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FULL PAPER

5-[2-(N-(substituted phenyl) acetamide)] amino-1,3,4-thiadiazole-2-sulfonamides as selective carbonic anhydrase II inhibitor with neuroprotective effects

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Abstract: In this study, 22 novel compounds were designed and synthesized by acetamide bridge chains, among which 5a-5k were mono-substituted compounds, and 6a-6k were di-substituted. A series of biological evaluations was then carried out to determine the carbonic anhydrase inhibitory activity, neuroprotective effects and cytotoxicity of 5a-5k and 6a-6k. The results showed that some compounds could protect PC12 cells from sodium nitroprusside (SNP)-induced damage. In terms of the neuroprotection and inhibitory activity against carbonic anhydrase II, mono-substituted compounds were better than di-substituted. Compound **5c** exhibited better protective effect in PC12 cells than that of edaravone and **5c** also showed less cytotoxicity. In addition, compound **5c** was the most effective selective carbonic anhydrase II inhibitor (IC_{50}=16.7 nM, CAI/CAII=54.3), which was comparable to the inhibitory effect of acetazolamide. Moreover, the selectivity of compound **5c** was better than that of acetazolamide (IC_{50}=12.0 nM, CAI/CAII=20.8). Molecular docking presented that the binding effect of compound **5c** with carbonic anhydrase II was superior to that of **5c** with carbonic anhydrase I and IX, which was consistent with the inhibitory results. Based on above findings, compound **5c** may be a potential candidate for selective carbonic anhydrase II inhibitor, and it had obviously neuroprotective effect and great advantages in drug safety.

Introduction

Glaucoma is one of the main causes of irreversible blindness in the world.[1, 2] By 2040, the number of glaucoma patients worldwide is predicted to reach 111.8 million.[3, 4] The clinically used drugs for the management of glaucoma are carbonic anhydrase (CA) inhibitors such as acetazolamide, methazolamide, and ethoxzolamide.[5-7]

CAs is widespread in organisms, varying with respect to tissue distribution and subcellular localization.[8, 9] Fifteen α-isozymes (CA I–XV) have been isolated and characterized so far, but only 12 of them are catalytically active.[10-12] CA II is the most widely expressed isoform and is identified in the anterior chamber of the eye, and that is also responsible for the production of bicarbonate and the main constituent of the aqueous humour.[13] CAI and II are ubiquitous CA isoforms, and both exist in the cytoplasm. CAII is associated with epilepsy,[14] glaucoma[15] and neurodegenerative disease,[16] while CAI is responsible for multiple side effects of CA inhibitors.[17,18] Therefore, compared with the off-target CA I isoform, it would have made more sense the evaluation of hCA II selectivity. hCA IX is a membrane-bound CAs that is overexpressed in hypoxic tumor cells and associated with many types of human cancers, which is widely used for screening tumor targets.[19]

The sulfonamide inhibitors can inhibit all human CA isoforms. However, they are not safe and limited due to their non-specificity and side effects, such as gastrointestinal disorders, acidosis, myopia, depression and sleepiness.[17, 20, 21] Therefore, it was a key task to develop a novel CA II inhibitor with specific selectivity and minimal side effects.

In fact, glaucoma is a degenerative optic nerve disease. And higher intraocular pressure could damage the optic nerve and cause its function loss. Recently, many neuroprotectants have been used for treatment of glaucoma by the means of lowering the intraocular pressure to improve the nerve function.[22-25] Hence, neuroprotection has become an important method for the management of glaucoma.

In this study, our aim was to discover a selective carbonic anhydrase II inhibitor with neuroprotective effects. The research firstly used acetazolamide as the lead compound to design and synthesize 22 novel compounds through acetamide bridge chains. Then it examined the inhibitory activity against carbonic anhydrase isoforms I, II and IX, neuroprotective effects on PC12 and the cytotoxicity of compounds **5a-5k** and **6a-6k** . At last, the research also performed docking and ADME properties prediction

experiments on optimal compounds to find potential candidates.

Results and Discussion

Chemistry

Optimization of reaction conditions

According to previous literature,[26, 27] mono- or di-substituted products cannot be obtained simultaneously in a reaction. After optimizing the reaction conditions by the study, mono- and di-substituted compounds were obtained in a reaction. The synthetic route was shown in Scheme 1. First, 5-amino-1,3,4-thiadiazole-2-sulfonamide **(2)** and 2-bromo-N-(4-fluorophenyl) acetamide **(4c)** served as reactants, with DMF as solvent,[28, 29] and reaction was carried out at room temperature. Results discovered that only di-substituted products were formed. Thus, the reaction conditions were optimized by controlling the reaction temperature, equivalent ratio of substrates, type and amount of alkali to improve the yield of mono-substituted product. The analysis was performed by using HPLC (Fig.1).

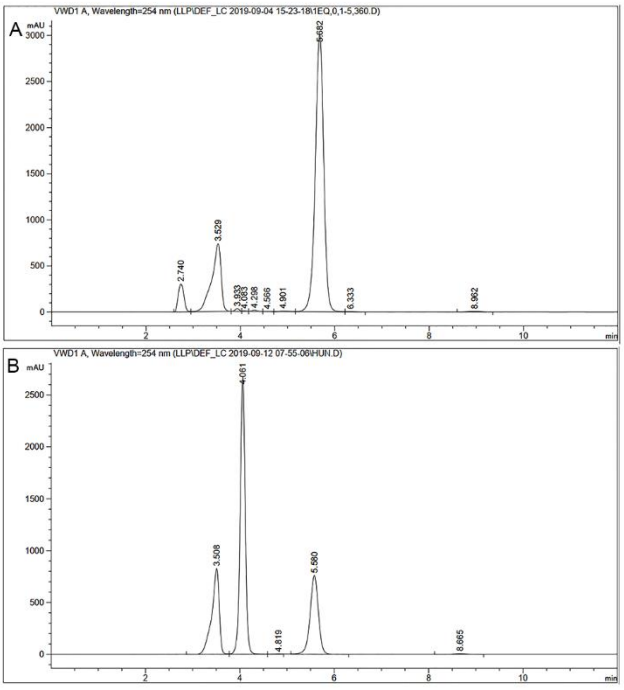


Fig.1 HPLC of 4c, 5c and 6c (A: 4c+5c+6c, B: Reaction solution)

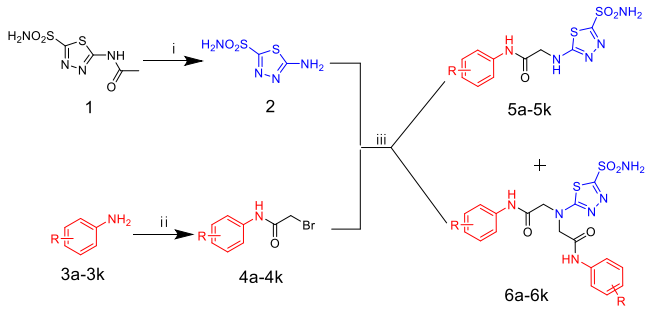
(ⅰ) Temperature: Reactions were carried out at 25, 0, -20 and -40℃, with 1 eq K_2CO_3 and the equivalent ratio of **4c:2** of 1:1. Results found that the lower the temperature was, the slower the reaction proceed. At 25℃, although reaction was completed in 2 h, only 3% mono-substituted product was produced. At 0℃, the reaction obtained 5% of mono-substituted products and the remaining 59% of raw materials. At -20℃, it obtained 21% of mono-substituted products and the remaining 56% of raw materials, and no products were formed at -40℃. As the data of the reaction shown, the reaction would control the temperature at -20℃ and simultaneously control other conditions to maximize production of mono-substituted and di-substituted products (Table 1, entries1-4).

(ⅱ) Equivalent ratios of **4c:2**: Reactions were carried out with 1 eq K_2CO_3 and the equivalent ratios of **4c:2** were 1:1, 1:3 and 1:5 at -20℃. Results discovered that the ratio of mono- and di-substituted products increased with the equivalence ratio of **4c:2** increasing. When the equivalent ratio of **4c:2** was changed to 1:5, raw materials were completely transformed. As a result, mono-substituted products have increased, which accounted for 22% of mono- and di-substituted products (Table 1, entries 4-6).

(ⅲ) Types of alkali: Compared with the reaction of catalyst $KHCO_3$, Na_2CO_3, CH_3COONa or no alkali, the reaction of catalyst K_2CO_3 acquired higher ratio of mono-substituted and di-substituted products, what's more, it was carried out completely. Therefore, K_2CO_3 was the optimal catalyst (Table 1, entries 6-10).

(ⅳ) Alkali dosage: Reactions were carried out at -20℃, with 1 eq, 0.5 eq, 0.3 eq of K_2CO_3, and **4c:2** of 1:5. The amount of K_2CO_3 was 0.3 eq and 0.5 eq, the reaction retained 69% and 17% of the raw materials respectively. When the amount of K_2CO_3 was 1eq, the reaction went on completely. Meanwhile, the reaction rate of 1 eq K_2CO_3 was high than that of 0.3 eq and 0.5 eq (Table 1, entries 6, 11-12).

Based on above experimental datas, the optimal reactions were carried out at -20℃, with 1 eq of K_2CO_3, and **4c:2** of 1:5.



Scheme 1. Reaction scheme for synthesis of compounds **5a-5k** and **6a-6k**. Reagents and conditions: (i) 3mol/L Hydrochloric acid, reflux, 3h; (ii) Glacial acetic acid, 5% sodium acetate, bromoacetyl bromide, room temperature, 1 h; (iii) Dimethyl sulfoxide, potassium carbonate, 4h.

Table 1. Optimization of reaction conditions

Entry	Base	Alkali (eq)	4c/2	T (℃)	5c/6c	(5c+6c)%
1	K_2CO_3	1	1/1	25	3/97	100
2	K_2CO_3	1	1/1	0	5/95	41
3	K_2CO_3	1	1/1	-20℃	21/79	44
4	K_2CO_3	1	1/1	-40℃	/	/
5	K_2CO_3	1	1/3	-20℃	15/85	97
6	**K_2CO_3**	**1**	**1/5**	**-20℃**	**22/78**	**100**
7	$KHCO_3$	1	1/5	-20℃	22/78	51
8	Na_2CO_3	1	1/5	-20℃	13/87	79
9	CH_3COONa	1	1/5	-20℃	/	/
10	no base	1	1/1	25	/	/
11	K_2CO_3	0.5	1/5	-20℃	13/87	83
12	K_2CO_3	0.3	1/5	-20℃	4/96	31

Characterization of structures

The structures of target products were verified by NMR, MS, UV and IR. The purity of these compounds was determined by HPLC. The difference between two series can be detected with ^{1}HNMR, for example, mono-substituted product (**5c**) had N-H signals with ^{1}HNMR (Fig. 2A), while di-substituted product (**6c**) had not (Fig. 2B).

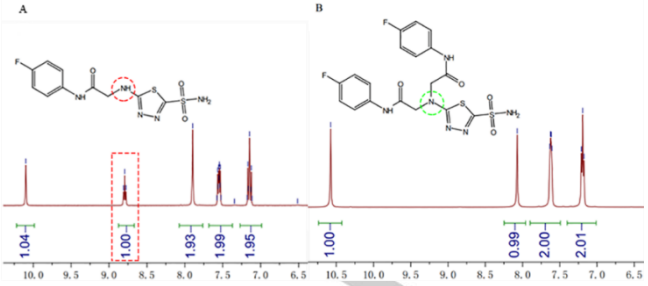


Fig.2 ^{1}HNMR of (A) 5c and (B) 6c

Biological tests

Carbonic anhydrase inhibition

The values of inhibition by compounds **5a-5k** and **6a-6k** against hCA I, II and IX were determined by an esterase assay[30-31]. Results are shown in Table 2.

(i) Compounds **5a-5k** and **6a-6k** had moderate inhibitory activity against hCA I. IC_{50} values of compounds **5a-5k** ranged from 907.3 to 2683.0 nM, and those of compounds **6a-6k** ranged from 2664 to 7080 nM.

(ii) Compounds **5a-5k** had significant inhibitory effect against hCA II which IC_{50} values of compounds **5a-5k** ranged from 16.7 to 516.3 nM, in contrast, most of compounds **6a-6k** exhibited moderate-weak inhibitory activity which compounds **6a-6k** ranged from 45.9 to 4698 nM. Compounds **5c** (F-substituted product) was the most active, with an IC_{50} of 16.7 nM.

(iii) Different phenyl ring substituents played a key role in inhibitory activity against CA II. IC_{50} values of compounds **5a, 5c, 5h, 5i, 5j** and **5k** were less than 100 nM. For F-substituted products, para-F product was strongest (para-F (**5c**) > meta-F (**5j**) > ortho-F (**5k**), with IC_{50} values of 16.7 nM, 54.7 nM, and 73.4 nM, respectively). When the halogen atom was at para-position of benzene ring, 4-F product was the most effective (4-F (**5c**) >4-Cl (**5a**) >4-Br (**5d**) > 4-I (**5g**), with IC_{50} values of 16.7 nM, 90.6 nM, 212.8 nM, and 516.3 nM, respectively).

(iv) For compound **5c**, IC_{50} values against hCA I and hCA II were 907.3 nM and 16.7 nM, and the selectivity of hCA I/CA II was 54.3. Otherwise, for acetazolamide as a positive control drug, IC_{50} values against hCA I and hCA II were 250.0 nM and 12.0 nM, and the selectivity of hCA I/CA II was 20.8. Compound **5c** had an IC_{50} of 16.7 nM for CA II, which was comparable to the inhibitory effect of acetazolamide, but the selectivity of **5c** against hCA I/CA II was 2.6 times as strong as that of AZA. Therefore, **5c** may have fewer side effects than acetazolamide.

(v) Compounds **5a-5k** and **6a-6k** had weak inhibitory activity against hCA IX. Except for compounds **5a, 5e, 5g, 5h** and **5i,** the IC_{50} values of rest compounds were all more than 10000 nM. Acetazolamide, a broad and non-selective CA inhibitor, had an IC_{50} of 25 nM for CAIX. The difference was that compound **5c** had poor selectivity for CAIX and it only targeted CAII.

Table 2. Inhibitory activity of target compounds on carbonic anhydrase I, II and IX

Entry	R	hCAI (IC_{50}, nM)[1,2]		hCA II (IC_{50}, nM)[1,2]		hCAIX (IC_{50}, nM)[1,2]	
		Comps 5	Comps 6	Comps 5	Comps 6	Comps 5	Comps 6
a	4-Cl	1611.0	3964.0	90.6	392.7	＞10000	＞10000
b	4-OCH_3	2060.0	7080.0	305.0	1100.0	＞10000	＞10000
c	4-F	907.3	2664.0	16.7	45.9	4757	＞10000
f	4-Br	1492.0	4276.0	212.8	732.9	＞10000	＞10000
e	4-CF_3	1543.0	4661.0	309.5	926.8	4499	＞10000
f	4-CH_3	1123.0	5781.0	221.6	730.2	＞10000	＞10000
g	4-I	2683.0	4886.0	516.3	4698.0	2300	＞10000
h	4-$C(CH_3)_3$	1214.0	3861.0	74.2	157.4	5471	＞10000
i	4-H	1188.0	4130.0	209.8	629.8	2285	＞10000
j	3-F	1058.0	2789.0	54.7	89.3	＞10000	＞10000
k	2-F	1244.0	34480.	73.4	178.2	＞10000	＞10000
AZA[3]		250.0		12.0		25.0	

[1] IC_{50} values were determined by the 4-nitrophenyl acetate (4-NPA) esterase assay.

[2] Mean from 3 different assays, by an esterase assay (errors were in the range of ± 5-10% of the reported values).

[3] Acetazolamide as positive reference material.

Cell viability assay

The excessive release of NO can cause neurotoxicity and many neurodegenerative diseases[32, 33] by mediating oxidative stress in human body. SNP is widely used as a direct donor of NO in experiments.[34, 35] Rat pheochromo cytoma PC12 cells are commonly used as nerve cells in study of neurodegenerative diseases.[36] First, SNP-induced PC12 cell injury model was established. The PC12 cells were cultured with SNP at different concentrations ranging from 500 to 700 μM for 24 h, and SNP concentration which could result in cell viability up to 60% was obtained. As shown in Fig. 3A, SNP concentration of 650 μM was chosen.

Compounds **5a-5k** and **6a-6k** were preliminarily screened by MTT assay. Edaravone was used as a positive control (edaravone is known as a free radical scavenger and is used to treat stroke in Japan).[36] Preliminary screening result (Fig. 3B) displayed that compounds **6a-6k** not only exhibited less protective effects, but also produced cytotoxicity. While compounds **5a, 5b, 5c, 5g** and **5i** could effectively protect PC12 cells, and their activities were better than that of edaravone.

The protective efficacy of different concentrations of compounds **5a, 5b, 5c, 5g** and **5i** was assessed to further investigate and compare their potency by MTT assay. As shown in Fig. 3C, compounds **5a, 5b, 5c, 5g** and **5i** exhibited dose-dependent protective effects. The protective effects of

compounds **5a, 5b, 5c, 5g** and **5i** increased significantly at 20 μM better than 50 μM edaravone and 20 μM acetazolamide.

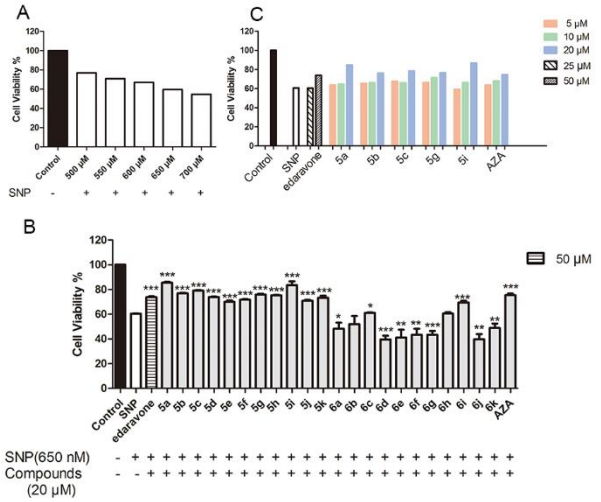


Fig. 3 PC12 cell viability (A) PC12 cells were treated with different doses of SNP followed by incubation for 24 h. (B) PC12 cells were pretreated with 50 μM edaravone and 20 μM target compounds and AZA for 2 h followed by treatment with 650 μM SNP for another 24 h. (C) PC12 cells were pretreated with 5, 10, 20 μM **5a, 5b, 5c, 5g, 5i** and 25, 50 μM edaravone for 24 h followed by treatment with 650 μM SNP for another 24 h and measured by MTT assay. (Data were expressed as the mean ± SD of three replicates ***p < 0.0005, **p < 0.005 and *p < 0.05 compared to SNP group).

Cytotoxicity assay

The cytotoxicity of compounds was an important index for evaluation of medicinal properties in drug research and development. The cytotoxicity of compounds **5a-5k** against HEK293 (human embryonic kidney normal cell line), LO2 (human normal hepatocytes) and PC12 (mouse pheochromo cytoma) cells was determined by MTT method in vitro (Table 3). In HEK293 cells, IC_{50} values of **5a-5k** ranged from 136.10 ± 2.72 μM to 163.50 ± 5.50 μM, which were all above 100 μM and higher than that of acetazolamide (IC_{50}=135.20 ± 1.74 μM). Thus, compounds **5a-5k** could be considered essentially non-toxic to HEK293 cells. For LO2 cells, IC_{50} values of **5a-5k** ranged from 162.40 ± 4.68 μM to 243.10 ± 7.53 μM, all of which were more than 100 μM. IC_{50} values of the rest compounds were higher than that of acetazolamide (IC_{50}=169.30 ± 3.33 μM), except **5g** and **5h**. For PC12 cells, IC_{50} values of **5a-5k** and acetazolamide were more than 200 μM. Therefore, compounds **5a-5k** were considered non-toxic to all three cell lines and showed a great advantage in safety over positive control acetazolamide.

Table 3. Cytotoxic activity of compounds **5a-5k** against HEK293, LO2 and PC12 cell lines

Comps	R	HEK293 IC_{50} (μM)	LO2 IC_{50}(μM)	PC12 IC_{50} (μM)
5a	4-Cl	139.2±3.3	183.8±4.5	＞200
5b	4-OCH_3	140.3±1.7	223.0±5.4	＞200
5c	4-F	157.5±0.2	243.1±7.5	＞200
5d	4-Br	144.8±4.7	216.5±4.9	＞200
5e	4-CF_3	136.1±2.7	198.3±4.8	＞200
5f	4-CH_3	137.9±3.5	176.2±6.5	＞200
5g	4-I	143.9±4.4	168.8±6.0	＞200
5h	4-$C(CH_3)_3$	1575±2.8	162.4±4.7	＞200
5i	4-H	163.5±5.5	221.8±3.45	＞200
5j	3-F	156.7±1.6	228.0±1.8	＞200
5k	2-F	152.6±3.6	188.8±2.4	＞200
AZA		144.7±19.9	169.3±3.3	＞200

Computational studies

Molecular docking

Molecular docking studies were conducted to better understand the interactions between ligands and receptors. Discovery Studio 2016 client was used to dock compound **5c** with CAI protein 5WLU,[37] CAII protein 5ULN,[38] and CAIX protein 5JN3 [39] (all from the RCSB PDB data bank), and conformation with the highest binding energy was selected for further analysis. The binding modes of compound **5c** with 5WLU, 5ULN, 5JN3 were observed by PyMol. His94, His96, His119 coordinate with Zn^{2+} to form all hCAs catalytic sites. The binding mode of compound **5c** in active site of CA I was stabilized by the four H-bonds interaction with Thr199, Thr200, Gln67, Ser65, and by the coordination of sulfonamide and zinc ion through negatively charged nitrogen (Fig. 4A-B). The active site residues Leu57, Ser73 were involved in hydrophobic interaction (Fig. 4A-B). Docking results of compound **5c** and CAII exhibited that compound **5c** interacted stably with Thr199, Leu198 and Asn67 by four H-bonds in the active site of CAII. And sulfonamide, through negatively charged nitrogen, coordinated to zinc ion, which also contributed to the stability (Fig. 4C-D). At the same time,

residue Phe131 interacted stably with benzene ring through π-π interaction. The active site residues Trp245, Phe131 and Asn62 participated in hydrophobic interactions. (Fig. 4C-D). At the active site of CA IX, compound **5c** formed three H-bonds interaction with Thr199 and His64, whereas residues Tyr7, Asn62, and Leu60 were involved in hydrophobic interaction (Fig. 4E-F).

In Figures 4C-D, the optimal position for compound **5c** binding to CAII was presented and demonstrated the ability to form an interaction network within the depths of nearby zinc ion catalytic sites. The sulfonamide group was one of the most vital functional groups in carbonic anhydrase inhibitors. The sulfonamide group of **5c** was in polar contact with the cavity of CAII. At the same time, **5c** formed hydrophobic interactions and factional interactions with multiple amino acids of CAII. Although **5c** also formed a hydrophobic interaction with the amino acids of CAI and IX, the sulfonamide group of **5c** was not strong enough to bind with CAI and IX cavities. This may explain why the inhibitory activity of **5c** on CAII was better than that of CAI and IX. Molecular docking results were consistent with those determined by an esterase method.[37-39] In addition, molecular docking experiment supported compound **5c** could become a new selective carbonic anhydrase II inhibitor.

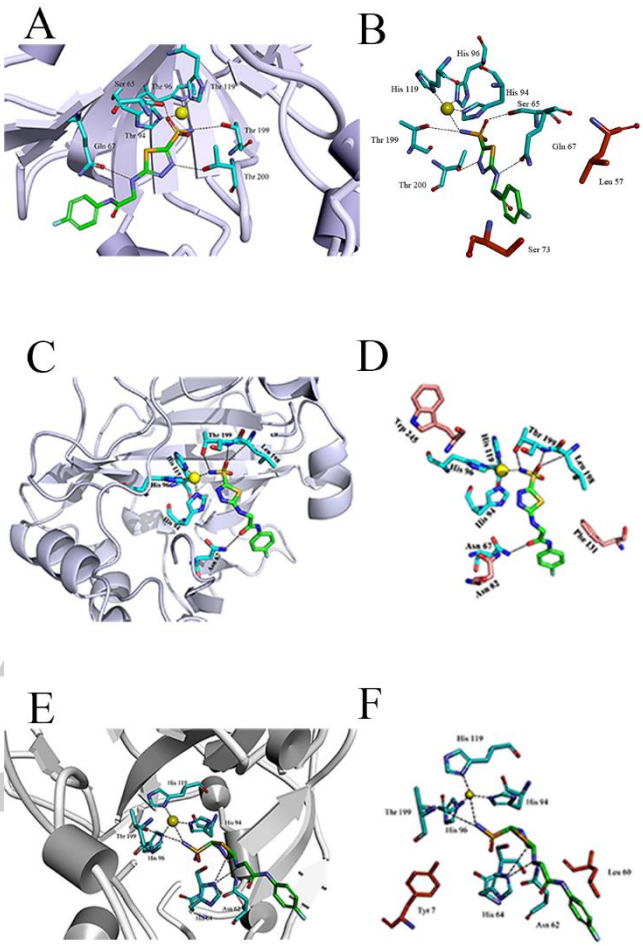


Fig. 4 Molecular Docking studies of compound 5c with CAI, CAII and CAIX: Cartoon view of (A) compound **5c** docked with CA I. (B) CAI active site residue interacted with compound **5c**. (C) compound **5c** docked with CA II. (D) CAII active site residue interacted with compound **5c**. (E) compound **5c** docked with CA IX. (F) CAIX active site residue interacted with compound **5c**.

Residues are shown with ball and stick and compound **5c** was shown with stick model. Hydrogen bonds were shown as broken lines (black).

ADME properties

ADME properties of drugs such as absorption, distribution, metabolism and excretion were the very important indicators for drug formulation. ADME properties of compound **5c** and positive control drug AZA were predicted by Molinspiration online calculation tool. Study of ADME can help researchers better understand properties of drugs. According to Table 4, compound **5c** exhibited good ADME properties, that met the

requirements with respect to TPSA, MW, miLogP, n-ON acceptors and n-OHNH donors. The results supported that compound **5c** was more likely to be a candidate for future drugs.

Table 4 The importance of pharmacokinetic parameters for good oral bioavailability of synthesized compound

Entry	%ABS	MW	MiLogP	TPSA(A^2)	n-ROTB	MV	n-ON acceptors	n-OHNH donors	Nviolations
Rule	—	≤500	≤5	＜140	—	—	＜10	＜5	≤1
5c	65.2	331.4	0.8	127.1	5	246.1	8	4	0
AZA	69.3	222.3	-1.2	115.1	2	157.1	7	3	0

MW: molecular weight, miLog P: logarithm of partition coefficient of compound between n-octanol and water, TPSA: topological polar surface area, n-ROTB: number of rotatable bonds, MV: molecular volume, n-ON acceptors: number of hydrogen bond acceptors, n-OHNH donors: number of hydrogen bonds donors.

Conclusion

The research synthesized a series of acetazolamide derivatives, mono-substituted compounds **5a-5k** and di-substituted compounds **6a-6k**. The inhibitory activities of all compounds on human carbonic anhydrases I, II and IX were studied. Mono-substituted compounds **5a-5k** were superior to di-substituted compounds **6a-6k** in biological evaluation. Compounds **5a-5k** displayed significant selective inhibition of CA II, and **5c** was the best (IC_{50}= 16.7 nM), which was comparable to the inhibitory effect of acetazolamide. What's more, the selectivity for hCA I against hCA II, 54.3, was superior to that of lead compound AZA. In addition, compounds **5a, 5b, 5c, 5g** and **5i** showed the better neuroprotection compared with the edaravone in the SNP-induced PC12 model. And compounds **5a-5k** had weaker cytotoxicity activity against HEK293, LO2 and PC12. Molecular docking presented that the binding effect of compound **5c** with carbonic anhydrase II was superior to carbonic anhydrase I and IX, which was consistent with the inhibitory results. Besides, ADME properties of **5c** indicated that **5c** may have medicine properties. In conclusion, compound **5c** may be the potential candidate drug for novel selective carbonic anhydrase II inhibitor with neuroprotective ability.

Experimental Section

Chemistry.

Acetazolamide (AZA) was purchased from Macklin. Bromoacetyl bromide was purchased from Aladdin. Anhydrous solvents, and all of the amines were obtained from Aladdin or Macklin. 4-Nitrophenyl acetate (4-NPA), HEPES, Brij and sodium chloride were all obtained from Aladdin. Buffer solution (Brij) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 15 mmol/L, pH=7.4) and tetraethylene glycol monododecyl ether (0.01%) and NaCl (100 mmol/L) was utilized. hCA I was purchased from R&D Systems Inc. hCA II and IX were purchased from Sino Biological Inc. NMR spectra were measured by 500 or 400 MHz nuclear magnetic resonance (Bruker). Mass spectrometry information was determined by a Thermo mass spectrometer. All of UV spectra were determined by an ultraviolet spectrophotometer (Shimadzu 2600). Thin layer chromatography (TLC) was carried out on GF254 silica gel (Sijia Biochemical Plastics Factory).

General procedure for the synthesis of 5-amino-1,3,4-thiadiazole-2-sulfonamide (2)

Acetazolamide (3.00 g, 13.5 mmol) was added to a 250 mL round bottom flask containing hydrochloric acid (3 M, 23.4 mL) and refluxed at 110 ℃ for 3 h. Mixture was neutralized with NaOH (4 M, 17.6 mL) and extracted with EtOAc (3 x 200 mL). The combined organic layers were then washed with brine (1 x 200 mL) and concentrated in a vacuum to obtain 5-amino-1,3,4-thiadiazole-2-sulfonamide. White solid; Yield 60%; ^{1}H NMR (400 MHz, DMSO-d6) δ 8.05 (s, 2H, -SO_2NH_2),7.80(s, 2H, -NH_2); ^{13}C NMR (101 MHz, DMSO-d6) δ 172.32, 158.42.

General procedure for the synthesis of 4a-4k

The proper substituted anilines (30 mmol) was added to 12 mL of saturated sodium acetate solution and then 12 mL acetic acid solution was added to form a suspension. Suspension was placed in an ice bath, and bromoacetyl bromide (30 mmol) was added dropwise when the temperature was less than 5 ℃. After addition, mixture was removed from the ice bath and stirred for 2 h at the room temperature. The precipitates were filtered, washed with distilled water and dried in a vacuum to obtain crude products **4a-4k,** which were recrystallized from anhydrous ethanol.[40,41]

2-bromo-N-(4-chlorophenyl) acetamide (4a): White solid; Yield 90%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.05 (s, 1H), 7.46-7.38 (m, 2H), 7.29-7.18 (m, 2H), 3.95 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.37, 135.49, 130.35, 129.19, 121.28, 29.35.

2-bromo-N-(4-methoxyphenyl)acetamide (4b): White solid; Yield 89%; ^{1}H NMR (400 MHz, Chloroform-d) δ 8.08 (s, 1H), 7.55-7.37 (m, 2H), 7.03-6.79 (m, 2H),4.04 (s, 2H),3.82 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-d) δ 163.30, 157.10, 129.97, 122.05, 114.28, 55.50, 29.49.

2-bromo-N-(4-fluorophenyl)acetamide (4c): White solid; Yield 89%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.16 (s, 1H), 7.52 (dd, *J* = 9.0, 4.6 Hz, 2H), 7.07 (t, *J* = 8.6 Hz, 2H), 4.04 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.45, 159.92 (d, J = 244.9 Hz), 132.90 (d, J = 2.7 Hz), 122.04 (d, J = 8.1 Hz), 115.87 (d, J = 22.7 Hz), 29.39.

2-bromo-N-(4-bromophenyl)acetamide (4d): White solid; Yield 85%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.07 (s, 1H), 7.45-7.33 (m, 4H), 3.95 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.30, 136.00, 132.15, 121.54, 117.98, 29.36.

2-bromo-N-(4-(trifluoromethyl)phenyl)acetamide (4e): White solid; Yield 88%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.27 (s, 1H), 7.68 (d, J = 8.5 Hz, 2H), 7.62 (d, J = 8.6 Hz, 2H), 4.04 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-d) δ 163.59, 139.94, 127.07(q, J = 33.33 Hz), 126.42(q, J = 4.0 Hz), 124.46(q, J = 272.7 Hz), 119.62, 29.26.

2-bromo-N-(p-tolyl)acetamide (4f): White solid; Yield 90%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.14 (s, 1H), 7.50-7.37 (m, 2H), 7.18 (d, *J* = 8.1 Hz, 2H), 4.03 (s, 2H), 2.35 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.33, 134.98, 134.37, 129.62, 120.20, 29.57, 20.92.

2-bromo-N-(4-iodophenyl)acetamide (4g): White solid; Yield 85%; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.10 (s, 1H), 7.83-7.57 (m, 2H), 7.48-7.27 (m, 2H), 4.01 (s, 2H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 163.36, 138.11, 136.72, 121.79, 88.68, 29.38.

2-bromo-N-(4-(tert-butyl)phenyl)acetamide (4h): White solid; Yield 87%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.16 (s, 1H), 7.44 (d, *J* = 8.8 Hz, 2H), 7.42-7.30 (m, 2H), 4.00 (s, 2H), 1.30 (s, 9H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.46, 148.32, 134.29, 125.97, 119.99, 34.48, 31.35, 29.56.

2-bromo-N-phenylacetamide (4i): White solid; Yield 90%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.37-8.06 (m, 1H), 7.78-7.46 (m, 2H), 7.36 (t, *J* = 8.0 Hz, 2H), 7.24-7.08 (m, 1H), 4.02 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.44, 136.92, 129.15, 125.26, 120.09, 29.53.

2-bromo-N-(3-fluorophenyl)acetamide (4j): White solid; Yield 83%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.30 (s, 1H), 7.48 (dt, *J* = 10.6, 2.3 Hz, 1H), 7.42-7.26 (m, 1H), 7.18 (ddd, *J* = 8.1, 2.0, 0.9 Hz, 1H), 6.86 (tdd, *J* = 8.3, 2.5, 1.0 Hz, 1H), 4.01 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ163.76, 162.95 (d, J = 229.3 Hz), 138.43 (d, J = 10.8 Hz), 130.26 (d, J = 9.3 Hz), 115.36 (d, J = 2.9 Hz), 112.00 (d, J = 21.3 Hz), 107.60 (d, J = 26.4 Hz), 29.33.

2-bromo-N-(2-fluorophenyl)acetamide (4k): White solid; Yield 85%; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.41 (s, 1H), 8.25 (tt, *J* = 7.8, 1.2 Hz, 1H), 7.22-7.03 (m, 3H), 4.05 (s, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.48, 152.73 (d, J = 244.4 Hz), 125.57 (d, J = 10.2 Hz), 125.35 (d, J = 7.7 Hz), 124.65 (d, J = 3.8 Hz), 121.57, 115.03 (d, J = 19.0 Hz), 29.31.

General procedure for synthesis of 5a-5k and 6a-6k

First, the mixture of 5-amino-1,3,4-thiadiazole-2-sulfonamide **2** (20 mmol) and potassium carbonate (4 mmol) was added

to a 100 mL round-bottom flask, and 20 mL of DMF was added to form a suspension. After 5-amino-1,3,4-thiadiazole-2-sulfonamide **2** was completely dissolved, the suspension was cooled to -20 ℃ and stirred for 5 minutes before adding benzoyl bromides **4a-4k**. Reaction progress was monitored by TLC. At the end of reaction, 150 mL of water was added to quench reaction, and products were extracted with ethyl acetate (3 x 150 mL). The combined organic layers were washed with water, dried by the anhydrous sodium sulfate and filtered to obtain the mixture which was purified by column chromatography to give mono-substituted compounds **5a-5k** and di-substituted compounds **6a-6k**.

5-N-[2-(N-(4-chlorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5a): White solid; yield: 21%; purity (HPLC): 99.88%; m.p. 217-219; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H, -CONH-), 8.81 (s, 1H, -NH-), 7.90 (s, 2H, -SO_2NH_2-), 7.69-7.49 (m, 2H, Ar-H), 7.49-7.25 (m, 2H, Ar-H), 3.91 (s, 2H, -CH_2-); ^{13}CNMR (101 MHz, DMSO-d_6) δ 172.46, 166.74, 155.68, 138.04, 129.19, 127.46, 121.18, 46.26; IR (KBr): 3447, 3335, 3263, 3202, 1684, 1610, 1595, 1359 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9ClN_5O_3S_2$: 345.9835 [M-H]$^+$; found: 345.9842.

5-N-[2-(N-(4-methoxyphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5b): White solid; yield: 23%; purity (HPLC)：99.81%; m.p. 218-220; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.89 (s, 1H, -CONH-), 8.75 (s, 1H, -NH-), 7.89 (s, 2H,-SO_2NH_2-), 7.47-7.38 (m, 2H, Ar-H), 6.96-6.80 (m, 2H, Ar-H), 3.86 (s, 2H, -CH_2-), 3.72 (s, 3H, -OCH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.46, 165.99, 155.81, 155.76, 132.19, 121.26, 114.36, 55.63, 46.20,40.66; IR (KBr): 3420, 3314, 3270, 3142, 1682, 1602, 1575, 1361 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{11}H_{12}N_5O_4S_2$: 342.0331 [M-H]$^+$; found: 342.0339.

5-N-[2-(N-(4-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5c): White solid; yield: 20%; purity (HPLC): 98.93%; m.p. 203-205; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (s, 1H, -CONH-), 8.79 (t, *J* = 6.0 Hz, 1H, -NH-), 7.90 (s, 2H, -SO_2NH_2-), 7.71-7.46 (m, 2H, Ar-H), 7.24-7.04 (m, 2H, Ar-H), 3.89 (d, *J* = 5.9 Hz, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.46, 166.47, 158.55 (d, J = 239.9 Hz), 155.73, 135.46 (d, J = 2.4 Hz), 121.46 (d, J = 7.9 Hz), 115.83 (d, J = 22.3 Hz), 46.21; IR (KBr): 3430, 3335, 3283, 3159, 1661, 162, 1512, 1361 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9ClN_5O_3S_2$: 330.0131 [M-H]$^+$; found: 330.0137.

5-N-[2-(N-(4-bromophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5d): White solid; yield: 22%; purity (HPLC): 99.62%; m.p. 198-200; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H, -CONH), 8.80 (s, 1H, -NH-), 7.89 (s, 2H, -SO_2NH_2-), 7.60-7.39 (m, 4H, Ar-H), 3.90 (s, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.46, 166.76, 155.71, 138.45, 132.08, 121.58, 115.52, 46.30; IR (KBr): 3435, 3333, 3269, 3193, 1675, 1606, 1591, 1388 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9BrN_5O_3S_2$: 389.9330 [M-H]$^+$; found: 389.9339.

5-N-[2-(N-(4-trifluoromethyl phenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5e): White solid; yield:19%; purity (HPLC): 99.31%; m.p. 225-227; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.41 (s, 1H, -CONH), 8.84 (s, 1H, -NH-), 7.90 (s, 2H, -SO_2NH_2-), 7.75 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.68 (d, *J* = 8.7 Hz, 2H, Ar-H), 3.96 (s, 2H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.48, 167.30, 155.72, 142.61, 126.61 (d, J = 3.8 Hz), 124.79 (d, J = 272.2 Hz), 123.90 (d, J = 44.1 Hz), 119.56, 46.33; IR (KBr): 3440, 3367, 3263, 3129, 1688, 1612, 1528, 1330 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{11}H_9F_3N_5O_3S_2$: 380.0099 [M-H]$^+$; found: 380.0104.

5-N-[2-(N-(p-methylphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5f): White solid; yield: 25%; purity (HPLC): 99.72%; m.p. 211-213; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.94 (s, 1H, -CONH-), 8.75 (d, *J* = 5.8 Hz, 1H,-NH-), 7.89 (s, 2H, -SO_2NH_2-), 7.47-7.33 (m, 2H, Ar-H), 7.23-7.01 (m, 2H, Ar-H), 3.88 (d, *J* = 4.5 Hz, 2H, -CH_2-), 2.25 (s, 3H, -CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.46, 166.24, 155.76, 136.56, 132.85, 129.62, 119.69, 46.24, 20.92; IR (KBr): 3433, 3359, 3275, 3120, 1684, 1610, 1514, 1363 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{11}H_{12}N_5O_3S_2$: 326.0382 [M-H]$^+$; found: 326.0390.

5-N-[2-(N-(4-iodophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5g): White solid; yield: 27%; purity (HPLC) : 99.86%; m.p. 228-230; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.14 (s, 1H, -CONH), 8.79 (s, 1H, -NH-), 7.89 (s, 2H, -SO_2NH_2-), 7.77-7.56 (m, 2H, Ar-H), 7.50-7.30 (m, 2H, Ar-H), 3.90 (s, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ

172.46, 166.75, 155.70, 138.91, 137.91, 121.84, 87.47, 46.33; IR (KBr): 3429, 3326, 3289, 3127, 1682, 1611, 1543, 1386 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9IN_5O_3S_2$: 437.9191 $[M-H]^+$; found: 437.9201.

5-N-[2-(N-(4-tert-butylphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5h): White solid; yield: 23%; purity (HPLC)：99.78%; m.p. 201-203; ^1H NMR (400 MHz, DMSO-d_6) δ 9.96 (s, 1H, -CONH-), 8.76 (s, 1H, -NH-), 7.89 (s, 2H, -SO_2NH_2-), 7.53-7.40 (m, 2H, Ar-H), 7.40-7.24 (m, 2H, Ar-H), 3.88 (s, 2H, -CH_2-), 1.25 (s, 9H, -CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.45, 166.28, 155.76, 146.25, 136.48, 125.85, 119.50, 46.21, 34.49, 31.66; IR (KBr): 3431, 3353, 3270, 3143, 1679, 1606, 1514, 1380 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{14}H_{18}N_5O_3S_2$: 368.0851 $[M-H]^+$; found: 368.0860.

5-N-[2-(N-phenylacetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5i): White solid; yield: 22%; purity(HPLC): 97.61%; m.p. 185-187; ^1H NMR (400 MHz, DMSO-d_6) δ 10.03 (s, 1H, -CONH-), 8.78 (s, 1H, -NH-), 7.90 (s, 2H, -SO_2NH_2-), 7.66-7.47 (m, 2H, Ar-H), 7.31 (t, *J* = 7.9 Hz, 2H, Ar-H), 7.06 (t, *J* = 7.3 Hz, 1H, Ar-H), 3.91 (s, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.46, 166.53, 155.79, 139.07, 129.26, 123.93, 119.67, 46.28; IR (KBr): 3432, 3351, 3277, 3128, 1684, 1606, 1512, 1359 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_{10}N_5O_3S_2$: 312.0225$[M-H]^+$; found: 312.0234.

5-N-[2-(N-(3-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5j): White solid; yield: 23%; purity (HPLC)：99.9%; m.p. 188-190; ^1H NMR (400 MHz, DMSO-d_6) δ 10.26 (s, 1H, -CONH-), 8.82 (s, 1H, -NH-), 7.90 (s, 2H,-SO_2NH_2-), 7.52 (dt, *J* = 11.6, 2.3 Hz, 1H, Ar-H), 7.35 (td, *J* = 8.2, 6.7 Hz, 1H, Ar-H), 7.25 (dt, *J* = 8.4, 1.3 Hz, 1H, Ar-H), 7.01 – 6.78 (m, 1H, Ar-H), 3.92 (s, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.48, 166.97, 162.58 (d, J = 241.4 Hz), 155.71, 140.77 (d, J = 11.0 Hz), 130.94 (d, J = 9.5 Hz), 115.40 (d, J = 2.3 Hz), 110.42 (d, J = 21.1 Hz), 106.47 (d, J = 26.3 Hz), 46.29; IR (KBr): 3440, 3351, 3273, 3120, 1688, 1616, 1516, 1357 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9FN_5O_3S_2$: 330.0131[M-H]$^+$; found: 330.0140.

5-N-[2-(N-(2-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (5k): White solid;yield: 22%; purity (HPLC)：99.75%; m.p. 185-187; ^1H NMR (400 MHz, DMSO-d_6) δ 9.83 (s, 1H, -CONH-), 8.80 (s, 1H, -NH-), 7.89 (s, 2H, -SO_2NH_2-), 7.86-7.76 (m, 1H, Ar-H), 7.34-7.19 (m, 1H, Ar-H), 7.22-7.04 (m, 2H, Ar-H), 3.98 (s, 2H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.48, 167.14, 155.68, 153.93 (d, J = 245.1 Hz), 126.11 (d, J = 11.6 Hz), 125.88 (d, J = 7.4 Hz), 124.87 (d, J = 3.4 Hz), 124.38, 115.98 (d, J = 19.3 Hz), 45.99; IR (KBr): 3420, 3345, 3275, 3131, 1690, 1620, 1495, 1359 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{10}H_9FN_5O_3S_2$: 330.0131[M-H]$^+$; found: 330.0139.

5-N,N-bis[2-(N-(4-chlorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6a): White solid; yield: 65%; purity (HPLC): 98%; m.p. 216-218; ^1H NMR (500 MHz, DMSO-d_6) δ 10.60 (s, 2H, -CONH-), 8.06 (s, 2H, -SO_2NH_2), 7.62 (d, *J* = 8.1 Hz, 4H, Ar-H), 7.41 (d, *J* = 8.3 Hz, 4H, Ar-H), 4.32 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 167.40, 153.30, 137.86, 129.31, 127.75, 121.26,52.98; IR (KBr): 3412, 3290, 3186, 1677, 1604, 1547, 1367 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}Cl_2N_6O_4S_2$: 512.9973 $[M-H]^+$; found: 512.9983.

5-N,N-bis[2-(N-(4-methoxyphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6b): White solid; yield: 67%; purity (HPLC): 95.17%; m.p. 233-235; ^1H NMR (400 MHz, DMSO-d_6) δ 10.47 (s, 2H, -CONH-), 8.07 (s, 2H, -SO_2NH_2), 7.53 (d, *J* = 8.6 Hz, 4H, Ar-H), 6.93 (d, *J* = 8.7 Hz, 4H, Ar-H), 4.28 (s, 4H, -CH_2-), 3.74 (s, 3H，-OCH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.82, 166.80, 156.09, 153.32, 132.09, 121.35, 114.54, 55.71, 53.42; IR (KBr): 3396, 3292, 3122 ,1673, 1620, 1512, 1357 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{20}H_{21}N_6O_6S_2$: 505.0964 $[M-H]^+$; found: 505.0974.

5-N,N-bis[2-(N-(4-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6c): White solid; yield: 60%; purity (HPLC): 97.66%; m.p. 216-218; ^1H NMR (500 MHz, DMSO-d_6) δ 10.57 (s, 2H, -CONH-), 8.07 (s, 2H, -SO_2NH_2-), 7.62 (dd, *J* = 8.8, 4.9 Hz, 4H, Ar-H), 7.19 (t, *J* = 8.7 Hz, 4H, Ar-H), 4.31 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 167.21, 158.70 (d, J = 240.2 Hz), 153.24, 135.31(d, J = 2.4 Hz), 121.48 (d, J = 7.8 Hz), 115.99 (d, J = 22.3 Hz), 53.08; IR (KBr): 3349, 3273, 3161, 1663, 1624, 1510, 1373 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}F_2N_6O_4S_2$: 481.0564 $[M-H]^+$; found: 481.0574.

5-N,N-bis[2-(N-(4-bromophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6d): White solid; yield: 68%; purity (HPLC)：97.61%; m.p. 234-236; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.58 (s, 2H, -CONH-), 8.06 (s,2H, -SO_2NH_2-), 7.55 (q, *J* = 8.8 Hz, 8H, Ar-H), 4.31 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.03, 167.40, 153.84, 137.87, 132.07, 122.28, 116.30, 53.43; IR (KBr): 3439, 3298, 3179, 1669, 1610, 1547, 1369 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}Br_2N_6O_4S_2$: 602.8942 [M-H]$^+$; found: 602.8953.

5-N,N-bis[2-(N-(4-trifluoromethyl phenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6e): White solid; yield: 70%; purity(HPLC)：97.7%; m.p. 222-224; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.77 (s, 2H, -CONH-), 8.06 (s, 2H, -SO_2NH_2-), 7.88-7.51 (m, 8H, Ar-H), 4.38 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 167.87, 153.37, 142.47, 126.73 (q, J = 3.8 Hz), 124.78 (q, J = 272.16 Hz), 124.15 (q, J = 31.5 Hz), 119.64, 52.84; IR (KBr): 3477, 3326, 3185, 1677, 1610, 1551, 1324 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{20}H_{15}F_6N_6O_4S_2$: 581.0500 [M-H]$^+$; found: 581.0511.

5-N,N-bis[2-(N-(p-methylphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6f): White solid; yield: 67%; purity (HPLC)：97%; m.p. 225-227; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.48 (s, 2H, -CONH-), 8.08 (s, 2H, -SO_2NH_2-), 7.50 (d, *J* = 8.0 Hz, 4H, Ar-H), 7.15 (d, *J* = 8.1 Hz, 4H, -H), 4.29 (s, 4H, -CH_2-), 2.27 (s, 6H, -CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 167.07, 153.20, 136.43, 133.15, 129.75, 119.70, 53.33, 20.94; IR (KBr): 3378, 3294, 3190, 1673, 1614, 1553, 1363 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{20}H_{21}N_6O_4S_2$: 473.1066 [M-H]$^+$; found: 473.1075.

5-N,N-bis[2-(N-(4-iodophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6g): White solid; yield: 60%; purity (HPLC): 99.77%; m.p. 211-213; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.55 (s, 2H, -CONH-), 8.05 (s, 2H, -SO_2NH_2-), 7.68 (d, *J* = 8.4 Hz, 4H, Ar-H), 7.43 (d, *J* = 8.4 Hz, 4H, Ar-H), 4.30 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.80, 167.42, 153.29, 138.73, 138.04, 121.89, 87.79, 53.02; IR (KBr): 3435, 3298, 3175, 1677, 1608, 1539, 1369 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}I_2N_6O_4S_2$: 696.8686 [M-H]$^+$; found: 696.8696.

5-N,N-bis[2-(N-(4-tert-butylphenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6h): White solid; yield: 67%; purity (HPLC): 99.17%; m.p. 258-260; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.55 (s, 2H, -CONH-), 8.09 (s, 2H, -SO_2NH_2-), 7.53 (d, *J* = 8.0 Hz, 4H, Ar-H), 7.36 (d, *J* = 8.3 Hz, 4H, Ar-H), 4.29 (s, 4H, -CH_2-), 1.27 (s,18H, -CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 167.16, 153.14, 146.54, 136.36, 125.99, 119.50, 53.42, 34.54, 31.66; IR (KBr): 3422, 3361, 3130, 1677, 1608, 1549, 1351 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{26}H_{33}N_6O_4S_2$: 557.2005 [M-H]$^+$; found: 557.2015.

5-N,N-bis[2-(N-phenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6i): White solid; yield: 66%; purity(HPLC): 98.42%; m.p. 226-228; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.55 (s, 2H, -CONH-), 8.08 (s, 2H, -SO_2NH_2-), 7.62 (d, *J* = 7.9 Hz, 4H, Ar-H), 7.35 (t, *J* = 7.7 Hz, 4H, Ar-H), 7.10 (t, *J* = 7.4 Hz, 2H, Ar-H), 4.32 (s, 4H, -CH_2-); ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.82, 167.32, 153.24, 138.94, 129.39, 124.19, 119.69, 53.30; IR (KBr): 3381, 3318, 3108, 1665, 1602, 1551, 1375 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{17}N_6O_4S_2$: 445.0753 [M-H]$^+$; found: 445.0762.

5-N,N-bis[2-(N-(3-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6j): White solid; yield: 66%; purity (HPLC)：96.50%; m.p. 230-232; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 2H, -CONH-), 8.05 (s, 2H, -SO_2NH_2-), 7.57 (dt, *J* = 11.5, 2.3 Hz, 2H, Ar-H), 7.39 (td, *J* = 8.2, 6.6 Hz, 2H, Ar-H), 7.30 (dt, *J* = 8.5, 1.3 Hz, 2H, Ar-H), 7.02-6.82 (m, 2H, Ar-H), 4.33 (s, 4H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.83, 167.59, 162.61 (d, J = 241.6 Hz), 153.33, 140.60 (d, J = 11.0 Hz), 131.10 (d, J = 9.5 Hz), 115.44(d, J = 2.3 Hz), 110.68 (d, J = 21.1 Hz), 106.51 (d, J = 26.4 Hz), 52.86; IR (KBr): 3392, 3271, 3146, 1679, 1614, 1563, 1363 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}F_2N_6O_4S_2$: 481.0564 [M-H]$^+$; found: 481.0575.

5-N,N-bis[2-(N-(2-fluorophenyl)acetamide)]-5-amino-1,3,4-thiadiazole-2-sulfonamide (6k): White solid; yield:65%; purity (HPLC): 97.99%; m.p. 230-232; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.24 (s, 2H, -CONH-), 8.05 (s, 2H, -SO_2NH_2-), 7.92 (td, *J* = 7.8, 3.5 Hz, 2H, Ar-H), 7.28 (ddd, *J* = 11.1, 6.8, 3.1 Hz, 2H, Ar-H), 7.18 (dq, *J* = 6.6, 3.8, 3.2 Hz, 4H, Ar-H), 4.38 (s, 4H, -CH_2-); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.82, 167.68, 153.82 (d, J = 245.4 Hz), 153.28, 126.09 (d, J = 11.5 Hz), 125.94 (d, J = 7.3 Hz), 124.90 (d, J

= 3.3 Hz), 124.20, 116.04 (d, J = 19.2 Hz), 52.83; IR (KBr): 3461, 3267, 3137, 1675, 1622, 1547, 1373 cm^{-1}; HRMS (ESI): m/z calcd. for $C_{18}H_{15}F_2N_6O_4S_2$: 481.0564 $[M-H]^+$; found: 481.0574.

Biological assays

Carbonic anhydrase inhibition assay: According to previously reported method,[34] 4-NPA can be converted into 4-nitrophenol with catalysed by carbonic anhydrase, and the reaction rate was determined by the spectrophotometry at 405 nm with an enzyme labelling instrument.[42,43] Human carbonic anhydrases I, II and IX were prepared in 250 ng/μL reserve solutions for the subsequent usage. Buffer was used to dilute hCA I, hCA II and hCA IX protein reserve solutions to 2.5 ng/μL, 5 ng/μL and 10 ng/μL, respectively, and they were then added to 384-well reaction plates. The experiment was divided into three groups: blank group, positive control group (AZA) and sample group. All inhibitors were diluted into seven different concentrations in a gradient and added to 384-well reaction plates in triplicate to form enzyme inhibitor (E-I) complexes; the complexes were incubated at 25℃ for 15 minutes. The substrate 4-NPA was added to the E-I solution and the incubation continued for 1 h (CA I), 1 h (CA II) and 4 h (CA IX), respectively. The absorbance of solution was measured at 405 nm by microplate reader (BioTek, Epoch, USA). IC_{50} value was obtained with GraphPadPrism 5.0 software. The experiments were repeated at least 3 times.

Cell culture: PC12 cells were cultured in DMEM containing 5% FBS, 5% HS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ and 5% CO_2. HEK293 and LO2 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ and 5% CO_2. When the cells were 80%-90% confluent, they were digested with 0.25% trypsin -0.02% EDTA and passaged.

Cell viability assay: Cell viability was measured by MTT assay. Briefly, the PC12 cells were seeded in 96-well plates (1.5×10^4 cells/well) and cultured for 24 h. The cells were then pre-treated with the indicated concentrations of target compounds for 2 h, and incubated with 650 μM SNP for 24 h. 10 μL of 5 mg/mL MTT were added to pre-treated cells for 4 h at 37 °C in the presence of 5% CO_2. The supernatant was discarded, 100 μL of DMSO was added to the wells of 96-well plates, and the solutions were mixed thoroughly. The absorbance was recorded at 570 nm using a microplate reader. The experiments were repeated at least 3 times.

Cytotoxicity assay: The cells (HEK293, LO2 and PC12) were respectively seeded into 96-well plates (5×10^3 cells/well) and cultured at 37℃ and 5% CO_2 for 24 h. The medium was removed, and 100 μL of new medium containing different concentrations of compounds was added and treated for 48 h. MTT (5 mg/mL, 10 μL) was added to each well and treated for 4 h at 37℃ and 5% CO_2. The supernatant in each well was removed, and 100 μL of DMSO were added to the wells of 96-well plates, and the solutions were mixed thoroughly. The absorbance was recorded at 570 nm using a microplate reader. IC_{50} values of compounds were calculated by GraphPad Prism 5.0 software. The experiments were repeated at least 3 times.

Computational assays

Molecular docking: The structures of ligands were drawn by ChembioDraw Ultra 14.0 software and saved in SDF format. The crystal structure of human CA I (PDB code: 5WLU),[37] human CA II (PDB code: 5ULN)[38] and human CA IX (PDB code: 5JN3)[39] were derived from RCSB PDB data bank, with 1.39 Å, 1.35 Å and 1.60 Å resolution. The proteins were treated with the process where all water was removed and hydrogen bonds were added. PyMol [The PyMol Molecular Graphics System, version 1.1 Schrödinger LLC] software was used to dock protein and compounds and to find the best docking posture for CA II and compound **5c**.

Prediction of ADME Properties: %ABS (percentage absorption), MW (molecular weight), miLog P (logarithm of partition coefficient of compound between n-octanol and water), TPSA (topological polar surface area), n-ROTB (number of rotatable bonds), n-ON acceptors (number of hydrogen bond acceptors), and n-OHNH donors (number of hydrogen bond donors) were calculated by Molinspiration Online Property Calculation Toolkit (Molinspiration,2015).[44] Absorption (% ABS) was calculated by using the equation % ABS = 109-(0.345 x TPSA).[45,46]

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Conflict of interest

The authors declare no conflict of interest.

Keywords: Acetazolamide derivatives • carbonic anhydrase II (CA II) • inhibitor • neuroprotection

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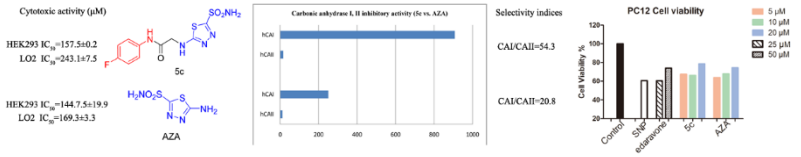

Compound **5c** was the most effective selective carbonic anhydrase II inhibitor (IC_{50}=16.7 nM, CAI/CAII=54.3), which was comparable to the inhibitory effect of acetazolamide. Moreover, the selectivity of compound 5c was better than that of acetazolamide (IC_{50}=12.0 nM, CAI/CAII=20.8). Compound **5c** exhibited a better protective effect for PC12 cells and showed less cytotoxicity than that of acetazolamide.