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Letter
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Design, Synthesis, and X-ray of Selenides as New Class of Agents for Prevention of Diabetic Cerebrovascular Pathology

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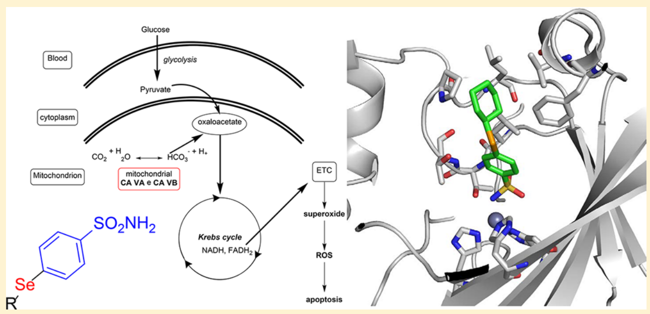
Supporting Information

ABSTRACT: A series of novel selenides bearing benzenesulfonamide moieties was synthesized and investigated for their inhibition on six human (h) carbonic anhydrase (CA, EC 4.2.1.1) isoforms such as the physiologically relevant hCA I, II, VA, VB, VII, and IX and the X-ray complex in adduct with hCA II for some of them investigated. These enzymes are involved in a variety of diseases including glaucoma, retinitis pigmentosa, epilepsy, arthritis, metabolic disorders, and cancer. The investigated compounds showed potent inhibitory action against hCA VA, VII, and IX, in the low nanomolar range, thus making them of interest for the development of isoform-selective inhibitors and as candidates for various biomedical applications.



KEYWORDS: *Carbonic anhydrase inhibitors (CAIs), diabetic pathology, selenium, metalloenzymes, organoselenium*

Diabetes is a chronic disease, and the number of people in the world with the disease has risen from 108 million in 1980 to 422 million in 2014.[1] Several complications of diabetes associated with hyperglycemia lead to disrupt the microvasculature of insulin-insensitive tissues such as the eye, nerve, and brain.[2] These complications are caused by oxidative stress and lead to pericyte loss,[3,4] disruption of the blood−brain barrier (BBB),[5,6] and cognitive decline.[7−9] The BBB is composed largely of endothelial cells in the microvasculature of the central nervous system. In close proximity to endothelial cells one finds cerebral pericytes, another type of cells vital for the integrity of the BBB.[10] Pericyte cells are especially susceptible to oxidative stress, which leads to cell death by apoptosis.[11] One cause of oxidative stress is the overproduction of superoxide as a byproduct during excess respiration caused by produced influx of glucose in insulin sensitive tissues.[2] Mitochondrial human carbonic anhydrases hCA VA and VB (CAs, EC 4.2.1.1) play a central role in the metabolism of pyruvate, by sustaining the rate of respiration[12] as described in Figure 1.

Pyruvate is the end-product of the glycolytic pathway obtained by glucose in the cytosol. Pyruvate enters the mitochondria where it is carboxylated to oxaloacetate. In this step, the production of bicarbonate anion required for carboxylation is produced by mitochondrial hCAs through reversible reaction between water and carbon dioxide ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$).[17] Oxaloacetate, when entering the Krebs cycle, produces $FADH_2$ and NADH, which are carried out to the electron transfer chain (ETC) to generate ATP. O_2^- is a byproduct of ETC reactions, and it is the precursor of all ROS. The high activity of the Krebs cycle produces a high potential of mitochondrial membrane, which inhibits the transport of electrons to complex III, increasing the half-life of the free radicals of the coenzyme Q and reducing O_2 to O_2^-. Mitochondrial CA isoform VA was identified as one of the major contributors to brain diabetic disease;[13] moreover, it was discover that hCAs IX and XII are recently implicated in ischemia-induced cerebrovascular pathology,[14] making these enzymes attractive drug targets for obtaining agents that can interfere with these deleterious processes. This may lead to novel strategies to prevent diabetic complications in the brain and possibly in other insulin-insensitive tissues. Sulfonamides are the most widely investigated class of CAIs; among these, the most investigated are acetazolamide (**AAZ**), zonisamide (**ZNS**), and topiramate (**TPM**) as pharmacologic inhibitors of mitochondrial hCAs[15−17] as outlined in Figure 2.

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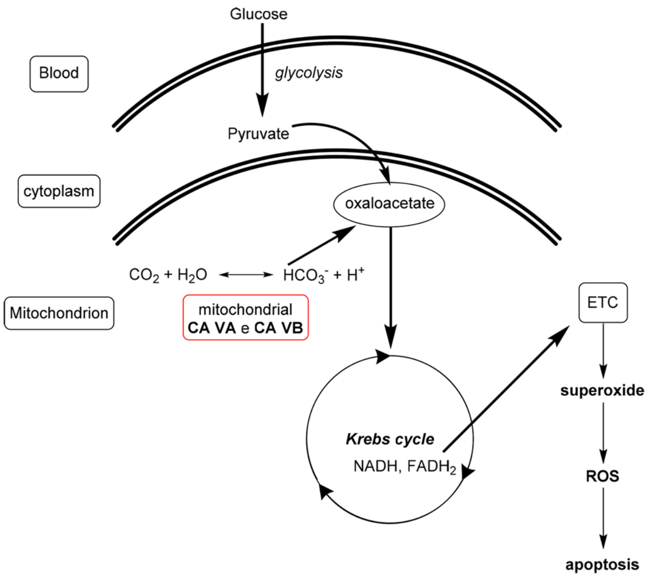
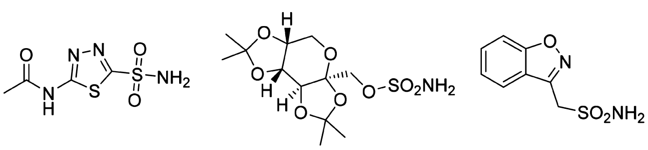


Figure 1. Mitochondrial CAs in ROS production and their role in brain pericytes apoptosis.



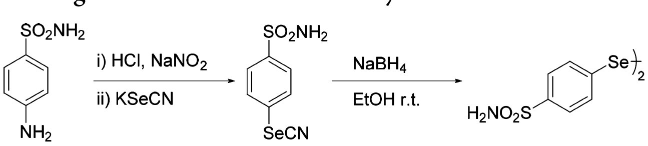
AAZ **TPM** **ZNS**

Figure 2. Structures of pharmacologic mitochondrial hCAs inhibitors: acetazolamide (**AAZ**), topiramate (**TPM**), and zonisamide (**ZNS**).

Topiramate, however, had several and serious side effects owing to its ability to inhibit other metabolic pathways,[18,19] among which, there are nonmitochondrial CA,[20] and in many cases, it is not well tolerated by patients. Thus, it is essential to develop newer drugs with more selectivity and fewer side effects.

Compound Design and Synthesis. The drug design, in this study, starts with the idea to combine the sulfonamide moiety as a typical zinc binding group (ZBG) with the interesting antioxidant propriety of organo-selenium scaffolds.[21−23] In the present study, we investigated different selenides incorporating a benzenesulfonamide moiety as a carbonic anhydrase inhibitor (CAI). The synthetic approach toward the synthesis of diselenide **3** is shown in Scheme 1. The diazonium salt of sulfanilamide was prepared by reaction of **1** with sodium nitrite in the presence of acid (Sandmeyer reaction) and used as a key intermediate for the synthesis of compound **2**. Successively, the selenocyanate derivative **2** was converted easily into the diselenide **3** as reported previously by our group[24] and outlined in Scheme 1.

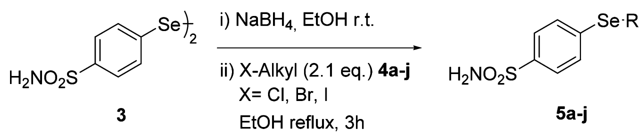
Scheme 1. Synthesis of Selenocyanate 2 and Diselenide 3 Bearing Benzenesulfonamide Moiety



1 **2** **3**

Thereafter, **3** was reduced with $NaBH_4$ to the corresponding selenolate, which in turn was treated *in situ* with the appropriate halo-alkyl moieties **4a−j**, affording the selenides **5a−j** in good yields (Table 1).

Table 1. Synthesis of Selenides Bearing Benzenesulfonamide Moieties, 5a−j



Entry	X-Alkyl	Product	Yield (%)[a]
1	**4a**	**5a**	68
2	**4b**	**5b**	69
3	ICH_3 **4c**	**5c**	60
4	**4d**	**5d**	68
5	**4e**	**5e**	54
6	**4f**	**5f**	50
7	**4g**	**5g**	53
8	**4h**	**5h**	60
9	**4i**	**5i**	72
10	**4j**	**5j**	87

[a]Yields refer to isolated products.

As a further investigation, in order to propose an alternative way to access the target compounds, we sought to achieve selenides from the selenocyanate **2**, thus avoiding the synthesis of the diselenide **3**. We were pleased to observe that selenides **5a−j** were obtained by nucleophilic substitution with the

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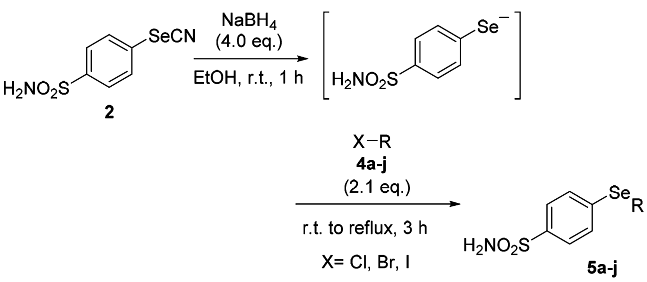
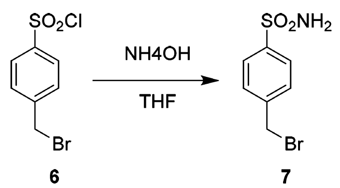
selenolate, *in situ* generated by reducing **2**, as reported in Scheme 2.

Scheme 2. Synthesis of Selenides 5a−j Bearing Benzenesulfonamide Moieties



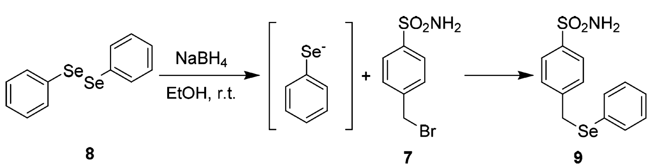
In order to access a different benzenesulfonamide with a seleno aromatic tail (**9**), the procedure started with synthesized 4-(bromomethyl)benzenesulfonamide **7** as reported in Scheme 3. Compound **7** was synthesized following the method of Naganawa et al.[25]

Scheme 3. Synthesis of 4-(Bromomethyl)benzenesulfonamide 7



Finally, the selenide **9** was obtained from the reduction of diphenyldiselenide **8** with $NaBH_4$ and was added *in situ* to sulfonamide **7** to afford in good yield the selenide **9** as outlined in Scheme 4.

Scheme 4. Synthesis of 4-((Phenylselanyl)methyl)benzenesulfonamide 9



Carbonic Anhydrase Inhibition. In this study was investigated the CA inhibition profile compounds **2**, **3**, **5a−j**, and **9** against six human isoforms such as the physiologically relevant CA I, II, VA, VB, VII, and IX by means of the stopped-flow carbon dioxide hydration assay[26] after a period of 15 min of incubation of the enzyme and inhibitor solutions.[27−31] Their activities were compared to the standard CAIs acetazolamide (AAZ) and topiramate (TPM) (Table 2).

One goal of this study was to generate compounds containing organoseleno scaffolds with antioxidant properties to improve selectivity of the targeting of mitochondrial hCA (VA and VB) compared with the clinically used topiramate inhibitor (**TPM**). For the preliminary investigation, we have tested the key intermediates **2** and **3**. We observed that the replacement of a selenocyanate group by a diselenide was well tolerated except for hCA I, so compound **3** displayed a constant of inhibition in the micromolar range (K_i = 1522.7 nM). The following structure−activity relationships (SARs) for selenides **5a−j** and **9** were observed by analyzing CA inhibition profile in Table 2. The inhibition profile of hCA I was comparable to standards **AAZ** and **TPM**, except for **5d−f** and **5i**. These compounds showed an activity more than 10 times greater for **5i** and over 25 times greater for **5d−f**. The other dominant cytosolic isoform, hCA II, was inhibited by almost all selenides with an inhibition constant in the medium nanomolar range, except for compound **5a** in the high nanomolar range (K_i = 6.3 to 355 nM). An interesting inhibition profile was observed for the mitochondrial isoform hCA VA. All compounds were potent inhibitors with an activity in the low nanomolar range (K_i = 2.9 to 8.8 nM). However, the small and cyclic moieties found in **5c**, **5d**, and **5i** led to less active inhibitors. This potency was substantially higher than those for the reported standards; thus, these compounds were excellent candidates for follow-up cell-based studies. In addition, the second mitochondrial isoform hCA VB was also well inhibited by almost all selenides reported here. An interesting case was offered by compound **5a** with **5j**. The difference in structure is a hydroxyl group versus a methoxy moiety, respectively. However, compound **5a** proved to be nearly 10-fold less potent than **5j**. The last cytosolic human isoform here studied, hCA VII, was also strongly inhibited by selenides **5a−j** with inhibition constants spanning from the subnanomolar (K_i = 0.24 nM) to the medium nanomolar range (K_i = 27.4 nM). Compound **5a**, this time, showed a potency of inhibition 35-fold less than that of the compound incorporating the methoxyl tail (**5j**). The transmembrane isoform hCA IX was effectively inhibited by all compounds here reported with K_is in the range of 2.2−12 nM. The SAR is almost absent in this case, as all selenides showed a very comparable behavior as potent hCA IX inhibitors. In addition, hCA IX is not only implicated in ischemia-induced cerebrovascular pathology[14] but its expression is upregulated in a wide type of hypoxic tumors. Thus, several of these compounds may have potential as anticancer therapeutics.

Table 2. Inhibition Data of Human CA Isoforms I, II, IV, VA, VB, VII, and IX with Compounds 2, 3, 5a−j, 9, AAZ, and TPM by a Stopped Flow CO_2 Hydrase Assay[26]

	K_i (nM)[a]					
Compound	hCAI	hCA II	hCAVA	hCAVB	hCA VII	hCAIX
2	95.6	53.1	8.2	5.7	7.1	9.3
3	1522.7	7.9	7.3	5.7	40.5	2.7
5a	338.3	355.3	5.4	46.8	27.1	2.4
5b	256.8	9.3	5.1	36.0	0.31	8.7
5c	352.2	73.2	7.3	31.3	4.6	2.7
5d	9.7	69.9	7.1	5.7	0.28	2.4
5e	5.2	36.5	6.9	45.1	0.27	2.6
5f	7.3	9.3	5.8	22.8	0.25	2.7
5g	293.5	7.6	7.1	16.8	2.0	2.2
5h	297.1	70.8	2.9	12.3	0.38	2.6
5i	21.9	6.3	8.8	12.7	0.24	2.6
5j	261.7	41.2	4.0	5.5	0.77	12.0
9	226.1	53.0	5.4	19.1	6.6	2.7
AAZ	250	10	63	54	2.5	25.8
TPM	250	10	63	30	9.0	58

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of 5−10% of the reported values).

Complex of hCA II/Selenides Ligand Complex. The protein complex with compound **5e** was determinate by X-ray crystal structure, and we had obtained ligand−protein

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interactions at the atomic level (Figure 3). hCA II was selected as a model isoform for crystallization because it easily forms

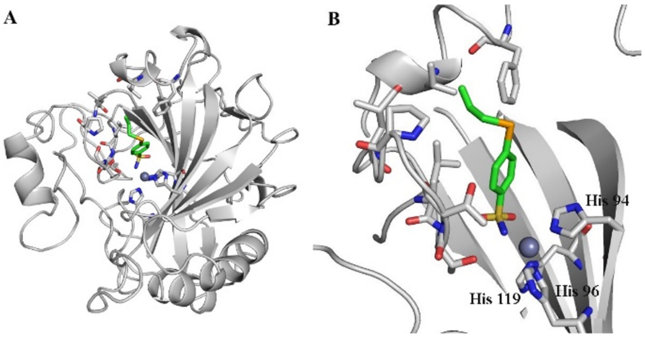

Figure 3. Whole protein hCA II with **5e** bound (A) and active site region of the hCA II/**5e** complex (B) (PDB code: 6CEH).

crystals, and many studies have been reported on its structure with different classes of inhibitors.[32]

Active site analysis of selenide **5e** showed that the sulfonamide moiety of inhibitor was tightly coordinated to the Zn(II) ion by a binding mode to the protein similar to other CAIs containing the same ZBG.[32] Additionally, hydrophobic residues that are in relatively close proximity of the inhibitor scaffold are shown around the alkyl chain of compound **5e** (Figure 3b).

Biological Assays. Some selected compounds (**3**, **5a**, **5d**, **5h**, and **5j**), chosen among those possessing an interesting inhibitory profile against the mitochondrial hCA VA and VB, were tested to evaluate their effects on the viability of rat brain endothelial (RBE4) cells, cultured in oxidative stress conditions in the presence of glucose oxidase (GOx). First of all, we evaluated the cytotoxicity of the tested compounds against RBE4, and the cell viability is shown in Figure 4.

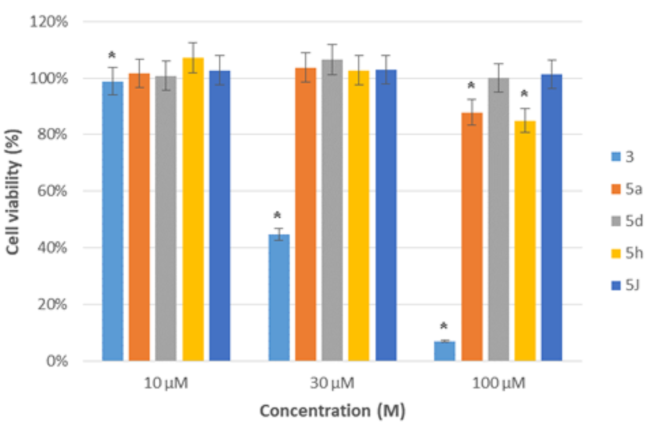

Figure 4. RBE4 cell viability (4×10^4 cell/well) following 24 h incubation with compounds **3**, **5a**, **5d**, **5h**, and **5j** at 10, 30, and 100 μM. *p < 0.001 versus control.

Diselenide **3** was significantly cytotoxic, inducing a mortality of about 50% at a concentration of 30 μM. All other selenides here reported proved to be safe at low concentrations. One should note that a decrease of cell viability was measured at high concentrations (100 μM), after 24 h treatment with compounds **5a** and **5h**. The safe compounds **5a**, **5d**, **5h**, and **5j** were evaluated as possible scavengers of oxidative stress by GOx. RBE4 cells were incubated with glucose oxidase in two concentrations (0.1 and 0.3 U/mL) and selenides, and the results are shown in Figure 5.

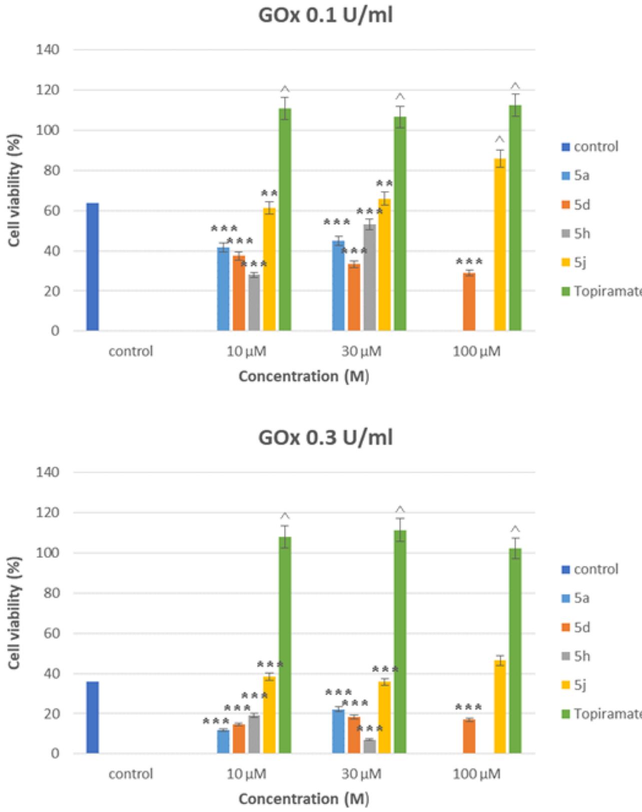

Figure 5. RBE4 cells (4×10^4 cell/well) were incubated 1 h with GOx (0.1, 0.3 U/mL) and **5a**, **5d**, **5h**, and **5j** (10 and 30 μM, also 100 μM, when it was not cytotoxic), and the following 24 h, they recovered in culture medium with the compounds at the same concentrations. **p < 0.01, ***p < 0.001 versus control; ^p < 0.05 vs GOX 0.1 U/mL.

Cell viability of control decreases up to 40% with enhancement of GOx. Among the tested compounds, only selenide **5j** showed good protection of cerebral endothelial cells from GOx damage at the higher dose (100 μM). The other selenides did not show any significant scavenger activity in these assay conditions.

Conclusion. We have designed, synthesized, and obtained an X-ray crystallographic structure of a novel series of selenides bearing the benzenesulfonamide moieties, which behave as potent mitochondrial CA inhibitors. Compound **5j** proved to be effective as a scavenger agent on RBE4 cell line. Selective binding to mitochondrial CA is an important undertaking for targeting diabetic cerebrovascular pathology and is challenging with classical pharmacologically using sulfonamide/sulfamate CA inhibitors. The findings reported here are of substantial interest and highlight the potential of selenides bearing benzenesulfonamide groups to be exploited for the discovery of potent, mitochondrial-selective CA inhibitors. Thus, in the future we will design new selenides with more selectivity and protector attributes for viability of rat brain endothelial (RBE4) cells.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00076.

Synthetic procedures, characterization of compounds, in vitro kinetic procedure, X-ray crystallography, and biological assay (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BBB, blood−brain barrier; CAs, carbonic anhydrases; ZBG, zinc binding group; AAZ, acetazolamide; ZNS, zonisamide; TPM, topiramate; RBE4, rat brain endothelial cell; GOx, glucose oxidase; ETC, electron transfer chain

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