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Synthesis of some natural sulphonamide derivatives as carbonic anhydrase inhibitors

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Abstract: Carbonic anhydrase inhibitors are both in clinical use as antiglaucoma, diuretics, antiepileptics and management of altitude sickness, and under investigation as anticancer, anticonvulsant and antiobesity agents. Sulphonamides have been known for decades as carbonic anhydrase inhibitors and are in clinical use. Sulphonamide derivatives of *p*-hydroxybenzoic acid and *3,4,5*-trihydroxybenzoic acid (gallic acid) were synthesized and their inhibition values over hCA I and hCA II isozymes, purified from human erythrocyte cells by Sepharose-4B-L-tyrosine-sulphanilamide, were determined. Compounds synthesized showed efficient carbonic anhydrase inhibition activity at low nM levels.

Keywords: Carbonic anhydrase inhibitor; sulphonamides; *p*-hydroxybenzoic acid; *3,4,5*-trihydroxybenzoic acid (gallic acid).

1. Introduction

Although since the first synthesis as a chemotherapeutic agent in the early 1930s, sulphonamides have been withdrawing increasing interest,[1] they found limited applications on human therapy, possibly due to the advancements in antibiotic area and some of their toxicological effects.[2-3] As they are relatively cheap and effective toward many bacterial infections, they are widely used as veterinary drugs. Moreover, its active side "-SO_2NH_2" has been incorporated into various actively used drugs on human therapy.[4-7]

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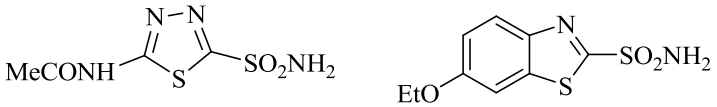
Conversion of carbon dioxide (CO_2) and water (H_2O) into bicarbonate (HCO_3^-) and proton (H^+) is a simple reaction catalyzed by a super family of zinc containing metallo enzymes, called carbonic anhydrases (CAs, EC 4.2.1.1).[8-11] Carbonic anhydrases, having medium-sized molecular weight, are ubiquitous metallo enzymes present either in prokaryotes or eukaryotes and, so far, six genetically distinct families have been acknowledged.[12-14] There are fifteen human carbonic anhydrases, belonging to α-class, have been recognized. They differ from each other with their kinetic properties, inhibition and their expression in various tissues and cellular localization, such that CAs I, II, III, VII, and XIII are cytosolic, IV, IX, XII, and XIV are associated with the cell membrane, VA and VB are located in the mitocondria, VI, secreted in saliva and milk, show enzymatic activity, and VIII, X and XI are CA-related proteins (CARPs) without any catalytic activity.[15-18]

Carbonic anhydrases play crucial roles in a variety of physiological processes such as electrolyte secretion in a variety of tissues and organs, calcification, biosynthetic reactions (lipogenesis, ureagenesis, and gluconeogenesis are among them), pH and CO_2 homeostasis, bone resorption and tumorigenicity, and many others.[21-23] Inhibition of carbonic anhydrases are becoming increasingly popular as a research subject, which is due to their abnormal levels associated with various diseases such as glaucoma, cancer, osteoporosis and some neurological disorders.[15]

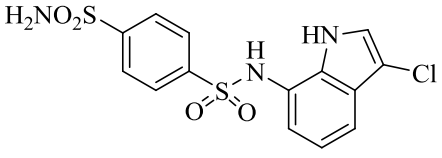
Sulphonamides, phenols and coumarins are three examples of CA inhibitors acting through different mechanisms, (i) displacing zinc-bound water/hydroxide ion by coordination to the Zn(II) ion in the enzyme active side and leading to a tetrahedral geometry, or by addition to the metal coordination sphere and leading to a trigonal bipyramidal geometry, (ii) hooking up to the Zn(II)-bound water/hydroxide ion and (iii) by obstructing the active site cavity of the enzyme, respectively.[17,22]

Primary sulphonamides are well known carbonic anhydrase inhibitors, first examples of which were derivatives of clinically used ones such as acetazolamide (AAZ), ethoxzolamide (EZA) and indisulfam (IND) (Figure 1.).[23] Today, it is acknowledged that when designing therapeutic agents, selective inhibition of CAs or, at least, organ specific targeting is the main goal.[15] However, since there are at least fifteen carbonic anhydrase isozyme in human, their spread localization in many tissues and organs, and none of the currently sulphonamide or sulfamate CA inhibitors in clinical use is selective for a specific isozyme is a high barrier to achieve.[24,25] Although, it was believed that amine group of sulphonamide should be primary to act as carbonic anhydrase inhibitor, recent studies indicated that secondary and even tertiary sulphonamides can act as affective carbonic anhydrase inhibitors and they can be selective.[26-38]



AAZ

EZA



IND

Figure 1. Some sulphonamides on clinical use, AAZ, EZA and IND

The natural phenolic acids, gallic (*3,4,5*-trihydroxybenzoic) acid and *p*-hydroxybenzoic acid, found in many plants and used as preservatives in food, possess antibacterial, anticarcinogenic, antioxidant, antifungal and antimutagenic activities, and cytotoxicity toward tumor cells.[29-31] Innocenti

et al. showed that *p*-hydroxybenzoic acid had better inhibition properties on hCA I and hCA II compare with the natural product polyphenols and phenolic acids.[22] In this study, we reported secondary sulphonamides synthesized from *p*-hydroxybenzoic acid and *3,4,5*-trihydroxybenzoic acid (Scheme 1), and investigated synergy of sulphonamides and polyphenols as carbonic anhydrase inhibitors on the hCA I and hCAII isozymes purified from human erythrocyte cells by Sepharose-4B-L-tyrosine-sulphanilamide.

2. Experimental

2.1. Materials and apparatus

All starting materials and reagents were purchased from commercial suppliers. Progress of the reactions were monitored by TLC, using silica gel-60 F254 plates with detection by short wave UV fluorescence (λ = 254 nm) and staining with ceric sulphate. Silica gel flash chromatography was performed using silica gel 60 Å (230-400 mesh). Purity of compounds determined by HPLC at 270 nm. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 600 MHz spectrometer at 25 ^{o}C. Chemical shifts for ^{1}H and ^{13}C NMR spectra obtained in DMSO-d_6 were reported in ppm relative to the residual solvent proton (δ = 2.50 ppm) and carbon (δ = 39.52 ppm) signals, respectively. Multiplicity was indicated as follows: s (singlet); d (doublet); m (multiplet). Coupling constants were reported in hertz (Hz). High-resolution mass spectra (HRMS) were recorded on BrukerMicrOTOF-Q at positive electro spray ionization (ESI+) mode. ^{1}H and ^{13}C NMR, and HRMS spectra of compounds are provided in Supporting Information.

Table 1. hCA I and hCA II inhibiton values of the compounds

	IC_{50} (nM)		K_I (nM)	
Compounds	hCA I	hCA II	hCA I	hCA II
Sulfachloropyridazine	**1253.24**	**750.71**	**5259±177.8**	**361.32±83.15**
p-Acetoxybenzamide (**5a**)	9.97	47.24	7.82±0.02	87.09±0.51
p-Hydroxybenzamide (**6a**)	16.15	51.56	8.04±0.01	64.55±0.29
3,4,5-Triacetoxybenzamide (**5b**)	34.65	49,51	55.72±34.01	48.51±13.76
3,4,5-Trihydroxybenzamide (**6b**)	8.76	36.69	9.03±0.02	47.37±0.05
Sulfacetamide	**1563.17**	**1471.16**	**1820±260.22**	**2040.13±431.13**
3,4,5-Triacetoxybenzamide (**5c**)	27.72	11.01	20.95±1.51	7.09±0.32
Sulfadimethoxine	**63.01**	**138.61**	**76.16±5.82**	**106.77±6.01**
3,4,5-Triacetoxybenzamide (**5d**)	14.14	9.63	13.65±3.16	8.41±0.90
***p*-Hydroxybenzoic acid (1a)**	46.21	57.75	35.69±12.03	37.96±1.48
p-Acetoxybenzoic acid (**2a**)	53.31	31.51	39.11±3.41	41.29±29.92
***3,4,5*-Trihydroxybenzoic acid**(**1b**)	57.75	31.51	32.12±5.85	38.16±12.05
3,4,5-Triacetoxybenzoic acid (**2b**)	46.21	99,01	57.75±7.71	104.61±30.64
AZA[*]	21.95	15.17	19.92±0.16	9.76±0.03

[*]Acetazolamide (AZA) was used as a standard inhibitor for both CA isoenzyme

*2.2. General procedure for acetyl protection of hydroxyl groups on phenolic acids (**2**):* To a stirred mixture of hydroxyl substituted benzoic acid **1** (20 mmol) and acetic anhydride (120 mmol) in round bottom flask was added a few drops of concentrated H_2SO_4, and the after all the solid was dissolved, the mixture was left stirring for an hour. Then water (100 mL) was added to remove any excess acetic anhydride left and the stirring was continued for further 2.5 h. A white crystalline product was filtered and washed with water (2 X 50 mL). The solid product was dried on filter by air suction for 10 minutes and then high vacuum for overnight.

*2.3. General procedure for preparing acyl chlorides (**3**):* A mixture of acetyl protected phenolic acid **2** (20 mmol) and thionyl chloride (300 mmol) was refluxed for 5 hours. Excess of thionyl chloride was

distilled and the remaining crude product was diluted with acetone (20 mL) of acetone and used for the next step without any further purification.

*2.4. General procedure for the synthesis of N-(sulfonamide)-acetoxybenzamide (**5**):* Acyl chloride **3** (20 mmol) in acetone 20 mL was added dropwise to a solution of sulphonamide (20 mmol) and pyridine (20 mmol) in acetone (50 mL) at 0 ^{o}C. Then, the reaction mixture was stirred at room temperature for overnight. When a precipitate was obtained, it was filtered. Otherwise the solvent was evaporated under reduced pressure and the crude product was purified by recrystallization from ethanol or flash column chromatography using ethyl acetate/hexane (1:1) as a mobile phase.

*2.5. General procedure for deacetylation of N-(sulfonamide)-acetoxybenzamide (**6**):* N-(sulfonamide)-acetoxybenzamide **5** was added to a solution of tetrahydrofuran (10 mL), methanol (10 mL) and concentrated hydrochloric acid (5 mL). The mixture was stirred at 60 ^{o}C for one hour. The solvent was evaporated under reduced pressure and the crude product was purified by recrystallization from ethanol or flash column chromatography using ethyl acetate/hexane (1:1) as a mobile phase.

*p-Acetoxybenzoic acid (**2a**):* Yield 90%. ^{1}H NMR (DMSO-d_6, 600 MHz) δ (ppm): 7.99 (d, *J* = 8.6 2H), 7.26 (d, *J* = 8.6 2H), 2.28 (s, 3H).

*3,4,5-Triacetoxybenzoic acid (**2b**):* Yield 85%. ^{1}H NMR (DMSO-d_6, 600 MHz) δ (ppm): 7.72 (s, 2H), 2.30 (s, 3H), 2.27 (s, 6H).

*N-(Sulfachloropyridazine)-p-acetoxybenzamide (**5a**):* Yellowish powder, yield 45%, m.p. 235-236 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.63 (s, 1H), 8.00 (d, *J* = 8.7 Hz, 2H), 7.97 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 8.8 Hz, 2H), 7.78 (d, *J* = 9.5 Hz, 1H), 7.57 (broad, 1H), 7.31 (d, *J* = 8.7 Hz, 2H), 2.31 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 168.97, 165.30, 154.27, 153.22, 143.30, 131.95, 131.64, 129.42, 128.06, 122.60, 122.03, 119.85, 20.90. HRMS (ESI^{+}): *m/z* calculated for $C_{19}H_{15}ClN_4O_5S_1Na_1$ [M+Na^{+}]: 469.0344, found 469.0342.

*N-(Sulfachloropyridazine)-3,4,5-triacetoxybenzamide (**5b**):* Yellowish powder, yield 35%, m.p. 242-243 ^{o}C.^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.67 (s, 1H), 7.93-7.89 (m, 4H), 7.80 (s, 2H), 7.76 (d, *J* = 9.5 Hz, 1H), 7.50 (broad, 1H), 2.32 (s, 3H), 2.30 (s, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 168.84, 168.12, 167.06, 163.76, 154.32, 150.50, 145.50, 143.24, 139.16, 137.71, 132.45, 128.10, 120.83, 120.15, 119.99, 20.39, 19.94. HRMS (ESI^{+}): *m/z* calculated for $C_{23}H_{20}ClN_4O_9S_1$ [M+H^{+}]: 563.0634, found 563.0640.

*N-(Sulfacetamide)-3,4,5-triacetoxybenzamide (**5c**):* Off white powder, yield 43%, m.p. 220-221 ^{o}C ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 12.01 (s, 1H), 10.72 (s, 1H), 7.96 (d, *J* = 8.9 Hz, 2H), 7.89 (d, *J* = 8.9 Hz, 2H), 7.82 (s, 2H), 2.33 (s, 3H), 2.31 (s, 6H), 1.90 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 169.14, 168.46 , 167.39 , 164.14 , 143.79 , 143.61 , 138.10 , 134.17 , 132.78 , 129.19 , 121.23 , 120.33 , 23.65 , 20.77 , 20.32 . HRMS (ESI^{+}): *m/z* calculated for $C_{21}H_{21}N_2O_{10}S_1$ [M+H^{+}]: 493.0911, found 493.0927.

*N-(Sulfadimethoxine)-3,4,5-triacetoxybenzamide (**5d**):* White powder, yield 57%, m.p. 197-198 ^{o}C. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 10.71 (s, 1H), 7.98 – 7.93 (m, 4H), 7.82 (s, 2H), 5.96 (s, 1H), 3.80 (s, 3H), 3.77 (s, 3H), 2.35 (s, 3H), 2.33 (s, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 172.11, 168.42, 167.37, 164.69, 164.13, 160.31, 143.59, 143.48, 138.07, 134.78, 132.80, 128.79, 121.18, 120.47, 85.02, 54.97, 54.24, 20.72, 20.28. HRMS (ESI^{+}): *m/z* calculated for $C_{25}H_{25}N_4O_{11}S_1$ [M+H^{+}]: 589.1235, found 589.1235.

*N-(Sulfachloropyridazine)-p-hydroxybenzamide (**6a**):* Yellowish powder, yield 35%, m.p. 161-162 ^{o}C.^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): ^{1}H NMR (600 MHz, dmso) δ 10.34 (s, 1H), 10.18 (s, 1H), 7.96 (d, *J* = 8.8 Hz, 2H), 7.89-7.84 (m, 4H), 7.78 (d, *J* = 9.6 Hz, 1H), 7.52 (broad, 1H), 6.87 (d, *J* = 8.6 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 165.50, 160.91, 154.22, 143.79, 132.36,

129.95, 128.02, 124.78, 119.67, 115.01. HRMS (ESI^+): *m/z* calculated for $C_{17}H_{13}ClN_4O_4S_1Na$ $[M+Na^+]$: 427.0238, found 427.0237.

*N-(Sulfachloropyridazine)-3,4,5-trihydroxybenzamide (**6b**):* Yellowish powder, yield 40%, m.p. 175-176 ^oC.^1H NMR (600 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.90 (s, 1H), 7.94 (d, *J* = 8.8 Hz, 2H), 7.86 (d, *J* = 8.3 Hz, 2H), 7.78 (d, *J* = 9.4 Hz, 1H), 6.95 (s, 2H).^{13}C NMR (150 MHz, DMSO-d_6) δ 166.37, 154.62, 146.00, 145.13, 137.65, 131.36, 128.40, 124.69, 120.00, 119.50, 107.80. HRMS (ESI^+): *m/z* calculated for $C_{17}H_{13}ClN_4O_6S_1Na$ $[M+Na^+]$: 459.0137, found 459.0134.

2.6. Biochemistry

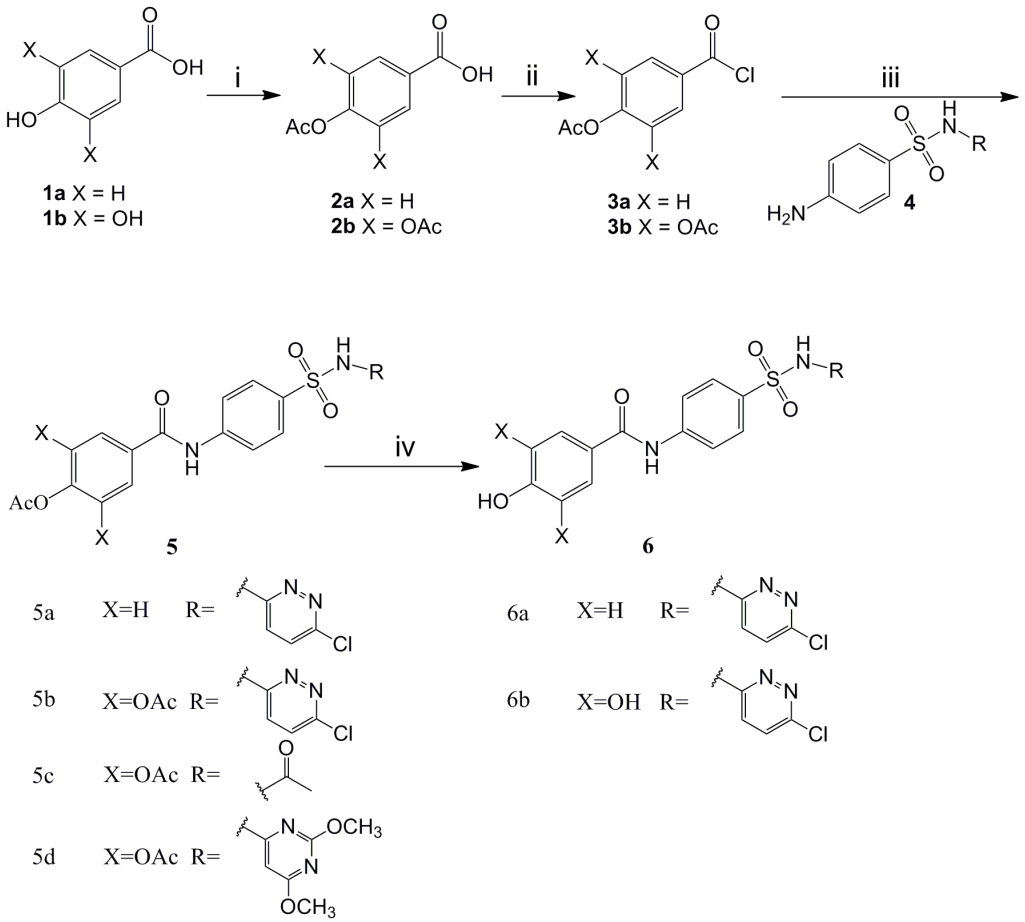
Both CA isoenzyme (hCA I and II) were purified by Sepharose-4B-L-tyrosine-sulphanilamide affinity chromatography in a single purification step.[37] The column material Sepharose-4B-L-tyrosine-sulphanilamide was prepared according to a reported method.[17] Then, the homogenate solution acidity was adjusted to 8.7 with a pH-meter using solid Tris and the supernatant was transferred to the Sepharose-4B-L tyrosine-sulphanilamide affinity column.[38] The proteins obtained from column was spectrophotometrically determined at 280 nm. The purity of the isozymes was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), where a single band was observed for each isozyme.[41] In this application, the imaging method was performed out in 10% and 3% acrylamide for the running and the stacking gel, respectively, with 0.1% SDS.[42]

Activities of the CA isozymes were determined applying the method reported by Verpoorte et al (1967).[43] Briefly, the absorbance, changing at 348 nm for *p*-nitrophenylacetate (NPA) to *p*-nitrophenolate (NP), was recorded over a 3 min period at the room temperature, using a spectrophotometer (Shimadzu, UV-VIS Spectrophotometer, UVmini-1240, Kyoto, Japan). The protein was measured quantitatively at 595 nm during the purification steps according to the Bradford method.[44] Bovine serum albumin (BSA) was used as a standard protein.[45] For determining the inhibition effect of each sulphonamide derivative, an activity (%)-[Sulphonamide] graph was plotted. To determine K_i values, three different concentrations of sulphonamide derivatives were tested. In these experiments, NPA was used as a substrate at five different concentrations and Lineweaver-Burk curves were plotted.

3. Results and discussion

Polyphenols are secondary metabolites ubiquitously occurring in all higher plants and are found largely in foods and beverages, such as fruits, vegetables, tea and coffee, which play multiple essential roles in plant physiology and have healthy effects on human organism associated to their anti-oxidant and metal chelator properties. In some cases it has been reported by some clinical and epidemiological studies that long term consumption of diets rich in plants comprise polyphenols offer protection against cancer, diabetes, cardiovascular and neurodegenerative diseases, hypertension and aging.[39,40] Synthesis of the sulphonamide derivatives **5a-d**, **6a,b** were started with acetyl protection of hydroxyl groups of the phenolic acids **1a,b** through an acid catalyzed reaction of acetic anhydride to obtain **2a,b**.[32] Conversion of the acid groups of **2a,b** using thionyl chloride to the acid chlorides **3a,b** was followed by addition of commercially available sulphonamides **4**, which yielded the products **5a,d**.[33] Acetyl protection of hydroxyl groups is a common approach in pharmaceutical industry to enhance oral bioavailability and increase the therapeutic concentration of the drug in bloodstream.

These protected compounds are called pro-drugs since they are hydrolyzed to the corresponding alcohol by carboxylesterases in blood.[34] The sulfachloropyridazines **6a,b** were then obtained *via* deacetylation of **5a,b**, applying an acid catalyzed reaction in tetrahydrofuran and methanol (Scheme 1).[33] It should be noted that compounds **5b**[35], **6a**[36] and **6b**[35] are known compounds but no carbonic anhydrase inhibition reported before.



Reagents and conditions. (i) Acetic anhydride, H_2SO_4, rt, 1 h; (ii) $SOCl_2$, 80 ^oC, 5 h; (iii) sulfonamide, acetone, pyridine, 0 ^oC - rt, 20 h; (iv) THF, MeOH, HCl, 60 ^oC, 1 h.

Scheme 1. Synthesis of sulphonamide derivatives

All synthesized derivatives showed highly effective carbonic anhydrase I and II inhibition at nano molar level and better inhibiton compared to their reference sulphonamides (Table 1.), where in case of sulfachloropyridazine for hCA I it is at least hundredfold much better. Except, *N*-sulfachloropyridazine-*3,4,*5-triacetoxybenzamide, all the compounds displayed better hCA I inhibition properties than their reference phenolic acids as well. The compound *N*-sulfachloropyridazine-*p*-acetoxybenzamide (5a) was found to be the best to inhibit cytosolic isozyme hCA I with a Ki value of 7.82±0.02 nM, where the drug in clinical use acetazolamide (AZA), had Ki value of 19.92±0.16 nM. The synergistic effect of polyphenols in combination with conventional antimicrobial agents against clinical multi-drug resistant microorganisms is discussed before[40] and here in this study the synergistic effect by combination of polyphenols or naturally phenolic acids with the sulfonamides -known as privileged class of medicinal chemistry- in one molecule is bring to light. Isozyme CA II, which has no disulfide, pendant sugar and phosphate groups, is frequently investigated inhibitor as it is inexpensive, widely available and has well known structure and active site geometry. The synthesized compounds displayed high inhibition toward hCA II at nano molar level compared with reference sulphonamides. Among all the compounds, *N*-sulfacetamide-*3,4,*5-triacetoxybenzamide (5c) showed the best inhibiton of hCA II with a Ki value of 7.09±0.32 nM, where acetazolamide (AZA) had Ki value of 9.76±0.03 nM. As an overall trend it can be seen that hydroxyl substitution performed better hCA II inhibition compared to acetoxy substitution.

Three mechanisms for CA inhibition reported in the literature. As our acetyl protected compounds has no hydroxyl or primary sulphonamide groups, possible mechanism could be the

blocking the active site cavity of the enzyme. According to the structure-activity relationship (SAR), unfortunately, it is hard to say anything on inhibiton properties related with the molecular structures of the compounds 5 and 6.

4. Conclusion

In conclusion, the novel sulphonamide derivatives were evaluated for their hCA I and hCA II isozymes inhibition activities. They were found to be sufficiently active at low nano molar levels. *N-sulfachloropyridazine-p-acetoxybenzamide (**5a**) and N-sulfacetamide-3,4,5-triacetoxybenzamide (**5c**)* were found to be the best inhibitors of hCA I (K_i: 7.82±0.02 nM) and hCA II (K_i: 7.09±0.32 nM), respectively.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/OC

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