfanilamide as reported in the literature;[29] compound **10** from acetazolamide by deacetylation;[30] imine **11** by deprotection of methazolamide with concentrated hydrochloric acid.[31] The sulfonamide derivatives **11**−**14** were prepared as described in ref 32. Perfluorosulfonyl halides, perfluoroacyl halides, 2,3,5,6-tetrafluorobenzoic acid, 1,2,4,5-tetrafluorobenzene, chlorosulfonic acid, thionyl chloride, cysteine, glutatione, cystine, and oxidized glutathione, as well as inorganic reagents were from Sigma-Aldrich (Milan, Italy). Compound **16** was prepared as described in ref 16 from 4-methoxyaniline and pentafluorophenylsulfonyl chloride. Acetonitrile, *N*,*N*-dimethylacetamide (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.

Synthesis of 2,3,5,6-Tetrafluorobenzoyl Chloride. An amount of 2 equiv of tetrafluorobenzoic acid was treated with 5 equiv of thionyl chloride in 50 mL of anhydrous benzene. The reaction mixture was heated at reflux for 5 h, then the solvent and excess thionyl chloride were removed in vacuo, and the acyl chloride was distilled under reduced pressure.

Synthesis of 2,3,5,6-Tetrafluorobenzenesulfonyl Chloride. Chlorosulfonic acid (97 g, 55.36 mL) was added to 1,2,4,5-tetrafluorobenzene (25 g), and the obtained solution was heated at 150 °C. After 2 h, the heating was stopped in order to allow the solution to reach room temperature and thionyl chloride (39.96 g, 24.30 mL) was poured to the solution. The resulting mixture was reheated for 3 h at 150 °C. The brown solution obtained was then poured dropwise with stirring into a mixture of 250 g of ice and 100 mL of water. The obtained suspension was extracted three times with 50 mL of ethyl acetate. The organic fractions were collected and dried over anhydrous sodium sulfate and then concentrated by solvent evaporation under vacuum. The obtained/resulting brown oil was collected and stored at −20 °C. The purity of the final compound was verified by TLC (MeOH/$CHCl_3$, 3/7).

General Procedure for the Preparation of the Fluorinated Sulfonamides. Method A (Schotten−Baumann Synthesis). An amount of 5 mmol of sulfonamide **1**−**15** (such as 5-amino-1,3,4-thiadiazole-2-sulfonamide **10** or 5-imino-4-methyl-2-sulfonamido-δ^2-1,3,4-thiadiazoline **11**) was dissolved in 15 mL of an aqueous 2.5 M solution of NaOH and cooled to 2−5 °C in a salt/ice bath. An amount of 5 mmol of sulfonyl/acyl chloride **23** and **25** were added in small portions concomitantly with 10 mL of a 2 M NaOH solution, maintaining the temperature under 10 °C. The reaction mixture was then stirred at room temperature for 5−10 h (TLC control), then the pH was adjusted to 2 with 5 N HCl, and the precipitated sulfonamides were filtered and recrystallized from aqueous ethanol.

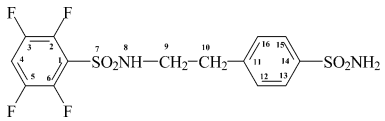
Method B. The aminosulfonamide **1**−**15** (1 g) was dissolved in *N*,*N*-dimethylacetamide (10 mL), and 2 equiv of 2,3,5,6-tetrafluorobenzenesulfonyl chloride **25** and 1 equiv of sodium bicarbonate were added to the solution, which was left on ice under stirring. After 60 min of stirring, water (50 mL) was added to the solution, which was then extracted three times with ethyl acetate (25 mL). The organic fractions were collected and extracted three times with a 1 N hydrochloric acid aqueous solution (15 mL). The organic phase was dried over magnesium sulfate. After charcoal treatment, the organic phase was dried under vacuum. The final compounds were recrystallized as mentioned above. The purity of the final compound was verified by TLC (MeOH/$CHCl_3$, 3/7).

Method C. The sulfonamide to be derivatized was suspended/dissolved in anhydrous acetonitrile (50 mL), and an amount of 2 equiv of pentafluorophenyl isocyanate **26** was added. The obtained mixture was stirred at room temperature for 60 min. The solution was dried under vacuum, and the obtained solid was recrystallized from aqueous ethanol. Purity of the final compound was verified by TLC (MeOH/$CHCl_3$, 3/7).

All compounds were extensively characterized. An example from each subclass is shown below.

4-Methyl-5-[2,3,5,6-tetrafluorophenylcarboximido]-δ^2-1,3,4-thiadiazoline-2-sulfonamide A11: white crystals, mp 202−3 °C; IR (KBr), cm^{-1}, 1148 (SO_2^{sym}), 1373 (SO_2^{as}), 1554 (amide II N−H), 1613 (amide I C═O), 3060 (NH), 3365 (NH_2 or N−H); ^{1}H NMR (DMSO-d_6), δ, ppm, 9.10 (br s, 1H, SO_2-NH), 8.13 (tt, H-4, 7.5, 10.3), 7.14 (br s, 2H, SO_2NH_2), 3.54 (s, 3H, Me); ^{13}C NMR (DMSO-d_6), δ, ppm, 31.5 (Me), 120.7, 124.3, 135.9, 164.91 (C-thiadiazoline), 164.94 (C-thiadiazoline); ^{19}F NMR (DMSO-d_6), δ, ppm, −137.8 (2,6-F_2), −158.2 (3,5-F_2). Anal. ($C_{11}H_7F_4N_3O_3S_2$) C, H, N.

4-[2,3,5,6-Tetrafluorobenzenesulfonylamidoethyl]-benzenesulfonamide B5: colorless crystals, mp 187−8 °C; IR (KBr) cm^{-1}, 1154 and 1163 (SO_2^{sym}), 1350 and 1378 (SO_2^{as}), 3365 (NH, NH_2).



^{1}H NMR (DMSO-d_6, δ ppm, J Hz), 8.79 (t, H-8, 5.5), 8.13 (tt, H-4, 7.5, 10.3), 7.69 (d, H-13−15, 8.4), 7.38 (d, H-12−16, 8.4), 7.30 (s, NH_2), 3.29 (bq, H-9, 5.5, 6.9), 2.83 (t, H-10, 6.9). H-4 is coupled with the two F from positions 3 and 5, J = 10.3 Hz, as well as with the two F from positions 2 and 6, J = 7.5 Hz. N−H is coupled with CH_2-9 (J = 5.5 Hz), whereas CH_2-9 appears as a broad quartet because it is also coupled with CH_2-10, which appears as a triplet (J(vic) = 6.9 Hz). ^{13}C NMR-(DMSO-d_6, δ ppm, J Hz), 120.87 (t, C-1, J(^{19}F−^{13}C) = 15.3), 110.74 (t, C-4, J(^{19}F−^{13}C) = 23.5), 145.63 (dddd, C-3, 4.4, 10.8, 15.2, 248.4), 142.85 (ddt, C-2, 3.3, 15.7, 254.5), 129.12 (C-12−16), 125.49 (C-13−15), 142.59 (C-11 or C-14), 142.21 (C-14 or C-11), 43.36 (C-9), 34.69 (C-10). C − 1 = 128.5 + 15.5 − 2 × 13.0 + 2 × 1.6 = 121.2; C − 2 = 128.5 + 34.8 − 13.0 + 1.6 − 4.4 = 147.5; C − 3 = 128.5 + 34.8 − 13.0 + 0.3 + 1.6 − 4.4 = 152.2; C − 4 = 128.5 − 2 × 13.0 + 2 × 1.6 + 3.1 = 108.8 ppm.

(The detailed spectrum is presented because these compounds show very complicated behavior because of the multiple couplings between the protons and fluorine and carbon atoms and no literature data for similar compounds are available as far as we know). Anal. ($C_{14}H_{12}F_4N_2O_4S_2$) C, H, N.

5-Perfluorophenylureido-1,3,4-thiadiazol-2-sulfonamide C10: white crystals, mp 268−9 °C; IR (KBr), cm^{-1}, 1151 and 1185 (SO_2^{sym}), 1360 and 1371 (SO_2^{as}), 3060 (NH), 3365 (NH_2); ^{1}H NMR (DMSO-d_6), δ, ppm, 9.17 (br s, 2H, NHCONH), 7.25 (br s, 2H, SO_2NH_2); ^{13}C NMR (DMSO-d_6), δ, ppm, 124.9, 127.5, 138.6, 159.7 (C-2 of thiadiazole), 170.5 (C-5 of thiadiazole); ^{19}F NMR (DMSO-d_6), δ, ppm, −134.3 (2,6-F_2), −143.9 (4-F), −158.6 (3,5- F_2). Anal. ($C_9H_4F_5N_5O_3S2_3$) C, H, N.

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.[33] Cell growth conditions were those described in ref 34, and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[35] 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 based on M_r = 28.85 kDa for CA I and 29.3 kDa for CA II.[36,37] bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[38]

Initial rates of 4-nitrophenylacetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically at 400 nm with a Cary 3 instrument interfaced to an IBM compatible PC.[27] 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.[27] Nonenzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate 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 to allow for the formation of the E−I complex. The inhibition constant K_I was determined as described by Pocker and Stone.[27] Enzyme concentrations were 3.3 nM for CA II, 10 nM for CA I, and 34 nM for CA IV (this isozyme has a decreased esterase activity,[39] and higher concentrations had to be used for the measurements).

Reaction of Fluorinated Sulfonamides with Thiol Reagents. An amount of 5 mL of a 0.1 mM solution of fluorinated sulfonamide in 10 mM phosphate buffer (pH 7.4) and 5 mL of 0.1 mM solution of cysteine (Cys) or glutathione (Glt) in the same buffer were incubated at 37 °C for 24 h. By means of HPLC, the amount of transformed Cys/Glt was followed (C_{18} reversed-phase μ-Bondapack (2.5 mm × 150 mm) column, 90% phosphate buffer/10% acetonitrile, 3 mL/min). Authentic cysteine and glutathione have been used as HPLC standards (retention times t_R of 5.45 min for Cys and 7.80 min for Glt in the conditions of the experiment). The same experiments have also been performed with cystine (Cys-Cys, t_R of 6.83 min) and oxidized glutathione (t_R of 8.63 min) instead of the two thiol reagents mentioned above, cases in which no chemical modifications could be evidenced. Blank experiments have also been performed by incubating the fluorinated sulfonamide solution (as above) with phosphate buffer without the thiol reagent/disulfides mentioned above. In these cases too, no reaction could be evidenced, and the starting sulfonamides have been isolated unchanged after the incubation, proving that these compounds are stable enough in solution (at least for 24 h).[28]

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. The animals were maintained on a 12 h/12 h light/dark cycle in a temperature-controlled room at 22−26 °C. Solutions of inhibitors (2%, by weight) were obtained in distilled−deionized water. The pH of these solutions was in the range 5.5−8.4.

IOP was measured using a Tono-Pen XL tonometer (Medtronic Solan) as described by Maren's group.[40,41] 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 mean values were reported. IOP was measured first immediately before drug administration and then at 30 min after the instillation of the pharmacological agent and then every 30 min for a period of 4−6 h. For all IOP experiments, the 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.[40,41] Ocular hypertension was elicited in the right eye of albino rabbits by the instillation of hydrocortisol (from Sigma) as described by Melena et al.[26] The IOP of treated animals was checked daily, and after approximately 3−4 weeks, an elevated pressure of 33−35 mmHg has been achieved. Such animals were used in the IOP measurement experiments.

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