

## *In Vitro* Inhibition Effects of some New Sulfonamide Inhibitors on Human Carbonic Anhydrase I and II

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A new series of aromatic and heterocyclic sulfonamides, including six new derivatives, 2-(3-cyclohexene-1-carbamido)-1,3,4-thiadiazole-5-sulfonamide (CCTS), 4-(3-cyclohexene-1-carbamido) methyl-benzenesulfonamide (CCBS), 2-(9-octadecenoylamido)-1,3,4-thiadiazole-5-sulfonamide (ODTS), 2-(4,7,10-trioxa-tetradecanoylamido)-1,3,4-thiadiazole-5-sulfonamide (TDTS), 2-(coumarine-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (COTS) and 2-(8-methoxycoumarine-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS), has been investigated. These sulfonamides were assayed for inhibition of human carbonic anhydrase I (hCA-I) and human carbonic anhydrase II (hCA-II) which were purified by affinity chromatography.

**Keywords:** Inhibition; Sulfonamides; Carbonic anhydrase isozymes

### INTRODUCTION

Fourteen different carbonic anhydrase (CA, EC 4.2.1.1) isozymes have been described up to now in higher vertebrates, including humans. These abundant zinc enzymes are involved in crucial physiological processes connected with respiration and transport of CO<sub>2</sub>/ bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, or biosynthetic reactions, such as lipogenesis, gluconeogenesis and ureagenesis among others<sup>1</sup>.

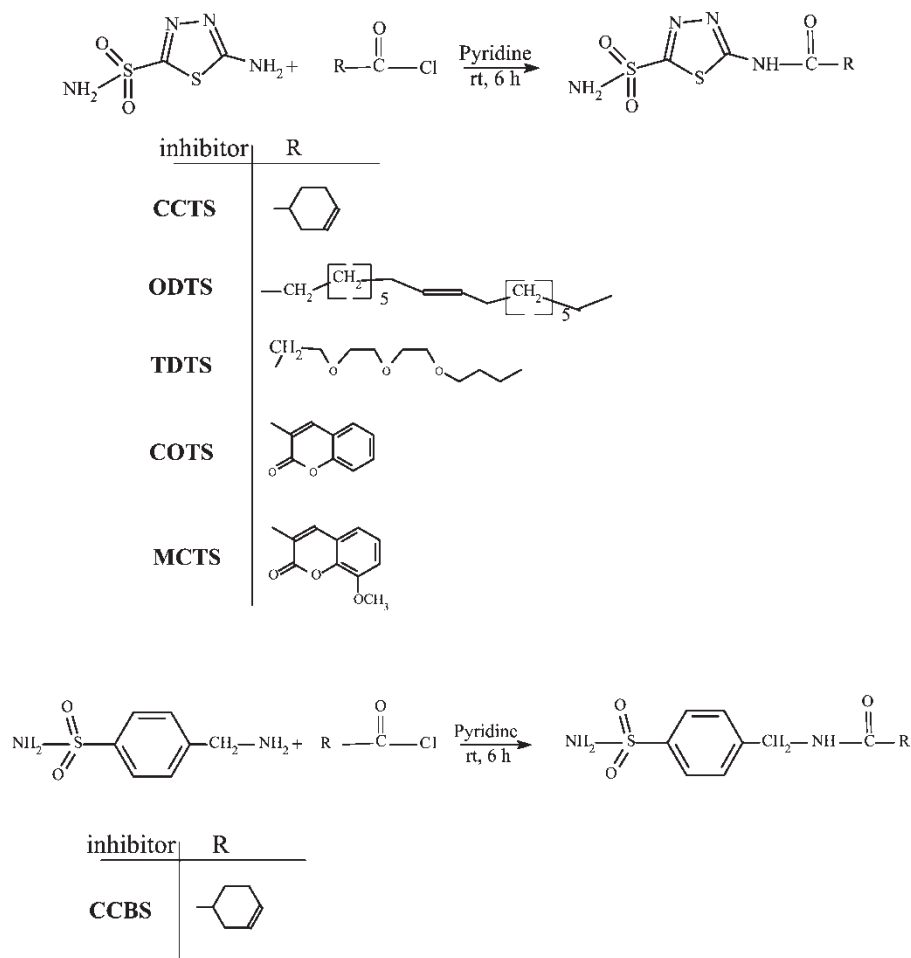
CA inhibition with sulfanilamide discovered by Mann and Keilin was the beginning of a great scientific adventure that led to important drugs such as the antihypertensives of the benzothiadiazine and high-ceiling diuretic type, the sulfonamides with CA inhibitory properties mainly used

as antiglaucoma agents, some anti-thyroid drugs, the hypoglycemic sulfonamides and ultimately to some novel types of anticancer agents.<sup>2</sup> In this study, a new series of aromatic and heteroaromatic sulfonamides were investigated. Also, these derivatives contain “tails,” which have not been investigated before for their effects on CA inhibition.<sup>3</sup> Among those derivatives, acyl substituted ones, such as 2-(3-cyclohexene-1-carbamido)-1,3,4-thiadiazole-5-sulfonamide (CCTS), 4-(3-cyclohexene-1-carbamido) methyl-benzenesulfonamide (CCBS), 2-(9-octadecenoylamido)-1,3,4-thiadiazole-5-sulfonamide (ODTS), 2-(4,7,10-trioxa-tetradecanoylamido)-1,3,4-thiadiazole-5-sulfonamide (TDTS), 2-(coumarin-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (COTS) and 2-(8-methoxycoumarin-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS) can be readily prepared through direct nucleophilic addition to the acyl carbonyl carbon using the amino group of the sulfonamide moiety as a relatively strong nucleophile.<sup>4</sup>

The parent sulfonamides both topical and systemic, lower intraocular pressure (IOP) by reducing HCO<sub>3</sub><sup>-</sup> formation in the ciliary process thus lowering Na<sup>+</sup> transport and flow of aqueous humor and this is the basis for their use in glaucoma.<sup>5,6</sup> Systemic therapy with the parent sulfonamides and their derivatives leads to significant side-effects.<sup>7</sup> The main problem of designing new inhibitors is therefore to make them more organ-selective or/and isozyme-selective by adjusting their pharmacokinetic properties.

The substitution reactions were achieved at the stage of mono acylation of the amino-sulfonamides

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SCHEME 1 Synthesis of the new sulphonamide inhibitors.

with acyl halides by controlling the reaction conditions (Scheme 1).

## MATERIALS AND METHODS

L-tyrosine and *p*-aminobenzenesulfonamide were from Merck Chem. Co. and the other chemicals were obtained from Sigma Chem. Co. and were of analytical grade. The blood samples with ACD (acid-citrate-dextrose) were obtained from human volunteers.

The structures of the newly synthesized inhibitors were established by IR spectra (Perkin Elmer Spectrum BX II), 400 MHz  $^1\text{H}$  NMR (Bruker GmbH DPX-400), 60 MHz  $^{13}\text{C}$  NMR (Bruker -AC), Mass spectra at 70 eV (Hitachi RMU-6E) and elemental analyses (Carlo Erba model 1200 instrument). Melting points were measured on an Electrothermal 9200 IA instrument.

### Preparation of Hemolysate

Human blood samples were anticoagulated with ACD (Acid-citrate-dextrose) and centrifuged at

1848  $\times$  g for 20 min at 4°C and the supernatant was removed. The packed red cells were washed with NaCl (0.9%) three times and the erythrocytes were hemolysed with cold water. The ghost and intact cells were removed by centrifugation at 18924  $\times$  g for 25 min at 4°C and the pH of the hemolysate was adjusted to 8.5 with solid Tris-base.<sup>8</sup> The hemolysate was applied to the affinity column containing Sepharose-4B-L-tyrosine-sulfonamide and equilibrated with 25 mM Tris-HCl/0.1 M  $\text{Na}_2\text{SO}_4$  (pH 8.5). The affinity gel was washed with a solution of 25 mM Tris-HCl/22 mM  $\text{Na}_2\text{SO}_4$  (pH 8.5). The human erythrocyte isozymes were eluted with a solution of 0.1 M  $\text{NaCH}_3\text{COO}$ /0.5 M  $\text{NaClO}_4$  (pH 5.6) for HCA-II and 0.1 M NaCl/25 mM  $\text{Na}_2\text{HPO}_4$  (pH 6.3) for HCA-I, respectively.

### Determination of Protein Content

After scanning at 280 nm the tubes with significant absorbance were pooled and a quantitative protein determination was done by the Coomassie brilliant blue G-250 method.<sup>9,10</sup>

## Enzyme Assay

Carbonic anhydrase activity was measured by the Maren method<sup>11</sup> which is based on determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO<sub>2</sub> hydration. Phenol red was added to the assay medium as the pH indicator, and the buffer was 0.5 M Na<sub>2</sub>CO<sub>3</sub>/0.1 M NaHCO<sub>3</sub> (pH 10.0). All solutions were chilled to 0°C before use. One unit of CA activity is defined as the amount of the enzyme that reduces by 50% the time of CO<sub>2</sub> hydration measured in the absence of enzyme. In the inhibition studies, the CO<sub>2</sub> concentration was 70 mM and five different inhibitor concentrations were used. I<sub>50</sub> values were calculated using computer regression analysis.<sup>12</sup>

## General Procedure for the Preparation of the New Compounds

Amino sulfonamide (5.5 mmol) dissolved in 1 mL pyridine and the appropriate acid chloride were stirred at 25°C for 6 h. The mixture was evaporated, and residue was crystallized from water.<sup>8</sup>

### 2-(3-Cyclohexene-1-carbamido)-1,3,4-thiadiazole-5-sulfonamide (CCTS)

3-Cyclohexene-1-methanoyl chloride and 2-amino-1,3,4-thiadiazole-5-sulfonamide were used as starting compounds. Yield, 60%; mp, 222°C; white crystals.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3290, 2915, 1675, 1615, 1535, 1395, 1200. <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 1.7 (dt, 2H, CH<sub>2</sub>-CH<sub>2</sub>), 2.3 (m, 2H, CH<sub>2</sub>-CH<sub>2</sub>), 2.9 (dd, 2H, HC-CH<sub>2</sub>), 3.2 (m, 1H, OC-CH), 5.8 (m, 2H, HC=CH). <sup>13</sup>C NMR (d-DMSO)  $\delta$  (ppm): 177, 168, 163, 133, 131.5, 45, 33, 30, 27. MS: m/z 288 (M<sup>+</sup>) 289 (M<sup>+</sup> + 1), 179.9 (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>S<sub>2</sub>N<sub>4</sub>), 78.8 (SO<sub>2</sub>NH). Anal. for C<sub>9</sub>H<sub>12</sub>N<sub>4</sub>O<sub>3</sub>S<sub>2</sub>. Calcd. C, 37.49; H, 4.19; N, 19.43; S, 22.24. Found: C, 37.43; H, 4.39; N, 19.55; S, 22.20%.

### 4-(3-Cyclohexene-1-carbamido) methylbenzenesulfonamide (CCBS)

3-Cyclohexene-1-methanoyl chloride and 4-amino-methyl benzenesulfonamide were used as starting compounds. Yield, 68%; mp, 185°C; pale ash-coloured solid.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3270, 2930, 1680, 1620, 1530, 1410, 1390. <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 1.75 (dt, 2H), 2.4 (m, 2H), 2.7 (dd, 2H), 3.3 (m, 1H, OC-CH), 4.4 (s, 2H, Ar-CH<sub>2</sub>), 5.8 (m, 2H, HC=CH), 7.5 (d, 2H, Ar-H), 7.8 (d, 2H, ArH). <sup>13</sup>C NMR (d-DMSO)  $\delta$  (ppm): 178, 168, 163, 147, 140, 133, 131, 129, 126, 51, 45, 33, 30, 27 MS: m/z 294 (M<sup>+</sup>), 295 (M<sup>+</sup> + 1), 170 (C<sub>7</sub>H<sub>8</sub>NO<sub>2</sub>S), Anal. for C<sub>14</sub>H<sub>18</sub>N<sub>2</sub>O<sub>3</sub>S. Calcd. C, 57.12; H, 6.16; N, 9.52; S, 10.89. Found: C, 57.01; H, 6.28; N, 9.72; S, 10.80%.

### 2-(9-Octadecenoylamido)-1,3,4-thiadiazole-5-sulfonamide (ODTS)

Oleoyl chloride and 2-amino-1,3,4-thiadiazole-5-sulfonamide were used as starting compounds. Yield, 71%; mp, 233–234°C; Yellow powder.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3280, 2935, 1685, 1610, 1540, 1395, 1190. <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 0.9–1.2 (m, 25H), 2.7 (m, 4H, CH<sub>2</sub>-CH=CH-CH<sub>2</sub>), 3.2 (t, 2H, OC-CH<sub>2</sub>), 5.6 (m, 2H, HC=CH). <sup>13</sup>C NMR (d-DMSO)  $\delta$  (ppm): 170, 164, 163, 131, 130, 35, 26–34, 10 MS: m/z 444 (M<sup>+</sup>), 445 (M<sup>+</sup> + 1), 179.9 (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>S<sub>2</sub>N<sub>4</sub>), 78.8 (SO<sub>2</sub>NH). Anal. for C<sub>20</sub>H<sub>36</sub>N<sub>4</sub>O<sub>3</sub>S<sub>2</sub>. Calcd. C, 54.02; H, 8.16; N, 12.60; S, 14.42. Found: C, 54.12; H, 8.20; N, 12.73; S, 14.30%.

### 2-(4,7,10-Trioxa-tetradecanoylamido)-1,3,4-thiadiazole-5-sulfonamide (TDTS)

4,7,10-Trioxa-tetradecanoyl chloride and 2-amino-1,3,4-thiadiazole-5-sulfonamide were used as starting compounds. Yield, 65%; mp, 212°C; white crystals.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3280, 2915, 1694, 1625, 1200, 1135, <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 0.9–1.1 (m, 7H), 3.5 (t, 2H, OC-CH<sub>2</sub>), 3.8 (m, 12H, CH<sub>2</sub>-O-CH<sub>2</sub>). <sup>13</sup>C NMR (d-DMSO)  $\delta$  (ppm): 168, 166, 163, 72, 70, 67, 37, 31, 22, 17. MS: m/z 421 (M<sup>+</sup>), 422 (M<sup>+</sup> + 1), 179.9 (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>S<sub>2</sub>N<sub>4</sub>), 78.8 (SO<sub>2</sub>NH). Anal. for C<sub>13</sub>H<sub>24</sub>N<sub>4</sub>O<sub>6</sub>S<sub>2</sub>. Calcd. C, 39.38; H, 6.10, N, 14.13; S, 16.17. Found: C, 39.23; H, 6.12; N, 14.25; S 16.1%.

### 2-(Coumarin-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (COTS)

Coumarin-3-methanoyl chloride and 2-amino-1,3,4-thiadiazole-5-sulfonamide were used as starting compounds. Yield, 60%; mp, 183–185°C; yellow powder.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3250, 2900, 1735, 1690, 1485, 1275, 1130, <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 7.3 (d, 1H, Ar-H), 7.4 (dd, 1H, Ar-H), 7.7 (dd, 1H, Ar-H), 7.8 (d, 1H, Ar-H), 8.7 (s, 1H, coumH). <sup>13</sup>C NMR (d-DMSO)  $\delta$  (ppm): 167, 163, 162, 160, 153, 150, 130, 128, 127, 126, 125, 120 MS: m/z 352 (M<sup>+</sup>), 353 (M<sup>+</sup> + 1), 179.9 (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>S<sub>2</sub>N<sub>4</sub>), 78.8 (SO<sub>2</sub>NH). Anal. for C<sub>12</sub>H<sub>8</sub>N<sub>4</sub>O<sub>5</sub>S<sub>2</sub>. Calcd. C, 40.91; H, 2.29; N, 15.90; S 18.20. Found: C, 40.85; H, 2.31; N, 15.87; S 18.18%.

### 2-(8-Methoxycoumarin-3-carbamido)-1,3,4-thiadiazole-5-sulfonamide (MCTS)

8-Methoxycoumarin-3-methanoyl chloride and 2-amino-1,3,4-thiadiazole-5-sulfonamide were used as starting compounds. Yield, 58%; mp, 234°C; yellow powder.

IR (KBr),  $\nu$  (cm<sup>-1</sup>): 3250, 2908, 1738, 1688, 1480, 1280, 1132, <sup>1</sup>H-NMR (d-DMSO),  $\delta$  (ppm) 4.0 (s, 3H), 7.4 (m, 2H, Ar-H), 7.9 (dd, 1H, Ar-H), 8.8 (s, 1H, coumH).

$^{13}\text{C}$  NMR (d-DMSO)  $\delta$  (ppm): 167, 163, 162, 160, 157, 153, 149, 140, 131, 125, 120, 114, 59 MS:  $m/z$  382 ( $\text{M}^+$ ), 383 ( $\text{M}^+ + 1$ ), 179.9 ( $\text{C}_2\text{H}_3\text{O}_2\text{S}_2\text{N}_4$ ), 78.8 ( $\text{SO}_2\text{NH}$ ). Anal for  $\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_6\text{S}_2$ . Calcd. C, 40.84; H, 2.64; N, 14.65; S, 16.77. Found: C, 40.74; H, 2.60; N, 14.58; S, 16.70%.

### Inhibition Studies

The inhibition of carbonic anhydrase by the sulfonamides CCTS, CCBS, ODTS, TDTS, COTS and MCTS was studied by determining their effects on the enzyme-catalyzed  $\text{CO}_2$  hydration rate at  $1^\circ\text{C}$ . The  $\text{CO}_2$  concentration was adjusted to 70 mM.  $\text{CO}_2$  hydration rates at  $1^\circ\text{C}$  were determined using five different inhibitor concentrations at this constant substrate concentration.<sup>13</sup>

In order to determine the  $I_{50}$  values, a regression analysis using a statistical package was carried out on the graph of percent inhibition values as a function of inhibitor concentration. The inhibitor concentration which reduced enzyme activity by 50%, was then determined from the graph.

## RESULTS AND DISCUSSION

Inhibitors of carbonic anhydrase play an important role in ophthalmology, where they are used to reduce elevated intraocular pressures. Apart from transient myopia and blurred vision, no adverse reactions from the eye have been described in spite of long-term treatment of patients over years.<sup>14</sup>

Acetazolamide (2-amino-1,3,4-thiadiazole-5-sulfonamide) is the inhibitor mostly used. Unfortunately, systemic therapy with sulfonamides and their derivatives leads to significant side-effects.<sup>3</sup> In this paper the amino-sulfonamide derivatives have been synthesized from different acid chlorides and 2-amino-1,3,4-thiadiazole-5-sulfonamide.

Therefore we focussed on finding the optimal reaction conditions for the above mentioned mono-addition type reactions and especially to synthesize new sulfonamide derivatives, which contain different functional groups (Scheme 1).

The acyl chlorides were easily prepared in the usual way by treating carboxylic acids with thionyl chloride ( $\text{SOCl}_2$ ). Addition of 2-amino-1,3,4-thiadiazole-5-sulfonamide to 3-cyclohexene-1-methanoyl chloride, 9-octadecenoyl chloride, 4,7,10-trioxa-tetradecanoyl chloride, coumarin-3-methanoyl chloride and 8-methoxy coumarin-3-methanoyl chloride gave the required derivatives in good yield.

The isozymes used in this study, CA-I and CA-II, were purified from human erythrocytes by Sepharose 4B-L-tyrosine-sulfonamide affinity column. The purified carbonic anhydrase isozymes

TABLE I  $I_{50}$  values for the synthesized compounds on carbonic anhydrase isozymes I and II

Inhibitor	$I_{50}$ , $\mu\text{M}$		
	hCA-I	hCA-II	hCA-I/hCA-II
CCTS	0.043	0.029	1.48
CCBS	0.055	0.017	3.23
ODTS	0.061	0.052	1.17
TDTS	0.024	0.017	1.41
COTS	0.057	0.06	0.95
MCTS	0.047	0.096	0.49

migrated as a single band during SDS polyacrylamide gel electrophoresis (data not shown). Inhibitors can be compared by their inhibitor-enzyme dissociation constants  $K_i$  and  $I_{50}$ . The results here are expressed as  $I_{50}$ , i.e., inhibitor concentration that reduces enzyme activity by 50% (Table I) using the  $\text{CO}_2$ -hydratase activity of carbonic anhydrase, which is the primary physiological function of this enzyme.

The *in vitro* inhibitory effects of CCTS, CCBS, ODTS, TDTS, COTS and MCTS on CA-I and CA-II, purified by affinity chromatography, are given in Table I. CCBS was a more potent inhibitor than CCTS with respect to CA-II. The inhibitory effect of TDTS on both CA isozymes is slightly similar compared against that for CCBS and CCBS is a weaker inhibitor than TDTS with respect to CA-I. The inhibitory effects of COTS and MCTS are similar on CA-I to these of CCBS and ODTS but these inhibitory effects are weaker on CA-II. As shown in Table I, COTS and ODTS had similar inhibitory effects on both CA isozymes.

The inhibitory effect of these compounds as shown in Table I are very similar to those for acetazolamide, which is used in the treatment of glaucoma.<sup>15</sup> The  $I_{50}$  value of CCBS, having  $\text{C}_6\text{H}_9\text{-CO-NH-CH}_2\text{-}$  as a side chain group has an approximately 30-fold higher inhibitory effect on CA-II than benzenesulfonamide.<sup>12</sup> This is the result of hydrophobic and van der Waals interactions between the heterocyclic/aromatic part of the inhibitor molecule and active site amino acid residues.<sup>2</sup>

CCBS is more selective inhibitor against CA-II, which is the target enzyme in glaucoma treatment and the new compound could be considered as a potential agent for treatment of glaucoma in animals.

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