European Journal of Medicinal Chemistry 125 (2017) 190–196

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Synthesis and evaluation of new benzodioxole-based dithiocarbamate derivatives as potential anticancer agents and hCA-I and hCA-II inhibitors

Mehlika Dilek Altıntop [a, *], Belgin Sever [a], Gülşen Akalın Çiftçi [b], Kaan Kucukoglu [c], Ahmet Özdemir [a], Seyedeh Sara Soleimani [d], Hayrunnisa Nadaroglu [e], Zafer Asım Kaplancıklı [a]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey
[b] Department of Biochemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey
[c] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey
[d] Department of Nano-Science and Nano-Engineering, Faculty of Engineering, Ataturk University, 25240, Erzurum, Turkey
[e] Department of Food Technology, Erzurum Vocational Training School, Ataturk University, 25240, Erzurum, Turkey

ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form
25 August 2016
Accepted 9 September 2016
Available online 10 September 2016

Keywords:
Dithiocarbamate
Benzodioxole
Cancer
Human carbonic anhydrase

ABSTRACT

In the current work, new benzodioxole-based dithiocarbamate derivatives were synthesized *via* the reaction of *N*-(1,3-benzodioxol-5-ylmethyl)-2-chloroacetamide with appropriate sodium salts of *N*,*N*-disubstituted dithiocarbamic acids. These derivatives were evaluated for their cytotoxic effects on A549 human lung adenocarcinoma and C6 rat glioma cell lines. *N*-(1,3-Benzodioxol-5-ylmethyl)-2-[4-(4-nitrophenyl)-1-piperazinylthiocarbamoylthio]acetamide (**10**) can be identified as the most promising anticancer agent against C6 cell line due to its notable inhibitory effect on C6 cells with an IC_{50} value of 23.33 ± 7.63 μg/mL when compared with cisplatin (IC_{50} = 19.00 ± 5.29 μg/mL). On the other hand, compound **10** did not show any significant cytotoxic activity against A549 cell line. The compounds were also tested for their *in vitro* inhibitory effects on hCA-I and hCA-II. Generally, the tested compounds were more effective on CAs than acetazolamide, the reference agent. Among these compounds, *N*-(1,3-benzodioxol-5-ylmethyl)-2-[(morpholinyl)thiocarbamoylthio]acetamide (**3**) and *N*-(1,3-benzodioxol-5-ylmethyl)-2-[(thiomorpholinyl)thiocarbamoylthio]acetamide (**4**) were found to be the most effective compounds on hCA-I with IC_{50} values of 0.346 nM and 0.288 nM, and hCA-II with IC_{50} values of 0.287 nM and 0.338 nM, respectively.

© 2016 Elsevier Masson SAS. All rights reserved.

1. Introduction

The metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) takes an important role in carbon dioxide hydration to bicarbonate ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$). CAs are involved in essential cellular processes such as respiration and pH homeostasis, electrolyte secretion, bone resorption, calcification in diverse tissues and organs and biosynthetic processes such as lipogenesis, gluconeogenesis and ureagenesis that require HCO_3^- as a substrate [1–4].

These abundant zinc enzymes have attracted a great deal of interest in medicinal chemistry as important targets in the treatment or prevention of a variety of disorders such as glaucoma, acid-base disequilibria, epilepsy, and other neuromuscular diseases, altitude sickness, edema and obesity. One of the outstanding applications belonging to CA inhibitors is in the treatment of hypoxic tumors. The involvement of CAs in tumor growth and metastasis has been investigated for so long and lots of researchers have described these enzymes as the key components for fighting acidosis during hypoxia [5,6]. Two CA isozymes (CA IX and CA XII) have been reported to be overexpressed in many tumors, whereas they show moderate-to-abundant expression in normal tissues. These two isozymes are associated with cancer progression, metastasis, and impaired therapeutic response. In particular, CA IX is expressed in various tumors such as carcinomas of cervix uteri, breast, lung, colon, brain, kidney and other types of neoplasia and CA IX is found in very limited amount in normal tissues just as

* Corresponding author.
E-mail address: mdaltintop@anadolu.edu.tr (M.D. Altıntop).

http://dx.doi.org/10.1016/j.ejmech.2016.09.035
0223-5234/© 2016 Elsevier Masson SAS. All rights reserved.

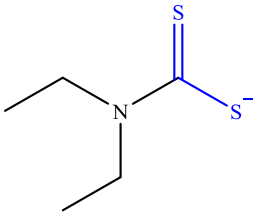


Fig. 1. *N,N*-diethyldithiocarbamate structurally important for the interaction with CAs [20].

gastrointestinal tract and it comprises epithelia of glandular stomach, small intestine and gallbladder [7–9]. CA XII is also present in many tumors but it is more findable in some healthy tissues [10]. CA I and II are the most abundant CAs in the body and they are also substantial drug targets [11]. CA I is reported to be involved in retinal and cerebral edema so its inhibition may be important to deal with these conditions [12]. Besides, CA II is associated with some disorders such as glaucoma, edema, epilepsy, and probably altitude sickness [13].

Dithiocarbamates (DTCs) have received considerable attention due to their diverse biological activities such as antifungal [14], antibacterial [15], and antioxidant activities [16]. In recent years, DTC has been identified as a pharmacophore to show anticancer activity [17]. DTCs are also capable of forming complexes with a variety of transition metal ions in diverse oxidation modes due to the presence of the anionic CS_2^- moiety [18]. They have been reported to act as efficient metalloenzyme inhibitors such as tyrosinase and CA inhibitors in various organisms [18,19]. Innocenti et al. reported the interaction of CAs with *N,N*-diethyldithiocarbamate containing a new zinc-binding group (CS_2^-) as shown in Fig. 1. DTCs have been found to inhibit several CA isoforms in the low micromolar or submicromolar range [20]. The discovery of CS_2^- as a new zinc-binding group has led to the design of new DTC derivatives as potent human carbonic anhydrase (hCA) inhibitors [18–20].

On the other hand, 1,3-benzodioxole has been found in a number of anticancer agents such as podophyllotoxin, steganacin and combretastatin A-2 (Fig. 2) due to its good bioavailability and low toxicity [21].

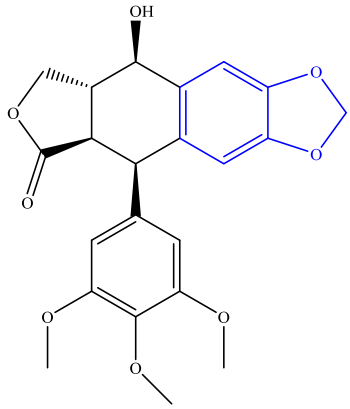
On the basis of afore-mentioned findings, in the present investigation we reported the synthesis and evaluation of a new series of benzodioxole-based dithiocarbamate derivatives as anticancer agents against A549 human lung adenocarcinoma and C6 rat glioma cell lines. The synthesized compounds were also tested for their *in vitro* inhibitory effects on hCA-I and hCA-II.

2. Results and discussion

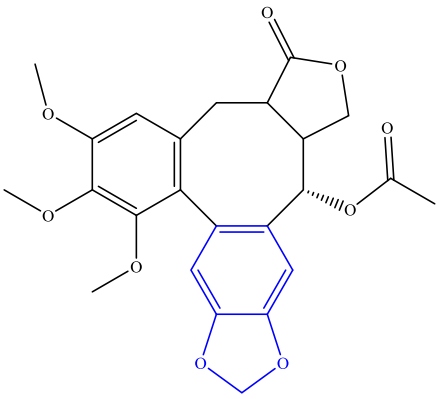
The synthesis of dithiocarbamate derivatives (**1**–**10**) was carried out according to the steps shown in Scheme 1. Sodium salts of *N,N*-disubstituted dithiocarbamic acids were obtained by the reaction of secondary amine with carbon disulfide in the presence of sodium hydroxide [22,23]. The reaction of *N*-(1,3-benzodioxol-5-ylmethyl)-2-chloroacetamide with sodium salts of *N,N*-disubstituted dithiocarbamic acids afforded novel dithiocarbamate derivatives (**1**–**10**).

MTT assay, which is based on the ability of metabolically active cells to convert the pale yellow MTT dye to a spectrophotometrically quantifiable blue formazan product, was carried out to determine the cytotoxic effects of the compounds on A549 human lung adenocarcinoma and C6 rat glioma cell lines [24]. According to the MTT assay, the tested compounds showed more potent inhibitory effects on C6 cells than A549 cells (Table 1). The effects of the heterocyclic rings on anticancer activity of the compounds against C6 cell line revealed the following potency order: Piperazine > Morpholine > Thiomorpholine > Piperidine.

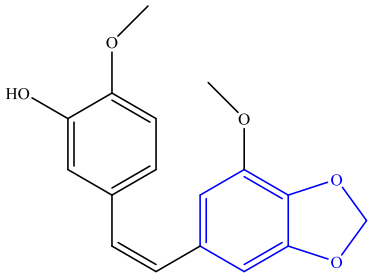
Considering the anticancer activity of piperidine substituted compounds **1** and **2**, it can be concluded that the methyl substituent at position 4 of the piperidine ring increased the cytotoxic effects on A549 and C6 cell lines.



Podophyllotoxin

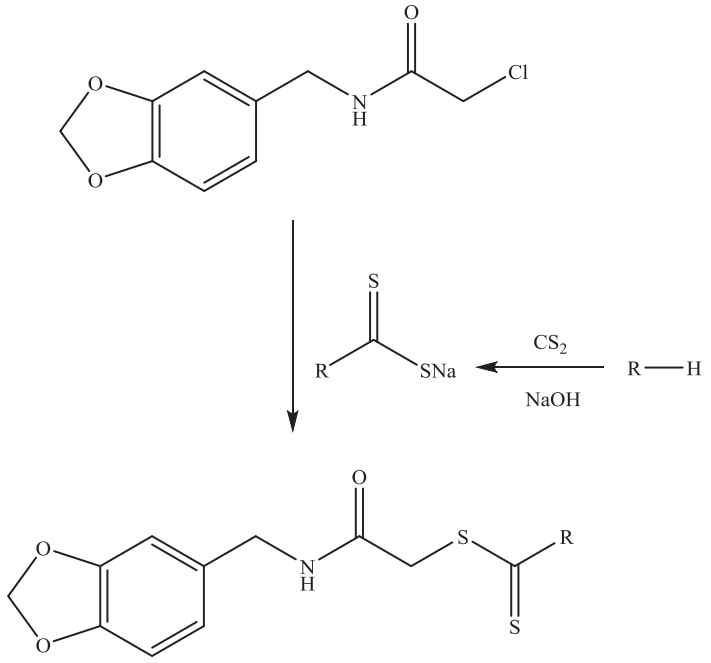


Steganacin

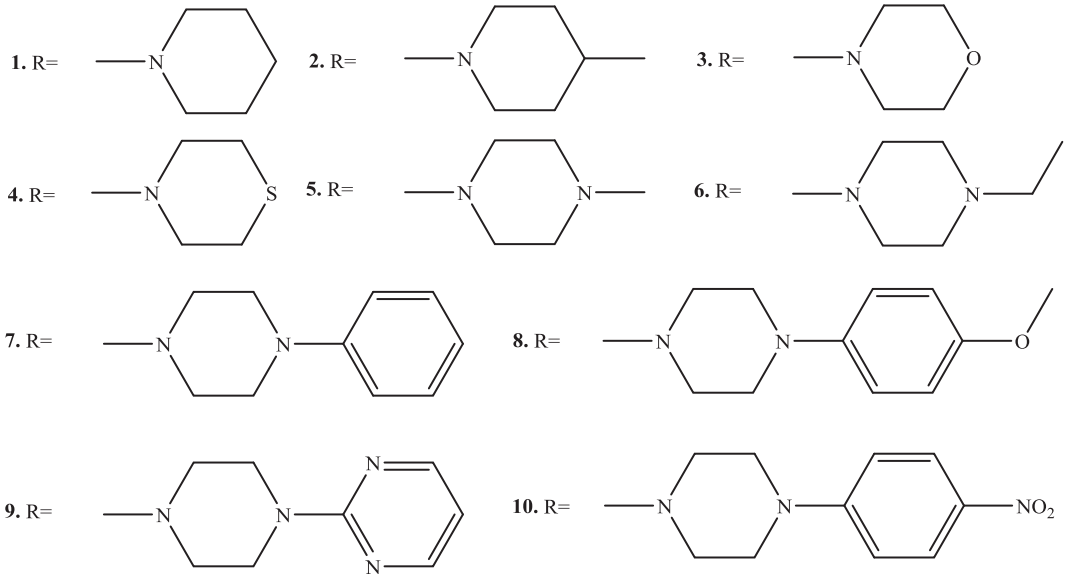


Combretastatin A-2

Fig. 2. Benzodioxole-based anticancer agents [21].



Compounds **1-10**



Scheme 1. The synthetic route for the preparation of the benzodioxole-based dithiocarbamate derivatives (**1**–**10**).

Compound **3** showed more inhibitory activity against A549 and C6 cell lines than compound **4**. This outcome indicated that morpholine ring increased the anticancer activity against A549 and C6 cell lines when compared with thiomorpholine ring.

Piperazine derivatives were more effective than piperidine, morpholine and thiomorpholine derivatives on C6 cells. Among piperazine derivatives, the most effective anticancer agents were found as compound **10** (IC_{50} = 23.33 ± 7.63 μg/mL), compound **9** (IC_{50} = 48.33 ± 7.64 μg/mL) and compound **8** (IC_{50} = 58.33 ± 14.43 μg/mL) when compared with cisplatin (IC_{50} = 19.00 ± 5.29 μg/mL). This outcome pointed out the importance of the alkyl/aryl/heteroaryl substituent at position 4 of the piperazine ring for the anticancer activity against C6 cell line. The effects of the substituents at position 4 of the piperazine ring on anticancer activity of the piperazine substituted compounds against C6 cell line revealed the following potency order: 4-Nitrophenyl > 2-Pyrimidinyl > 4-Methoxyphenyl > Ethyl > Phenyl > Methyl. Generally the aryl substituted compounds except compound **7** were more effective than alkyl substituted compounds on C6 cell line. Among aryl groups, electron withdrawing groups on benzene ring such as nitro group increased the anticancer activity against C6 cell line. The elongation of the alkyl chain at position 4 of the piperazine ring increased the anticancer activity of the alkyl substituted

Table 1
IC_{50} values of the compounds against A549 and C6 cells for 24 h.

Compound	IC_{50} (μg/mL)	
	A549	C6
1	875.00 ± 106.07	633.33 ± 208.17
2	810.00 ± 14.14	566.67 ± 76.38
3	700.00 ± 278.39	206.67 ± 11.55
4	>1000	220.00 ± 26.46
5	>1000	166.67 ± 23.09
6	850.00 ± 212.13	156.67 ± 20.82
7	>1000	163.33 ± 15.28
8	>1000	58.33 ± 14.43
9	833.33 ± 175.59	48.33 ± 7.64
10	>1000	23.33 ± 7.63
Cisplatin	27.00 ± 3.06	19.00 ± 5.29

compounds against A549 and C6 cell lines.

Compounds **1**–**10** were tested for their *in vitro* inhibitory effects on hCA-I and hCA-II (Table 2). Acetazolamide (AAZ) was used as the reference agent. Generally, the tested compounds were more effective on CAs than AAZ (IC_{50} = 5.8 nM). In particular, compounds **3** and **4** exhibited the most potent inhibitory effect on hCA-I with IC_{50} values of 0.346 and 0.288 nM, respectively. It is noteworthy to indicate that morpholine and thiomorpholine rings significantly increased the carbonic anhydrase inhibitory activity when compared with the other chemical structures. Compounds **1**, **5** and **7** revealed inhibitory effects on hCA-I with the range of 0.518–0.556 nM. Similarly, compounds **6**, **8** and **9** revealed inhibitory effects on hCA-I with the range of 0.624–0.699 nM. Although compounds **2** and **10** were more effective than AAZ on hCA-I, these compounds showed less inhibitory activity than other derivatives against hCA-I.

Similarly, compounds **3** and **4** showed the most potent inhibitory effect on hCA-II isozyme with IC_{50} values of 0.287 and 0.338 nM, respectively. Compounds **7** and **8** were found to be ineffective on hCA-II, whereas other derivatives showed notable inhibitory effect on hCA-II isozyme. The inhibitory effects of compounds **1** and **5** showed fairly close inhibition on hCA-II with IC_{50} values of 0.651 nM and 0.622 nM, respectively. On the other hand, compounds **6**, **9** and **10** inhibited hCA-II isozyme with the range of 0.799–0.855 nM when compared with AAZ (IC_{50} = 6.7 nM).

Compounds **1**, **2, 4, 5**, **6, 7, 8** and **9** exhibited more significant inhibitory effects on hCA-I than hCA-II, whereas compounds **3** and **10** showed more significant inhibitory effects on hCA-II than hCA-I. The methyl substitution at position 4 of the piperidine ring significantly decreased the CA inhibition on both hCA-I and hCA-II.

3. Conclusion

In the current research, new benzodioxole-based dithiocarbamate derivatives were synthesized. All synthesized compounds (**1**–**10**) were evaluated for their cytotoxicity against A549 human lung adenocarcinoma, and C6 rat glioma cell lines. Among these compounds, compound **10** can be considered as the most promising anticancer agent against C6 cell line (IC_{50} = 23.33 ± 7.63 μg/mL) when compared with cisplatin (IC_{50} = 19.00 ± 5.29 μg/mL). Moreover, compounds **1**–**10** were also investigated for their ability to inhibit human carbonic anhydrase isozymes (hCA-I and hCA-II). Although compounds **1**–**10** do not carry a sulfonamide group, an important pharmacophore for hCA inhibitory activity, all compounds (**1**–**10**) showed significant inhibitory effect on hCA-I. In addition, except compounds **7** and **8**, they also notably inhibited hCA-II as well. Among the synthesized compounds, compounds **3** and **4** can be identified as potential hCA-I and hCA-II inhibitors.

4. Experimental

4.1. Chemistry

N-(1,3-benzodioxol-5-ylmethyl)-2-chloroacetamide (97%) was purchased from Maybridge, whereas other reagents were purchased from Merck, Sigma-Aldrich and Acros. The melting points (M.p.) of the compounds were determined on an Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. IR spectra were recorded on an IRPrestige-21 Fourier Transform Infrared spectrophotometer (Shimadzu, Tokyo, Japan). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer (Bruker, Billerica, MA, USA). Liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization mode. Shimadzu's LCMS Solution software was used for data analysis. Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyzer (Perkin-Elmer, Norwalk, CT, USA) and the results were within ±0.4% of the theoretical values. Thin Layer Chromatography (TLC) was performed on TLC Silica gel 60 F_{254} aluminium sheets (Merck, Darmstadt, Germany) to check the purity of the compounds.

4.1.1. General procedure for the synthesis of the compounds

4.1.1.1. Sodium salts of N,N-disubstituted dithiocarbamic acids. Sodium hydroxide (10 mmol) was dissolved in ethanol (80 mL) with constant stirring. After addition of the secondary amine (10 mmol) the mixture was cooled in an ice bath and carbon disulfide (100 mmol) was added dropwise with stirring. The reaction

Table 2
The results obtained from regression analysis graphs for hCA-I and hCA-II in the presence of the tested compounds.

Compound	hCA-I inhibition[a] IC_{50}	hCA-II inhibition[a] IC_{50}	hCA-I/hCA-II
1	0.556	0.651	0.854
2	1.69	1.875	0.901
3	0.346	0.287	1.206
4	0.288	0.338	0.852
5	0.535	0.622	0.860
6	0.677	0.802	0.844
7	0.518	–	–
8	0.624	–	–
9	0.699	0.855	0.818
10	0.903	0.799	1.129
Acetazolamide (AAZ)	5.8	6.7	0.87

[a] They were determined as nM.

mixture was stirred for 2 h at room temperature. The products were afforded by filtration and washed with diethyl ether [22,23].

*4.1.1.2. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(substituted)-thiocarbamoylthio]acetamide (**1**–**10**).* A mixture of *N*-(1,3-benzodioxol-5-ylmethyl)-2-chloroacetamide (0.01 mol) and appropriate sodium salt of *N*,*N*-disubstituted dithiocarbamic acid (0.01 mol) was treated in acetone at room temperature for 6 h. The solvent was evaporated, the resulting solid was washed with water and crystallized from ethanol [23].

*4.1.1.2.1. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(piperidin-1-yl)thiocarbamoylthio]acetamide (**1**).* Yield: 79%. Mp 137.7 °C.

IR ν_{max} (cm^{-1}): 3263 (N–H stretching), 3068 (aromatic C–H stretching), 2929 (aliphatic C–H stretching), 2829 (O–CH_2 stretching), 1641 (C=O stretching), 1548, 1504, 1489, 1473 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 1.59–1.68 (m, 6H), 3.92 (s, 2H), 4.06 (s, 2H), 4.19 (d, J = 5.95 Hz, 4H), 5.97 (s, 2H), 6.72–6.74 (m, 1H), 6.82–6.84 (m, 2H), 8.52–8.55 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 23.98 ($3CH_2$), 52.00 ($2CH_2$), 40.81 (CH_2), 42.73 (CH_2), 101.25 (CH_2), 108.33 (CH), 108.40 (CH), 120.81 (CH), 133.57 (C), 146.48 (C), 147.68 (C), 166.98 (C), 193.58 (C).

For $C_{16}H_{20}N_2O_3S_2$ Calculated: C, 54.52; H, 5.72; N, 7.95; O, 13.62; S, 18.19. Found: C, 54.10; H, 5.23; N, 7.34.

HRMS (ESI) (*m/z*): $[M+Na]^+$ 375.08.

*4.1.1.2.2. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(4-methylpiperidin-1-yl)thiocarbamoylthio]acetamide (**2**).* Yield: 78%. Mp 154.4 °C.

IR ν_{max} (cm^{-1}): 3273 (N–H stretching), 3041 (aromatic C–H stretching), 2910 (aliphatic C–H stretching), 2833 (O–CH_2 stretching), 1637 (C=O stretching), 1541, 1504, 1490, 1473 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 0.92 (d, J = 6.25 Hz, 3H), 1.07–1.15 (m, 2H), 1.75–1.79 (m, 3H), 3.19–3.25 (m, 2H), 4.05 (s, 2H), 4.19 (d, J = 5.90 Hz, 2H), 4.46–4.49 (m, 1H), 5.23–5.25 (m, 1H), 5.97 (s, 2H), 6.73 (d, J = 8.20 Hz, 1H), 6.82–6.85 (m, 2H), 8.52–8.55 (m, 1H).

^{13}C NMR (100 MHz) (DMSO-d_6) δ (ppm): 21.01 (CH_3), 29.92 (CH), 33.21 (CH_2), 33.72 (CH_2), 40.37 (CH_2), 42.24 (CH_2), 50.13 (CH_2), 51.83 (CH_2), 100.74 (CH_2), 107.82 (CH), 107.89 (CH), 120.31 (CH), 133.05 (C), 145.98 (C), 147.18 (C), 166.48 (C), 193.22 (C).

For $C_{17}H_{22}N_2O_3S_2$ Calculated: C, 55.71; H, 6.05; N, 7.64. Found: C, 55.10; H, 6.23; N, 7.34.

HRMS (ESI) (*m/z*): $[M+Na]^+$ 389.10.

*4.1.1.2.3. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(morpholinyl)thiocarbamoylthio]acetamide (**3**).* Yield: 83%. Mp 131.5 °C.

IR ν_{max} (cm^{-1}): 3273 (N–H stretching), 3076 (aromatic C–H stretching), 2985 (aliphatic C–H stretching), 2910, 2854 (O–CH_2 stretching), 1643 (C=O stretching), 1552, 1489, 1454 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.67–3.68 (m, 4H), 3.94 (s, 2H), 4.09 (s, 2H), 4.19 (d, J = 5.95 Hz, 4H), 5.97 (s, 2H), 6.72–6.74 (m, 1H), 6.83–6.84 (m, 2H), 8.59 (s, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.58 (CH_2), 42.75 (CH_2), 50.67 ($2CH_2$), 66.06 ($2CH_2$), 101.26 (CH_2), 108.33 (CH), 108.41 (CH), 120.82 (CH), 133.53 (C), 146.49 (C), 147.68 (C), 166.79 (C), 195.54 (C).

For $C_{15}H_{18}N_2O_4S_2$ Calculated: C, 50.83; H, 5.12; N, 7.90. Found: C, 50.10; H, 5.23; N, 7.94.

HRMS (ESI) (*m/z*): $[M+H]^+$ 355.08.

*4.1.1.2.4. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(thiomorpholinyl) thiocarbamoylthio]acetamide (**4**).* Yield: 82%. Mp 160.4 °C.

IR ν_{max} (cm^{-1}): 3267 (N–H stretching), 3074 (aromatic C–H stretching), 2970, 2902 (aliphatic C–H stretching), 2837 (O–CH_2 stretching), 1635 (C=O stretching), 1552, 1504, 1489, 1460 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 2.74 (s, 4H), 4.09 (s, 2H), 4.19 (d, J = 5.95 Hz, 2H), 4.25 (br, 2H), 4.50 (br, 2H), 5.97 (s, 2H), 6.72–6.74 (m, 1H), 6.83–6.84 (m, 2H), 8.56–8.58 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 26.90 ($2CH_2$), 40.79 (CH_2), 42.75 (CH_2), 53.46 ($2CH_2$), 101.26 (CH_2), 108.33 (CH), 108.40 (CH), 120.80 (CH), 133.53 (C), 146.49 (C), 147.68 (C), 166.76 (C), 195.02 (C).

For $C_{15}H_{18}N_2O_3S_3$ Calculated: C, 48.63; H, 4.90; N, 7.56. Found: C, 48.10; H, 4.23; N, 7.54.

HRMS (ESI) (*m/z*): $[M+H]^+$ 371.06.

*4.1.1.2.5. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(4-methyl-1-piperazinyl)thiocarbamoylthio]acetamide (**5**).* Yield: 80%. Mp 163.2 °C.

IR ν_{max} (cm^{-1}): 3255 (N–H stretching), 3057 (aromatic C–H stretching), 2972, 2937, 2902 (aliphatic C–H stretching), 2833, 2785 (O–CH_2 stretching), 1635 (C=O stretching), 1546, 1504, 1463, 1460 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 2.21 (s, 3H), 2.39–2.41 (m, 4H), 3.93–3.99 (m, 2H), 4.07 (s, 2H), 4.19 (d, J = 5.90 Hz, 4H), 5.97 (s, 2H), 6.73 (d, J = 8.0 Hz, 1H), 6.82–6.84 (m, 2H), 8.55–8.57 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.76 (CH_2), 42.75 (CH_2), 45.57 (CH_3), 50.07 (CH_2), 51.57 (CH_2), 54.44 ($2CH_2$), 101.26 (CH_2), 108.34 (CH), 108.40 (CH), 120.82 (CH), 133.54 (C), 146.49 (C), 147.68 (C), 166.85 (C), 195.01 (C).

For $C_{16}H_{21}N_3O_3S_2$ Calculated: C, 52.30; H, 5.76; N, 11.43. Found: C, 52.10; H, 5.23; N, 11.54.

HRMS (ESI) (*m/z*): $[M+H]^+$ 368.11.

*4.1.1.2.6. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(4-ethyl-1-piperazinyl)thiocarbamoylthio]acetamide (**6**).* Yield: 82%. Mp 144.9 °C.

IR ν_{max} (cm^{-1}): 3250 (N–H stretching), 3066 (aromatic C–H stretching), 2972, 2895 (aliphatic C–H stretching), 2816, 2779 (O–CH_2 stretching), 1637 (C=O stretching), 1548, 1502, 1456, 1427 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 1.01 (t, J = 7.2 Hz, 3H), 2.36 (q, J = 7.2 Hz, 2H), 2.45 (t, J = 5.15 Hz, 4H), 3.93 (s, 2H), 4.07 (s, 2H), 4.19 (d, J = 5.95 Hz, 4H), 5.97 (s, 2H), 6.72–6.74 (m, 1H), 6.82–6.84 (m, 2H), 8.53–8.55 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 11.90 (CH_3), 40.20 (CH_2), 42.30 (CH_2), 49.70 (CH_2), 51.00 ($2CH_2$), 51.70 ($2CH_2$), 100.80 (CH_2), 107.80 (CH), 107.90 (CH), 120.30 (CH), 133.00 (C), 146.00 (C), 147.20 (C), 166.40 (C), 194.40 (C).

For $C_{17}H_{23}N_3O_3S_2$ Calculated: C, 53.52; H, 6.08; N, 11.01. Found: C, 53.10; H, 6.23; N, 11.04.

HRMS (ESI) (*m/z*): $[M+H]^+$ 382.13.

*4.1.1.2.7. N-(1,3-Benzodioxol-5-ylmethyl)-2-[(4-phenyl-1-piperazinyl)thiocarbamoylthio]acetamide (**7**).* Yield: 86%. Mp 137.2 °C.

IR ν_{max} (cm^{-1}): 3259 (N–H stretching), 3080 (aromatic C–H stretching), 2897 (aliphatic C–H stretching), 2819 (O–CH_2 stretching), 1672 (C=O stretching), 1647, 1600, 1560, 1500, 1452, 1429 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.29–3.31 (m, 4H), 4.11 (s, 4H), 4.19 (d, J = 5.90 Hz, 2H), 4.35 (br, 2H), 5.96 (s, 2H), 6.73–6.74 (m, 1H), 6.80–6.84 (m, 3H), 6.94–6.96 (m, 2H), 7.23–7.26 (m, 2H), 8.56 (s, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.20 (CH_2), 42.30 (CH_2), 47.50 ($2CH_2$), 51.00 ($2CH_2$), 100.70 (CH_2), 107.80 (CH), 107.90 (CH), 115.40 (2CH), 119.20 (CH), 120.30 (CH), 129.00 (2CH), 133.00 (C), 146.00 (C), 147.20 (C), 150.00 (C), 166.30 (C), 194.60 (C).

For $C_{21}H_{23}N_3O_3S_2$ Calculated: C, 58.72; H, 5.40; N, 9.78. Found: C, 58.10; H, 5.23; N, 9.74.

HRMS (ESI) (*m/z*): $[M+H]^+$ 430.13.

*4.1.1.2.8. N-(1,3-Benzodioxol-5-ylmethyl)-2-[4-(4-methoxyphenyl)-1-piperazinylthiocarbamoylthio]acetamide (**8**).* Yield: 84%. Mp 158.7 °C.

IR ν_{max} (cm^{-1}): 3302 (N–H stretching), 3074, 3003 (aromatic C–H stretching), 2902 (aliphatic C–H stretching), 2816 (O–CH_2 stretching), 1672 (C=O stretching), 1654, 1546, 1500, 1458, 1429 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.13 (s, 4H), 3.69 (s, 3H), 4.12 (s, 4H), 4.20 (d, J = 5.55 Hz, 2H), 4.36 (s, 2H), 5.96 (s, 2H), 6.74 (d, J = 7.8 Hz, 1H), 6.82–6.85 (m, 4H), 6.92 (d, J = 8.5 Hz, 2H), 8.57 (s, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.77 (CH_2), 42.79 (CH_2), 49.82 (2CH_2), 51.00 (2CH_2), 55.67 (CH_3), 101.26 (CH_2), 108.34 (CH), 108.41 (CH), 114.84 (2CH), 118.36 (2CH), 120.83 (CH), 133.54 (C), 144.89 (C), 146.50 (C), 147.69 (C), 153.89 (C), 166.86 (C), 195.14 (C).

For $C_{22}H_{25}N_3O_4S_2$ Calculated: C, 57.50; H, 5.48; N, 9.14. Found: C, 57.10; H, 5.43; N, 9.74.

HRMS (ESI) (*m/z*): $[M+H]^+$ 460.14.

*4.1.1.2.9. N-(1,3-Benzodioxol-5-ylmethyl)-2-[4-(2-pyrimidinyl)-1-piperazinylthiocarbamoylthio]acetamide (**9**).* Yield: 87%. Mp 179.1 °C.

IR ν_{max} (cm^{-1}): 3257 (N–H stretching), 3051 (aromatic C–H stretching), 2997 (aliphatic C–H stretching), 2893 (O–CH_2 stretching), 1645 (C=O stretching), 1585, 1550, 1489, 1462, 1440, 1421 (C=N, C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.88–3.97 (m, 4H), 4.08–4.11 (m, 4H), 4.19 (d, J = 5.85 Hz, 2H), 4.25–4.33 (m, 2H), 5.97 (s, 2H), 6.70 (t, J = 4.7 Hz, 1H), 6.74 (d, J = 8.0 Hz, 1H), 6.82–6.85 (m, 2H), 8.41 (d, J = 4.7 Hz, 2H), 8.57–8.59 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.74 (CH_2), 42.98 (CH_2), 49.78 (2CH_2), 51.19 (2CH_2), 101.25 (CH_2), 108.35 (CH), 108.41 (CH), 111.11 (CH), 120.84 (CH), 133.54 (C), 146.49 (C), 147.68 (C), 158.48 (2CH), 161.32 (C), 166.82 (C), 195.29 (C).

For $C_{19}H_{21}N_5O_3S_2$ Calculated: C, 52.88; H, 4.91; N, 16.23. Found: C, 52.10; H, 4.93; N, 16.24.

HRMS (ESI) (*m/z*): $[M+H]^+$ 432.12.

*4.1.1.2.10. N-(1,3-Benzodioxol-5-ylmethyl)-2-[4-(4-nitrophenyl)-1-piperazinylthiocarbamoylthio]acetamide (**10**).* Yield: 90%. Mp 197.2 °C.

IR ν_{max} (cm^{-1}): 3257 (N–H stretching), 3066 (aromatic C–H stretching), 2980 (aliphatic C–H stretching), 2875 (O–CH_2 stretching), 1643 (C=O stretching), 1597 (NO_2 stretching), 1552, 1496, 1442, 1423 (C=C stretching and N–H bending).

^{1}H NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.72 (s, 4H), 4.12 (s, 4H), 4.19 (d, J = 5.90 Hz, 2H), 4.36 (s, 2H), 5.97 (s, 2H), 6.74 (d, J = 8.0 Hz, 1H), 6.83–6.84 (m, 2H), 6.95 (d, J = 9.35 Hz, 2H), 8.10 (d, J = 9.25 Hz, 2H), 8.58–8.60 (m, 1H).

^{13}C NMR (125 MHz) (DMSO-d_6) δ (ppm): 40.66 (CH_2), 42.78 (CH_2), 45.18 (2CH_2), 48.84 (CH_2), 50.58 (CH_2), 101.26 (CH_2), 108.34 (CH), 108.41 (CH), 112.31 (2CH), 120.83 (CH), 126.26 (2CH), 133.53 (C), 137.34 (C), 146.50 (C), 147.69 (C), 154.18 (C), 166.78 (C), 195.30 (C).

For $C_{21}H_{22}N_4O_5S_2$ Calculated: C, 53.15; H, 4.67; N, 11.81. Found: C, 53.10; H, 4.63; N, 11.84.

HRMS (ESI) (*m/z*): $[M+H]^+$ 475.11.

4.2. Biochemistry

4.2.1. Cell culture and drug treatment

C6 Rat glioma cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Deisenhofen, Germany) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland). A549 Human lung adenocarcinoma cells were incubated in 90% RPMI supplemented with 10% fetal bovine serum (Gibco, Paisley, Scotland). All media were supplemented with 100 IU/mL penicillin-streptomycin (Gibco, Paisley, Scotland) and cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at 2×10^4 cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). The stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO; Sigma Aldrich, Poole, UK) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

4.2.2. MTT assay

The level of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) reduction was quantified as previously described in the literature with small modifications [24,25].

After 24 h of preincubation, the tested compounds and cisplatin (positive control) were added to give final concentration in the range 3.9–1000 μg/mL and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL and the cells were incubated for 4 h at 37 °C. After the medium was removed, the formazan crystals formed by MTT metabolism were solubilized by addition of 200 μL DMSO to each well and absorbance was read at 540 nm with a microtiter plate spectrophotometer (Bio-Tek plate reader). Every concentration was repeated in three wells. IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

4.2.3. Purification of human carbonic anhydrase isozymes (hCA-I and hCA-II) from human erythrocytes by affinity chromatography

Fresh human blood was obtained from the blood center, Ataturk University, it was stored at 4 °C used within 2–3 days at most. The blood samples were centrifuged to separate erythrocytes at 2500 rpm for 15 min and plasma and buffy coat were carefully removed. Then, underlying erythrocytes were washed with 0.9% NaCl solution twice and upper portions were also discarded. The erythrocytes were hemolyzed with distilled water at 0 °C, it was stirred for half an hour at 4 °C. The hemolysate was centrifuged at 20000 rpm for 30 min and cell membranes were separated. pH was adjusted to 8.7 with solid Tris. So, the hemolysate was recovered to be applied to the column [26,27].

The affinity gel was prepared on Sepharose-4B matrix. After Sepharose-4B was activated with CNBr, L-tyrosine was covalently fitted. Then sulfanilamide was coupled to tyrosine with diazotization reaction as a ligand. The hemolysate was applied to the prepared Sepharose-4B-L-tyrosine-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4 °C [28].

4.2.4. Determination of CA activity

4.2.4.1. Hydratase activity. Carbonic anhydrase activity was determined using the Wilbur-Anderson Method which was modified by Rickli et al. [28,29]. This method, as a result hydration of CO_2 is released H^+ ions and the pH changes were determined by means of bromine thymol blue indicator, based on measuring the elapsed time. Enzyme Unit (EU) was calculated using the equation $(t_o\text{-}t_c/t_c)$ where t_o and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

4.2.4.2. Inhibition assays. The inhibitory effects of compounds **1**–**10** and AAZ on the hydratase activity of hCA-I and hCA-II enzymes were investigated. IC_{50} values were calculated for the compounds at different concentrations while maintaining a constant substrate concentration. The activities of enzymes in the medium without inhibitors were used as 100% activity. The activity % values of enzymes were calculated by measuring the hydratase activity in the presence of different concentrations of inhibitors. The IC_{50} value was calculated by utilizing graphs of activity%-[I] for each inhibitor [29–31].

Declaration of interest

The authors report no conflicts of interest.

Acknowledgements

This study was supported by Anadolu University Scientific Research Projects Commission under the grant no: 1404S123.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.09.035.

References

[1] C.T. Supuran, Bioorg. Med. Chem. Lett. 20 (2010) 3467–3474.
[2] C.T. Supuran, A. Scozzafava, Eur. J. Med. Chem. 35 (2000) 867–874.
[3] A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Expert Opin. Ther. Pat. 14 (2004) 667–702.
[4] K.K. Sethi, S.K. Sahoo, J.N. Pichikala, P. Suresh, J. Enzyme Inhib. Med. Chem. 27 (2012) 97–100.
[5] D. Neri, C.T. Supuran, Nat. Rev. Drug Discov. 10 (2011) 767–777.
[6] S.A. Poulsen, Expert Opin. Ther. Pat. 20 (2010) 795–806.
[7] A. Thiry, J.M. Dogne, B. Masereel, C.T. Supuran, Trends Pharmacol. Sci. 27 (2006) 566–573.
[8] M. Zatovicova, L. Jelenska, A. Hulikova, L. Csaderova, Z. Ditte, P. Ditte, T. Goliasova, J. Pastorek, S. Pastorekova, Curr. Pharm. Des. 16 (2010) 3255–3263.
[9] P.C. McDonald, J.Y. Winum, C.T. Supuran, S. Dedhar, Oncotarget 3 (2012) 84–97.
[10] G.L. Almajan, A. Innocenti, L. Puccetti, G. Manole, S. Barbuceanu, I. Saramet, A. Scozzafava, C.T. Supuran, Bioorg. Med. Chem. Lett. 15 (2005) 2347–2352.
[11] M.K. Abdel-Hamid, A.A. Abdel-Hafez, N.A. El-Koussi, N.M. Mahfouz, A. Innocenti, C.T. Supuran, Bioorg. Med. Chem. 15 (2007) 6975–6984.
[12] V. Alterio, A. Di Fiore, K. D'Ambrosio, C.T. Supuran, G. De Simone, Chem. Rev. 112 (2012) 4421–4468.
[13] F. Carta, A. Akdemir, A. Scozzafava, E. Masini, C.T. Supuran, J. Med. Chem. 55 (2012) 1721–1730.
[14] S.M. Monti, A. Maresca, F. Viparelli, F. Carta, G. De Simone, F.A. Mühlschlegel, A. Scozzafava, C.T. Supuran, Bioorg. Med. Chem. Lett. 22 (2012) 859–862.
[15] H. Nabipour, S. Ghammamy, S. Ashuri, Z.S. Aghbolagh, Org. Chem. J. 2 (2010) 75–80.
[16] S. Kanchi, P. Singh, K. Bisetty, Arab. J. Chem. 7 (2014) 11–25.
[17] R.D. Li, H.L. Wang, Y.B. Li, Z.Q. Wang, X. Wang, Y.T. Wang, Z.M. Ge, R.T. Li, Eur. J. Med. Chem. 93 (2015) 381–391.
[18] M. Bozdag, F. Carta, D. Vullo, A. Akdemir, S. Isik, C. Lanzi, A. Scozzafava, E. Masini, C.T. Supuran, Bioorg. Med. Chem. 23 (2015) 2368–2376.
[19] F. Carta, M. Aggarwal, A. Maresca, A. Scozzafava, R. McKenna, C.T. Supuran, Chem. Commun. 48 (2012) 1868–1870.
[20] A. Innocenti, A. Scozzafava, C.T. Supuran, Bioorg. Med. Chem. Lett. 20 (2010) 1548–1550.
[21] H.H. Wang, K.M. Qiu, H.E. Cui, Y.S. Yang, Yin Luo, M. Xing, X.Y. Qiu, L.F. Bai, H.L. Zhu, Bioorg. Med. Chem. 21 (2013) 448–455.
[22] M.D. Altıntop, A.S. Gurkan-Alp, Y. Özkay, Z.A. Kaplancıklı, Arch. Pharm. Chem. Life Sci. 346 (2013) 571–576.
[23] M.D. Altıntop, A. Özdemir, Z.A. Kaplancıklı, G. Turan Zitouni, H.E. Temel, G. Akalın Çiftçi, Arch. Pharm. Chem. Life Sci. 346 (2013) 189–199.
[24] T. Mosmann, J. Immunol. Methods 16 (1983) 55–63.
[25] K. Keiser, C.C. Johnson, D.A. Tipton, J. Endod. 26 (2000) 288–291.
[26] B. Nalbantoglu, N. Demir, H. Ozdemir, O.I. Kufrevioglu, Turk. J. Med. Sci. 26 (1996) 163–166.
[27] Y. Demir, N. Demir, H. Nadaroglu, E. Bakan, Prep. Biochem. Biotech. 30 (2000) 49–59.
[28] Y. Demir, N. Demir, S. Yildirim, H. Nadaroglu, M. Karaosmanoglu, E. Bakan, Prep. Biochem. Biotechnol. 31 (2001) 291–304.
[29] K.M. Wilbur, N.G. Anderson, J. Biol. Chem. 176 (1948) 147–154.
[30] E.E. Rickli, S.A. Ghazanfar, B.H. Gibbons, J.T. Edsall, J. Biol. Chem. 239 (1964) 1065–1078.
[31] M.D. Altintop, A. Ozdemir, K. Kucukoglu, G. Turan-Zitouni, H. Nadaroglu, Z.A. Kaplancikli, J. Enzyme Inhib. Med. Chem. 30 (2015) 32–37.