Carbonic anhydrase inhibitors. Synthesis of a novel series of 5-substituted 2,4-dichlorobenzenesulfonamides and their inhibition of human cytosolic isozymes I and II and the transm...

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MAY 2014

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2014.05.039 · Source: PubMed

6 AUTHORS, INCLUDING:

Jarosław Sławiński
Medical University of Gdansk
85 PUBLICATIONS **606** CITATIONS
SEE PROFILE

Aneta Pogorzelska
Medical University of Gdansk
10 PUBLICATIONS **24** CITATIONS
SEE PROFILE

Beata Zolnowska
Medical University of Gdansk
16 PUBLICATIONS **35** CITATIONS
SEE PROFILE

Kamil Brożewicz
Medical University of Gdansk
13 PUBLICATIONS **30** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 82 (2014) 47–55

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Carbonic anhydrase inhibitors. Synthesis of a novel series of 5-substituted 2,4-dichlorobenzenesulfonamides and their inhibition of human cytosolic isozymes I and II and the transmembrane tumor-associated isozymes IX and XII

Jarosław Sławiński [a,*], Aneta Pogorzelska [a], Beata Żołnowska [a], Kamil Brożewicz [a], Daniela Vullo [b], Claudiu T. Supuran [b]

[a] Department of Organic Chemistry, Medical University of Gdańsk, Al. Gen. J. Hallera 107, 80-416 Gdańsk, Poland
[b] Dipartimento di Chimica, Universita degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form
16 December 2013
Accepted 8 May 2014
Available online 13 May 2014

Keywords:
2,4-Dichlorobenzenesulfonamides
Synthesis
Carbonic anhydrase isozymes I, II, IX and XII inhibitors

ABSTRACT

A series of novel 5-substituted 2,4-dichlorobenzenesulfonamides **5a–c, 6a–d, 7a–j** and **10a–i** have been synthesized and investigated as inhibitors of four isoforms of zinc enzyme carbonic anhydrase (CA.EC 4.2.1.1), that is the cytosolic CA I and II, and tumor-associated isozymes CA IX and XII. Against the human CA I investigated compounds displayed K_I values from 349 to 7355 nM, toward hCA II at range of 6.9 to 164 nM, while against hCA IX ranging from 2.8 to 76 nM and against hCA XII in the range of 2.7 to 95 nM. The excellent inhibitory activity against tumor-associated hCA IX was found. The twenty one new compounds displayed a powerful inhibitory potency toward hCA IX (K_I = 2.8–21.7 nM) in comparison with the clinically used CAIs **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (24–50 nM). Among them the most potent hCA IX inhibitor **7b** (K_I = 2.8 nM) was 8.5-fold stronger than **IND** (K_I = 24 nM). Toward tumor-associated hCA XII compounds **6c** and **10a** (K_I = 2.7 and 2.8 nM, respectively) showed a better inhibitory potency than reference sulfonamides **MZA** and **IND** (K_I = 3.4 nM).

1. Introduction

The innovative anticancer treatment strategies are based on the design of agents aimed at the non-classical performance targets, such as proteins and enzymes of the process of tumorigenesis, cell cycle regulators or oncogenes [1]. Carbonic anhydrases (CAs) are a family of enzymes found in a diversity of organisms and primarily responsible for catalyze the reversible hydratation of carbon dioxide. Currently at least fifteen humans CA isoforms belonging to the α-CA class are known [2]. The CAs are involved in many physiological and pathological processes, including pH homeostasis, electrolyte secretion in various tissues and organs, gluconeogenesis, lipogenesis, ureagenesis, bone resorption calcification and tumorigenicity [2–12] making that the inhibitors of these proteins can be considered as a therapeutic agents in prevention and treatment of various disease.

At the end of the last century, the new tumor-associated membrane carbonic anhydrase isozymes CA IX and CA XII have been identified [13–15]. Since then the function of CA IX in tumor physiology has been widely cognized [16–20]. This metalloenzyme is mainly involved in the regulation of pH dynamics in solid tumors [20,21]. However, the CA IX contributes to other cell processes essential for cancer such as adhesion, migration and proliferation [22]. Moreover the CA IX is overexpressed in a broad spectrum of hypoxic human tumors [23] and the relationship between CA IX expression and poor patient prognosis in many kind of cancer is now well-established [22]. Because of the undeniable role of CA IX in promote tumor cell survival and invasion, search for compounds selectively inhibit the CA IX is an important point of the development of new anticancer therapies.

Since discovered that sulfanilamide inhibits the activity of CA [25] many sulfa drugs were revealed as antiglaucoma agents [17,26–28], anti-thyroid drugs [27], the hypoglycemic sulfonamides [29] and, finally, novel types of anticancer agents [30]. It is believed that the latter is due to the inhibition of tumor-associated CA IX and CA XII [24,31].

* Corresponding author.
E-mail address: jaroslaw@gumed.edu.pl (J. Sławiński).

http://dx.doi.org/10.1016/j.ejmech.2014.05.039
0223-5234/

The first reports about inhibition of CA IX applied for a series of aromatic and heterocyclic sulfonamides including clinically used derivatives acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP** (Chart 1) [9]. Further showed that indisulam (E7070) **IND**, a novel sulfonamide anticancer agent in clinical development for the treatment of solid tumors [32], act as a strong CA II and CA IX inhibitor [33]. In the last decade many of inhibitors with strong selectivity toward tumor-associated hCA IX have been detected (structures **U-104**, **CAI17**and **I**–**III**, Chart 1) [34–37]. The potency of most of them compared to that of indisulam was several times higher. Moreover, it has been shown that **U-104** and **CAI17** [36,38,39] displayed *in vivo* significant inhibition of tumor growth constituting an interesting candidates for the development of novel antitumor agents. In addition it should be emphasized that this inhibitors have the ability to specifically target CA IX-expressing tumors [38].

In our previous study we examined the inhibition of hCA I, II, IX and XII with some *S*-substituted 4-chloro-2-mercapto-5- R- or 6-R^1-benzenesulfonamides [40–42]. Some of those compounds showed both strong acting as CAIs and the selectivity for the inhibition of the tumor-associated over the cytosolic CA isoforms [40–42]. These findings prompted us to further investigation and therefore, we reported herein on the design, synthesis and evaluations of inhibitory activity of novel class of 2,4-dichlorobenzenesulfonamides derivatives of type **IV-VII** which was modified in 5 position by moieties with a similar nature as well-known CA IX inhibitors (Chart 1).

2. Results and discussion

2.1. Chemistry

The synthesis of the desired new compounds **5a**–**c**, **6a**–**d**, **7c**–**j** and **10a**–**i** were presented at Schemes 1 and 2. Following the Scheme 1, semi- or thiosemicarbazides **5a**–**c** were obtained with good yield by the convenient one-step reaction of 2,4-dichloro-5-sulfamoylbenzhydrazide **4** with appropriate isothio- or isocyanate in dry tetrahydrofuran either at room temperature (**5a**, **5c**) or at reflux (**5b**).

In turn, starting from 2,4-dichloro-5-sulfamoylbenzoyl chloride **2** and the corresponding hydrazide the expected 2,4-dichloro-5-hydrazinecarbonylbenzenesulfonamides **6a**–**d** were prepared in 65–75% yields through a simple addition–elimination reaction. The reactions were carried out in dry tetrahydrofuran with the

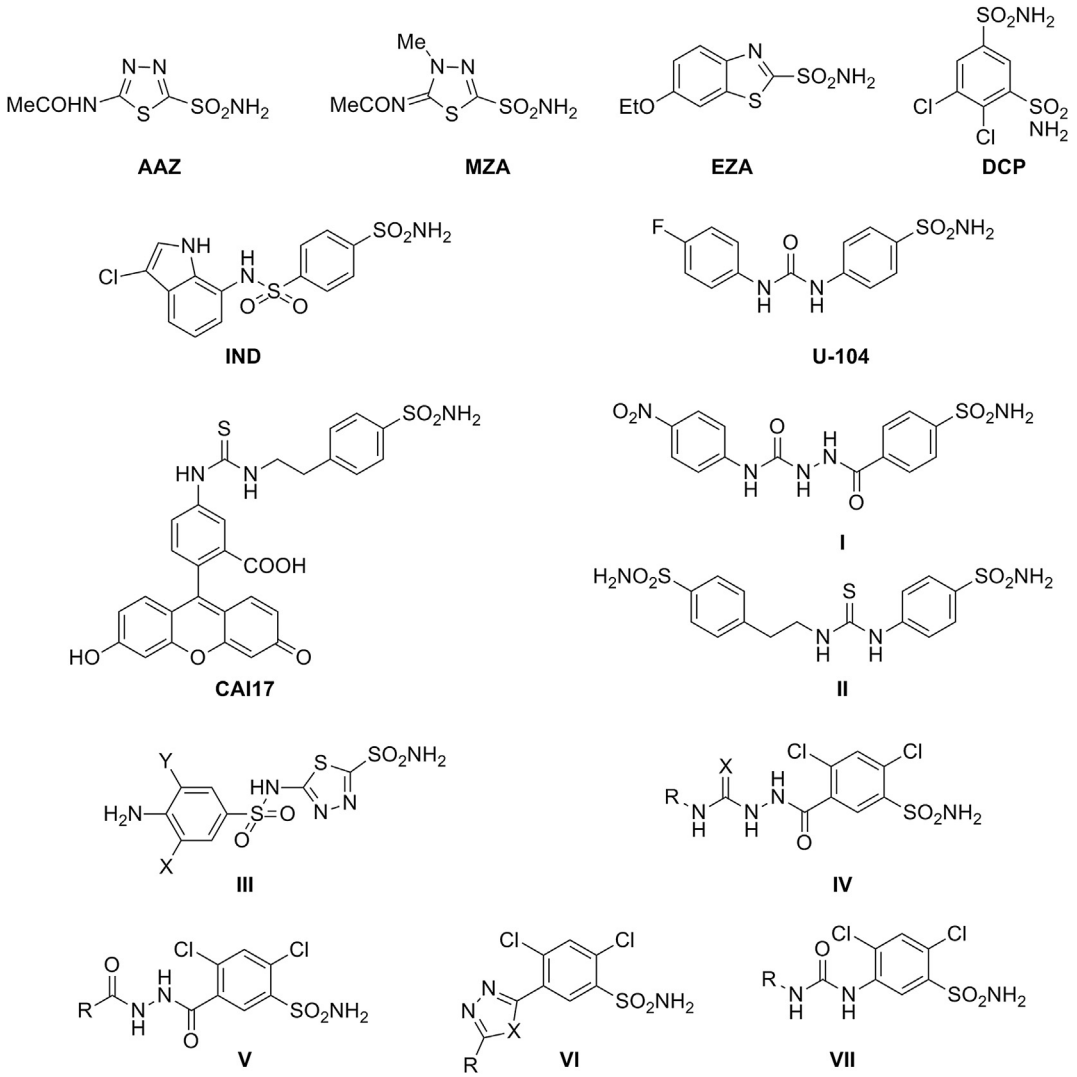
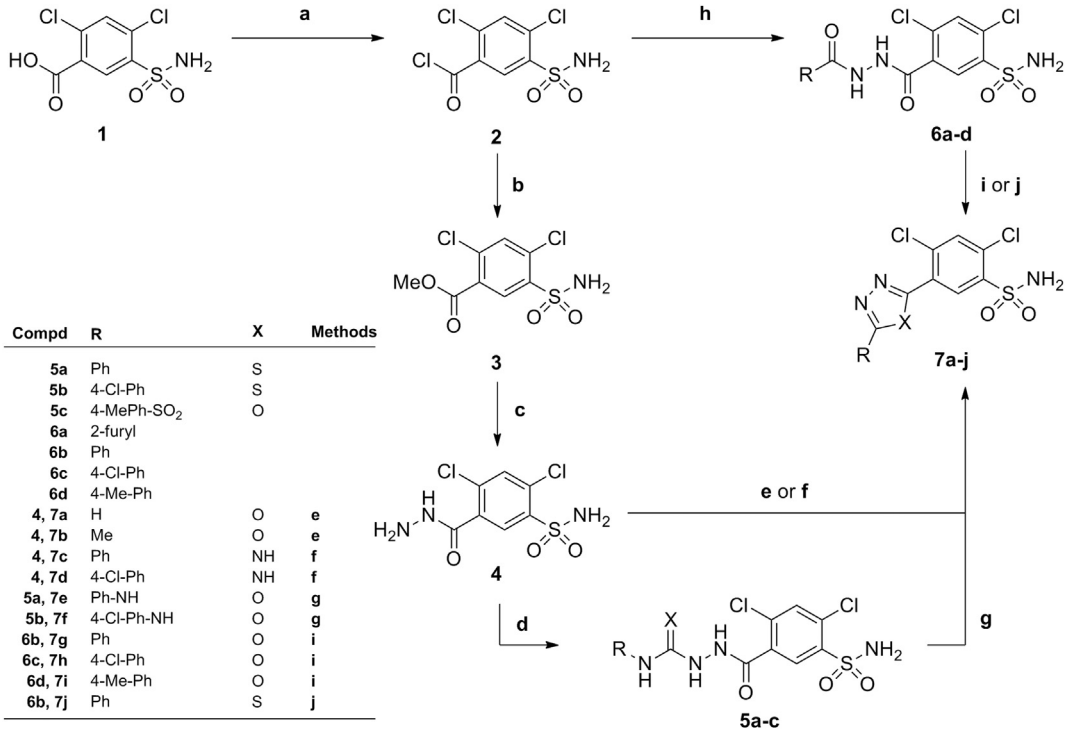


Chart 1. General structures of known clinically used sulfonamides **AAZ**, **MZA**, **EZA**, **DCP** and **IND** (standard CA inhibitors), highly potent CA inhibitors **U-104**, **CAI17** and sulfonamides **I**–**III** and novel 5-substituted 2,4-dichlorobenzenesulfonamides **IV**–**VII.**

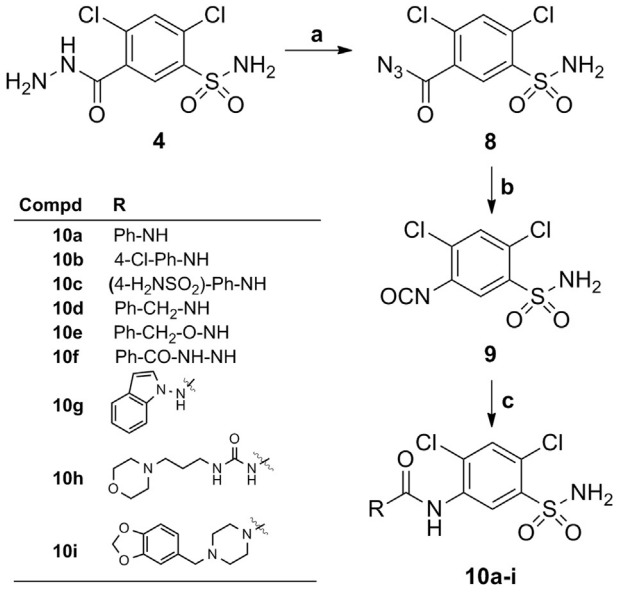


Compd	R	X	Methods
5a	Ph	S	
5b	4-Cl-Ph	S	
5c	4-MePh-SO_2	O	
6a	2-furyl		
6b	Ph		
6c	4-Cl-Ph		
6d	4-Me-Ph		
4, 7a	H	O	**e**
4, 7b	Me	O	**e**
4, 7c	Ph	NH	**f**
4, 7d	4-Cl-Ph	NH	**f**
5a, 7e	Ph-NH	O	**g**
5b, 7f	4-Cl-Ph-NH	O	**g**
6b, 7g	Ph	O	**i**
6c, 7h	4-Cl-Ph	O	**i**
6d, 7i	4-Me-Ph	O	**i**
6b, 7j	Ph	S	**j**

Scheme 1. Synthesis of *N*-(2,4-dichloro-5-sulfamoylbenzoyl)-*N*′′-R-semi- or thiosemicarbazides **5a**–**c**, 2,4-dichloro-5-(2-R-hydrazinecarbonyl)benzenesulfonamides **6a**–**d**, 2,4-dichloro-5-(5-R-1,3,4-oxa- or thiadiazol-2-yl)benzenesulfonamides **7a**,**b** and **7e**–**j**, and 2,4-dichloro-5-(5-R-1,2,4-triazol-3-yl)benzenesulfonamides **7c**,**d**. Reagents and conditions: a) $SOCl_2$, reflux, 4 h; b) dry MeOH, reflux, 16–18 h; Et_3N, H_2O, 1 h; c) 99% hydrazine hydrate (5 M eq.), EtOH, reflux, 4 h; d) RNCX, dry THF r.t. (**5a**, **5c**) or reflux (**5b**), 0.5–3 h; e) $RC(OEt)_3$ (6 M eq.), glacial AcOH, reflux, 7–12 h (**7a**,**b**); f) RC(OEt)NH*HCl, DBU, dry MeOH, reflux, 22–24 h (**7c**,**d**); g) TsCl, Py, acetonitrile, reflux, 3–8 h (**7e**,**f**); h) $RCO(NHNH_2)$, Et_3N (1–2 M eq.), dry THF, r.t., 40–90 h; i) $SOCl_2$, reflux, 6–7 h (**7g**–**i**); j) LR, dry THF, r.t., 24 h (**7j**).

addition of 0.5–1 eq. triethylamine at room temperature for 40–90 h.

The synthetic pathways of new 2,4-dichlorobenzenesulfonamides **7c**–**j**, modified at the position 5 by the azole-containing residues, were depicted in Scheme 1. As it was shown the compounds **7c**–**j** were synthesized according to four different methods (marked as **f**–**g**, **i** or **j**).



Compd	R
10a	Ph-NH
10b	4-Cl-Ph-NH
10c	(4-H_2NSO_2)-Ph-NH
10d	Ph-CH_2-NH
10e	Ph-CH_2-O-NH
10f	Ph-CO-NH-NH
10g	
10h	
10i	

Scheme 2. Synthesis of 2,4-dichloro-5-ureidobenzenesulfonamides **10a**–**i**. Reagents and conditions: a) $NaNO_2$, HCl (aq.), 0–5 °C; b) dry toluene 110 °C, 1 h; c) RH, dry toluene, 110 °C, 0.5–6 h.

Conversion of **4** into the final 2,4-dichloro-5-(1,2,4-triazol-3-yl) benzenesulfonamides **7c**–**d** with the appropriate ethyl benzimidiate hydrochloride (*Method **f***) was carried out in refluxing dry methanol in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).

The 5-(arylamino)-1,3,4-oxadiazole derivatives **7e**,**f** were obtained as a result of desulfurization of thiosemicarbazides **5a**,**b**, respectively, with 4-toluenesulfonyl chloride in the presence of pyridine (1.75 eq.) as a base (*Method **g***). The excellent reaction yields (above 90%) can be explained by both, a selective and rapid sulfonation of thiocarbonyl group of the **5a**,**b**, and the high reactivity of the resulting carbodiimide intermediate.

Alternatively, thionyl chloride (*Method **i***) was used as the dehydrating agent required to accomplish the dehydration of 5-(*N*′-benzoylhydrazinecarbonyl)benzenesulfonamide derivatives **6b**–**d** resulting in the formation of the desired 1,3,4-oxadiazole derivatives **7g**–**i**.

In turn, the 1,3,4-thiadiazole derivative **7j** was prepared by the reaction of diacylhydrazine with the Lawesson's reagent (LR, *Method **j***). Initially formed unstable derivative of **6b** (i.e., mono- or dithiocarbonylhydrazine) was dehydrated or desulfurized to form five-membered heterocyclic ring of **7j** as shown in Scheme 1.

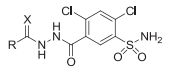
As shown in Scheme 2 the synthesis of 2,4-dichloro-5-ureidobenzenesulfonamides **10a**–**i** was achieved by three-stage process starting from 2,4-dichloro-5-sulfamoylbenzhydrazide **4**. At first, treatment of hydrazide **4** with nitrous acid ($NaNO_2$/HCl) at −5 °C afforded the corresponding azide **8**, which was converted to the isocyanate **9** by Curtius rearrangement. Then, the isocyanate **9** was reacted with the appropriate primary or secondary amines in refluxing dry toluene to furnish the desired urea derivatives **10a**–**i**.

The structures of all new compounds were confirmed by IR and NMR data and elemental analyses (see Experimental protocols).

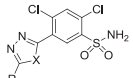
2.2. CA inhibition studies

The compounds **5a**–**c**, **6a**–**d**, **7a**–**j** and **10a**–**i** as well as standard, clinically used CAIs, such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP** and indisulam **IND** (Chart 1), have been tested for the inhibition of two cytosolic, ubiquitous isozymes of human origin, that is, hCA I and II, and two transmembrane tumor-associated isozymes: hCA IX and XII. The following structure–activity relationship (SAR) can be drawn from CA inhibitory data of Table 1:

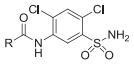
Table 1
Carbonic anhydrase inhibition data for compounds **5a**–**c**, **6a**–**d**, **7a**–**j** and **10a**–**i** and standard inhibitors against human isozymes hCA I, II, IX and XII by a stopped-flow, CO_2 hydration assay [46].



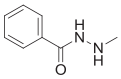
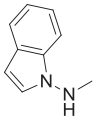
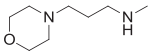
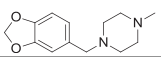
5a-c, 6a-d



7a-j



10a-i

Compd	R	X	K_I[a] (nM)			
			hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]
AAZ			250	12.0	25.0	5.7
MZA			780	14.0	27.0	3.4
EZA			25	8.0	34.0	22.0
DCP			1200	38.0	50.0	50.0
IND			31	15.0	24.0	3.4
5a	PhNH	S	6230	123.0	31.0	24.0
5b	4-ClPhNH	S	5490	87.0	27.0	30.0
5c	4-MePhSO$_2$NH	O	7200	63.0	15.3	8.4
6a	2-furyl	O	3470	13.5	3.9	4.7
6b	Ph	O	2750	24.0	10.2	5.0
6c	4-ClPh	O	3025	10.1	4.8	2.7
6d	4-MePh	O	2400	25.0	5.5	8.1
7a	H	O	643	13.1	3.1	7.6
7b	Me	O	718	8.7	2.8	6.3
7c	Ph	NH	1350	47.0	9.7	12.5
7d	4-ClPh	NH	1175	54.0	6.6	8.9
7e	PhNH	O	3200	41.0	13.8	7.1
7f	4-ClPhNH	O	2340	33.0	15.1	6.4
7g	Ph	O	568	28.0	13.2	8.0
7h	4-ClPh	O	671	15.1	10.9	13.6
7i	4-MePh	O	349	29.0	4.7	8.2
7j	Ph	S	1320	68.0	13.6	5.4
10a	PhNH		2340	44.0	7.0	2.8
10b	4-ClPhNH		3200	39.0	7.1	4.3
10c	4-H$_2$NSO$_2$PhNH		573	6.9	15.8	3.6
10d	BnNH		1165	57.0	21.7	23.0
10e	BnONH		934	40.0	18.9	33.0
10f			1260	79.0	12.5	24.0
10g			5430	84.0	26.0	32.0
10h			6400	115.0	38.0	44.0
10i			7355	164.0	76.0	95.0

[a] Errors in the range of ±5–10% of the reported value (from 3 different assays).
[b] Human (cloned) isozymes, by CO_2 hydration method.
[c] Catalytic domain of human, cloned isozymes [47], by the CO_2 hydration method.

a) The investigated 2,4-dichlorobenzenesulfonamides **5a**–**c**, **6a**–**d**, **7a**–**j** and **10a**–**i** displayed rather weak inhibitory properties against the slow cytosolic isoform hCA I with K_I in the range of 349–7355 nM. Thus, derivatives **5a**–**c** and **10g**–**i** showed weak inhibition of this isoform, with K_I in the range of 5430–7355 nM, being thus the weakest inhibitors among tested compounds, including the clinically used compounds **AAZ**–**IND** (Table 1). The compounds **7a**,**b**, **7g**–**i** and **10c** had a slightly increased affinity to the hCA I and stand out as the most active derivatives (K_I of 349–718 nM) in comparison with the other tested 2,4 -dichlorobenzenesulfonamides (Table 1).

b) The hCA II inhibitory activity (K_I in the range of 6.9–164 nM) was comparable to the reference compounds **AAZ**–**IND** (Table 1). However, low K_I values (8.7–29 nM) observed for derivatives **7a**,**b** and **7g**–**i** suggest that the insertion the oxadiazole ring system in *meta* position to the sulfamoyl moiety increase the inhibitory properties. Similarly, the substitution of benzenesulfonamide by *meta*-hydrazinocarbonyl group in the series **6a**–**d** resulted in increased hCA II inhibitory activity (K_I of 10.1–25 nM). In addition, it should be emphasized, that compound **10c** with K_I = 6.9 was the best hCA II inhibitor even in comparison with **EZA** (K_I = 8 nM) with the highest inhibitory activity in the reference group.

c) An excellent inhibition profile of the isoform hCA IX was found for novel 2,4-dichlorobenzenesulfonamides. All compounds, with the exception of **10i**, inhibited the activity of this isozyme similar or better (K_I of 2.8–31 nM) than the references **AAZ**–**IND** (K_I of 24–50 nM). The best inhibitory properties (K_I values in the range of 2.8–15.1 nM) was observed for derivatives of **6** and **7** series possessing hydrazinocarbonyl group (**6a**–**d**), oxa- or thiadiazoles (**7a**,**b**, **7e**–**i**) and triazoles (**7c**,**d**) moieties in *meta* position of benzenesulfonamide system. It should be pointed out that the twenty one new 2,4-dichlorobenzenesulfonamides were characterized by higher activity compared with **IND**, the most effective clinically used hCA IX inhibitor (see Table 1). Moreover, the most potent hCA IX inhibitor in the tested series was 2,4-dichloro-5-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonamide (**7b**, K_I = 2.8 nM) being thus 8.5-fold stronger than **IND** (K_I = 24 nM).

d) Similar results were obtained for the second tumor-associated isoform hCA XII. The inhibitory properties of tested compounds were comparable with the references **AAZ**–**IND** with the exception of **10i,** with the higher inhibition constant K_I = 95 nM. As in the case of the hCA IX, the good hCA XII inhibitory activity was observed mainly for the compounds of series **6** and **7** with K_I in the range of 2.7–13.6 nM. The most potent inhibitor **6c** with K_I = 2.7 nM was characterized by better hCA XII inhibition in comparison with clinically used **AAZ**–**IND.** However, the most promising inhibitory properties were found for 2,4-dichloro-5-[3-(4-R-phenyl)ureido]benzenesulfonamides **10a**–**c** (R = H, Cl, SO_2NH_2) with K_I: 2.8–3.6 nM. Moreover, compound **10a** exerted the highest selectivity toward hCA XII versus hCA II (hCA II/hCA XII = 15.7). Relatively good selectivity ratios towards isoform hCA XII and hCA II displayed also compound **7j** (hCA II/hCA XII = 12.6).

e) It should be noted that the presence of 4-R-phenyl group (R = H, Cl, SO_2NH_2) attached directly to the nitrogen atom *N*-3 of the urea moiety in the series **10a**–**i** resulted in increase of inhibitory potency against all tested CA isoforms. The rather significant decrease of inhibition of hCA I, II, IX and XII was found for **10h**–**i** possessing either 3-(3-morpholin-4-

ylpropyl)ureido moiety (**10h**) or 4-(benzodioxol-5-ylmethyl) piperazino-1-carboxamide substituent (**10i**) in *meta* position to the sulfamoyl group. Moreover, the relatively low activity against isoforms hCA I, II, IX and XII exhibited thiosemicarbazides **5a**,**b** (see Table 1).

3. Conclusions

We have developed methods for the preparation of novel series of 5-substituted 2,4-dichlorobenzenesulfonamides (thio- or semicarbazides, hydrazinocarbonyls, oxa- or thiadiazoles, triazoles and ureas). The 26 new sulfonamides have been assayed for the inhibition of four physiologically relevant CA isozymes, such as CA I and II, the tumor-associated isozymes CA IX and XII. A weak inhibitory activity against the human CA I was observed for all investigated compounds with K_I values from 349 to 7355 nM. However, in the case of the second physiological isoform hCA II the inhibitory activity of most of the tested benzenesulfonamides was comparable with reference compounds and their K_I values were in the range from 6.9 to 164 nM. Likewise, the inhibition of tumor-associated hCA XII by the tested benzenesulfonamides (K_I = 2.7–95 nM) was quite similar to the clinically used CA inhibitors **AAZ**–**IND**. It should be noted, however, that compounds **6c** and **10a**, with K_I = 2.7 and 2.8 nM, respectively, exhibited a better inhibitory potency than the best inhibitors in the references, **MZA** and **IND** (K_I = 3.4 nM). Moreover, compound **10a** exerted the highest selectivity ratios toward hCA XII versus hCA II (hCA II/hCA XII = 15.7). On the other hand, the excellent inhibitory activity against hCA IX should be stressed; this isoform was inhibited with K_I values from 2.8 to 76 nM. The twenty one new compounds displayed a powerful inhibitory potency toward hCA IX (K_I = 2.8–21.7 nM) in comparison with the clinically used CA inhibitors **AAZ**–**IND** (24–50 nM). Among them the most potent inhibitor **7b** (K_I = 2.8 nM) was 8.5-fold stronger than **IND** (K_I = 24 nM). The present studies indicate that the currently synthesized 2,4-dichloro derivatives with bulky and highly functionalized substituents at the 5 position of the benzenesulfonamide scaffold display excellent inhibition profile of the isoforms hCA IX and XII, similar or much better in comparison to the 2-mercapto-5- R- or 6-R^1-benzenesulfonamide analogs [40–42].

4. Experimental protocols

4.1. Synthesis

Melting points were determined with a Boethius PHMK apparatus. Infrared (IR) spectra were recorded with a Thermo Mattson Satellite FTIR spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) experiments were carried out on a Varian Unity Plus 500 MHz or Varian Gemini 200 apparatus at 200 and 50 MHZ respectively; chemical shifts are expressed in parts per million (ppm) relative to TMS as internal standard. The mass spectra were acquired on a Bruker Biflex III MALDI-TOF spectrometer after deposition on a 2,5-dihydroxybenzoic acid (DHB) matrix. The results of elemental analyses for C, H and N were in agreement with the theoretical values within ±0.4% range. The starting 2,4-dichloro-5-sulfamoylbenzoyl chloride **2** and 2,4-dichloro-5-sulfamoylbenzhydrazide **4** were obtained from commercially available 2,4-dichloro-5-sulfamoylbenzoic acid **1** according to methods described previously [43,44]. 2,4-Dichloro-5-(1,3,4-oxadiazol-2-yl)benzenesulfonamide **7a** and 2,4-dichloro-5-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonamide **7b** were obtained from **4** by known method [45].

4.1.1. Procedures for the preparation of N-(2,4-dichloro-5-sulfamoylbenzoyl)-thiosemicarbazides (**5a**,**b**)

To a suspension of **4** (1 mmol) in dry tetrahydrofuran (5 ml) corresponding isothiocyanate (1.05 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 1.5 h (**5a**) or heated at reflux for 3 h (**5b**). The desired products were filtered off and dried.

*4.1.1.1. N-(2,4-Dichloro-5-sulfamoylbenzoyl)-N″-(phenyl)thiosemicarbazide (**5a**).* Starting from **4** (1.0 g) and phenyl isothiocyanate (0.5 g) the desired **5a** was obtained (1.065 g, 72%): m.p. 193–195 °C; IR (KBr) 3318 (NH_2, NH), 1690 (C=O), 1587, 1561, 1496 (C=C), 1324, 1164 (SO_2) cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.19–7.21 (m, 1H, H arom.), 7.36–7.39 (m, 2H, H arom.), 7.48 (s, 2H, NH_2), 7.82 (s, 2H, H arom.), 7.99 (m, 1H, H-3), 8.31 (s, 1H, H-6), 9.89 (s, 2H, 2NH), 10.71 (s, 1H, NH) ppm; MALDI-TOF *m/z* obsd: 420.9, $[M + H]^+$, 442.9, $[M + Na]^+$ calcd: 419.9; Anal. ($C_{14}H_{12}Cl_2N_4O_3S_2$) C, H, N.

*4.1.1.2. N-(2,4-Dichloro-5-sulfamoylbenzoyl)-N″-(4-chlorophenyl) thiosemicarbazide (**5b**).* Starting from **4** (1.0 g) and 4-chlorophenyl isothiocyanate (0.6 g) the desired **5b** was obtained (1.053 g, 66%): m.p. 203–205 °C; IR (KBr) 3319 (NH_2, NH), 1695 (C=O), 1552, 1493 (C=C), 1321, 1164 (SO_2) cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.43(d, J = 8.79 Hz, 2H, H arom.), 7.51 (s, 2H, NH_2), 7.77–7.83 (m, 2H, H arom.), 7.99 (m, 1H, H-3), 8.34 (s, 1H, H-6), 10 (s, 2H, 2NH), 10.73 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 128.31, 128.49, 130.33, 132.45, 132.97, 133.51, 135.22, 138.38, 139.97, 164.19, 164.23. Anal. ($C_{14}H_{11}Cl_3N_4O_3S_2$) C, H, N.

4.1.2. Preparation of N-(2,4-dichloro-5-sulfamoylbenzoyl)-N″-tosylsemicarbazide (**5c**)

To a suspension of **4** (0.5 g) in dry tetrahydrofuran (8 ml) *p*-toluenesulfonyl isocyanate (0.5 g) was added. The reaction mixture was stirred at room temperature for 0.5 h and then evaporated under reduced pressure. 2% Hydrochloric acid solution (10 ml) and methanol (10 ml) were added, the mixture was cooled and the precipitation of *N*-(2,4-dichloro-5-sulfamoylbenzoyl)-*N*″-tosylsemicarbazide **5c** was collected by filtration, washed with water and dried (0.7 g, 81%): m.p. 214–216 °C; IR (KBr) 3320 (NH_2, NH), 1697, 1673 (C=O), 1582, 1547, 1505, 1452 (C=C), 1353, 1168 (SO_2) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6) δ 2.4 (s, 3H, CH_3), 7.42 (d, J = 8.05 Hz, 2H, H arom.), 7.77–7.83 (m, 5H, NH_2, H arom.), 7.97 (s, 1H, H-6), 8.84 (s, 1H, NH), 10.39 (s, 1H, NH), 11.2 (s, 1H, SO_2NH) ppm; MALDI-TOF *m/z* obsd: 482.8, $[M + H]^+$ calcd: 481.9; Anal. ($C_{15}H_{14}Cl_2N_4O_6S_2$) C, H, N.

4.1.3. Procedures for the preparation of 2,4-dichloro-5-hydrazinecarbonylbenzenesulfonamides (**6a**–**d**)

To a stirred suspension of the appropriate R-hydrazide (1 eq.) in dry tetrahydrofuran, triethylamine (0.5–1 eq.) was added and the mixture was cooled to 5 °C. A solution of **2** (1 eq.) in dry tetrahydrofuran was added dropwise and the reaction mixture was stirred at room temperature for 40–90 h. The solvents were evaporated under reduced pressure and the products **6a**–**d** were isolated as described below.

*4.1.3.1. 2,4-Dichloro-5-[2-(furan-2-carbonyl)hydrazinecarbonyl]benzenesulfonamide (**6a**).* Starting from furane-2-carbohydrazide (1.261 g, 10 mmol) in THF (20 ml), Et_3N (0.29 g, 5 mmol) and **2** (1.443 g, 5 mmol) in THF (20 ml), the crude **6a** (1.486 g) was obtained through the treatment of evaporated mixture by 18% hydrochloric acid (20 ml) and filtration of the precipitation. The crude **6a** was crystallized from methanol/water (1/9) and the title compound **6a** (1.323 g, 70%) was isolated: m.p. 218–219 °C; IR (KBr) 3385, 3302 (NH_2), 3242 (NHNH), 1710 (C=O), 1686 (C=O), 1350,

1167 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 6.67–6.7 (m, 1H, H-furyl-4), 7.27–7.29 (m, 1H, H-furyl-3), 7.88 (s, 2H, NH_2), 7.93–7.95 (m, 1H, H-furyl-5), 8.01 (s, 1H, H-3), 8.05 (s, 1H, H-6), 10.61 (s, 1H, NH), 10.64 (s, 1H, NH) ppm; MALDI-TOF *m/z* obsd: 379.8, $[M + H]^+$, 401.8, $[M + Na]^+$ calcd: 378.9; Anal. ($C_{12}H_9Cl_2N_3O_5S$) C, H, N.

*4.1.3.2. 2,4-Dichloro-5-(2-benzoylhydrazinecarbonyl)benzenesulfonamide (**6b**).* Starting from benzhydrazide (14.9 g, 0.11 mol) in THF (40 ml), Et_3N (6.5 g, 0.11 mol) and **2** (15.25 g, 0.055 mol) in THF (40 ml), the crude **6b** was obtained through the treatment of evaporated mixture by water, acidified with 18% hydrochloric acid and filtration of the precipitation. The crude **6b** washed with 10% aqueous solution of sodium bicarbonate (3 × 20 ml), ethanol and crystallized from DMF/water (1/1) and the title compound **6b** (15.35 g, 75%) was isolated: m.p. 279–281 °C; IR (KBr) 3390, 3291 (NH_2), 3182 (NHNH), 1674 (C═O), 1608 (C═O), 1358, 1164 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.49–7.65 (m, 3H, H arom.), 7.9–7.95 (m, 4H, H arom., NH_2), 8.02 (s, 1H, H-3), 8.11 (s, 1H, H-6), 10.69 (s, 1H, NH), 10.72 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 127.79, 128.82, 129.62, 132.31, 132.43, 132.67, 132.97, 133.93, 134.89, 140.22, 164.32, 165.78. Anal. ($C_{14}H_{11}Cl_2N_3O_4S$) C, H, N.

*4.1.3.3. 2,4-Dichloro-5-[2-(4-chlorobenzoyl)hydrazinecarbonyl]benzenesulfonamide (**6c**).* Starting from 4-chlorobenzhydrazide (1.5 g, 8.8 mmol) in THF (15 ml), Et_3N (0.26 g, 4.4 mmol) and **2** (1.27 g, 4.4 mmol) in THF (15 ml), the crude **6c** was obtained through the treatment of evaporated mixture by water and filtration of the precipitation. The crude **6c** crystallized from ethanol/water (7/3) and the title compound **6c** (1.209 g, 65%) was isolated: m.p. 287–290 °C; IR (KBr) 3355, 3251 (NH_2), 3183 (NHNH), 1687 (C═O), 1655 (C═O), 1321, 1165 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.62 (d, J = 8.3 Hz, 2H, H arom.), 7.89 (s, 2H, NH_2), 7.95 (d, J = 8.79 Hz, 2H, H arom.), 8.03 (s, 1H, H-3), 8.09 (s, 1H, H-6), 10.72 (s, 1H, NH), 10.82 (s, 1H, NH) ppm; MALDI-TOF *m/z* obsd: 423.8, $[M + H]^+$, 446.8, $[M + Na]^+$ calcd: 422.9; Anal. ($C_{14}H_{10}Cl_3N_3O_4S$) C, H, N.

*4.1.3.4. 2,4-Dichloro-5-[2-(4-methylbenzoyl)hydrazinecarbonyl]benzenesulfonamide (**6d**).* Starting from 4-methylbenzhydrazide (1.501 g, 10 mmol) in THF (30 ml), Et_3N (0.29 g, 5 mmol) and **2** (1.443 g, 5 mmol) in THF (20 ml), the crude **6d** was obtained through the treatment of evaporated mixture by water and filtration of the precipitation. The crude **6d** crystallized from methanol/water (4/3) and the title compound **6d** (1.45 g, 72%) was isolated: m.p. 272–274 °C; IR (KBr) 3346, 3186 (NH_2), 1682 (C═O), 1647 (C═O), 1325, 1164 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 2.38 (s, 3H, CH_3), 7.33 (d, J = 8.35 Hz, 2H, H arom.), 7.85 (d, J = 8.26 Hz, 2H, H arom.), 7.89 (s, 2H, NH_2), 8.02 (s, 1H, H-3), 8.1 (s, 1H, H-6), 10.64 (s, 2H, NHNH) ppm. Anal. ($C_{15}H_{13}Cl_2N_3O_4S$) C, H, N.

*4.1.4. Procedures for the preparation of 2,4-dichloro-5-(5-aryl-1,2,4-triazol-3-yl)benzenesulfonamides (**7c**,**d**)*

To a suspension of **4** (1.76 mmol) in dry methanol (10 ml) corresponding ethyl benzimidate hydrochloride (1.9 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 3.52 mmol) were added. The reaction mixture was stirred and heated at reflux for 22–24 h. The solvents were evaporated under reduced pressure and the residue was dissolved in dichloromethane and acidified with 2% hydrochloric acid (**7c**) or glacial acetic acid (**7d**). The precipitation of appropriate 2,4-dichloro-5-(5-aryl-1,2,4-triazol-3-yl)benzenesulfonamide was filtered off and dried.

*4.1.4.1. 2,4-Dichloro-5-(5-phenyl-1,2,4-triazol-3-yl)benzenesulfonamide (**7c**).* Starting from 4 (0.5 g) and ethyl benzimidate hydrochloride (0.35 g) the **7c** was obtained (0.36 g, 53%): m.p. 301–303 °C; IR (KBr) 3339, 3249 (NH_2), 3183 (NH), 3096, 3012, 2930 (C–H sp^2, sp^3), 1542, 1468 (C═C, C═N), 1349, 1171 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.55–7.58 (m, 3H, H arom.), 7.85 (s, 2H, NH_2), 8.03–8.09 (m, 3H, H arom.), 8.6 (s, 1H, H-6), 14.82 (s, 1H, NH) ppm. Anal. ($C_{14}H_{10}Cl_2N_4O_2S$) C, H, N.

*4.1.4.2. 2,4-Dichloro-5-[5-(4-chlorophenyl)-1,2,4-triazol-3-yl]benzenesulfonamide (**7d**).* Starting from **4** (0.5 g) and ethyl 4-chlorobenzimidate hydrochloride (0.43 g) the **7d** was obtained (0.36 g, 51%): m.p. 288–290 °C; IR (KBr) 3380, 3273 (NH_2), 3092, 2924 (C–H sp^2, sp^3), 1543, 1482 (C═C, C═N), 1346, 1174 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.66 (m, 2H, H arom.), 7.87 (s, 2H, NH_2), 8.07 (m, 2H, H arom.), 8.6 (s, 1H, H-6), 14.4 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 127.58, 128.14, 128.75, 129.43, 131.21, 131.54, 133.33, 134.76, 135.35, 140.32, 156.41, 159.99. Anal. ($C_{14}H_9Cl_3N_4O_2S$) C, H, N.

*4.1.5. Procedures for the preparation of 2,4-dichloro-5-[5-(arylamino)-1,3,4-oxadiazol-2-yl]benzenesulfonamides (**7e**,**f**)*

To a suspension of **5a**,**b** (1 mmol) in dry tetrahydrofuran (5 ml) 4-toluenesulfonyl chloride (1.2 mmol) and dry pyridine (2.1 mmol) were added and heated at reflux for 3–8 h. The solvents were evaporated under reduced pressure and the residue was treated with ethanol (5–10 ml), stirred and cooled for 30 min. The precipitation of adequate 2,4-dichloro-5-[5-(arylamino)-1,3,4-oxadiazol-2-yl]benzenesulfonamides **7e**,**f** was collected by filtration, washed with water and ethanol and dried.

*4.1.5.1. 2,4-Dichloro-5-[5-(phenylamino)-1,3,4-oxadiazol-2-yl]benzenesulfonamide (**7e**).* Starting from **5a** (0.42 g) the desired compound **7e** was obtained (0.37 g, 96%): m.p. 280–281 °C; IR (KBr) 3371, 3265 (NH, NH_2), 3092, 2926, 2855 (C–H sp^2, sp^3), 1680 (N–H), 1602, 1590, 1556 (C═C, C═N), 1343, 1163 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.04–7.07 (m, 1H, H arom.), 7.38–7.41 (m, 2H, H arom.), 7.63–7.64 (m, 2H, H arom.), 7.93 (s, 2H, NH_2), 8.14 (s, 1H, H-3), 8.5 (s, 1H, H-6), 10.89 (s, 1H, NH) ppm. Anal. ($C_{14}H_{10}Cl_2N_4O_3S$) C, H, N.

*4.1.5.2. 2,4-Dichloro-5-[5-(4-chlorophenylamino)-1,3,4-oxadiazol-2-yl]benzenesulfonamide (**7f**).* Starting from **5b** (0.45 g) the desired compound **7f** was obtained (0.37 g, 92%): m.p. 279–281 °C; IR (KBr) 3300 (NH, NH_2), 3099, 2928 (C–H sp^2, sp^3), 1648 (N–H), 1624, 1586, 1556 (C═C, C═N), 1337, 1163 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.44 (d, J = 8.79 Hz, 2H, H arom.), 7.63 (d, J = 8.79 Hz, 2H, H arom.), 7.92 (s, 2H, NH_2), 8.12 (s, 1H, H-3), 8.47 (s, 1H, H-6), 11.06 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 119.06, 122.04, 126.10, 129.28, 130.11, 133.29, 134.02, 135.06, 137.59, 140.66, 154.88, 160.28. Anal. ($C_{14}H_9Cl_3N_4O_3S$) C, H, N.

*4.1.6. Procedures for the preparation of 2,4-dichloro-5-(5-aryl-1,3,4-oxadiazol-2-yl)benzenesulfonamides (**7g**–**i**)*

The required 2,4-dichloro-5-hydrazinecarbonylbenzene sulfonamides **6b**–**d** (2.5 mmol) were heating with thionyl chloride (25 ml) at reflux for 6–7 h. After evaporation the crushed ice was added and the precipitated desired compounds **7g**–**i** were filtered off and crystallized.

*4.1.6.1. 2,4-Dichloro-5-(5-phenyl-1,3,4-oxadiazol-2-yl)benzenesulfonamide (**7g**).* Starting from **6b** (0.971 g), the title compound **7g** was obtained (0.461 g, 56%) and crystallized from methanol/water (2/1) : m.p. 216–218 °C; IR (KBr) 3382, 3280 (NH_2), 1589, 1550 (C═C, C═N), 1357, 1172 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.63–7.69 (m, 3H, H arom.), 7.98 (s, 2H, NH_2), 8.09. (d, J = 6.84 Hz, 2H, H arom.), 8.19 (s, 1H, H-3), 8.66 (s, 1H, H-6) ppm; ^{13}C NMR (DMSO-d_6)

δ 121.84, 123.19, 127.09, 129.84, 130.93, 132.71, 134.15, 134.41, 135.85, 140.86, 161.24, 164.89 ppm. Anal. ($C_{14}H_9Cl_2N_3O_3S$) C, H, N.

*4.1.6.2. 2,4-Dichloro-5-[5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl] benzenesulfonamide (**7h**).* Starting from **6c** (0.634 g), the title compound **7h** was obtained (0.254 g, 42%) and crystallized from ethanol: m.p. 240–241 °C; IR (KBr) 3369, 3261 (NH_2), 1589, 1542, 1483 (C=C, C=N), 1343, 1172 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.74 (d, $J = 8.79$ Hz, 2H, H arom.), 7.99 (s, 2H, NH_2), 8.12 (d, $J = 8.3$ Hz, 2H, H arom.), 8.21 (s, 1H, H-3), 8.67 (s, 1H, H-6) ppm; ^{13}C NMR (DMSO-d_6) δ 121.72, 122.10, 128.89, 130.03, 130.95, 134.18, 134.49, 135.87, 137.46, 140.87, 161. 37, 164.14. Anal. ($C_{14}H_8Cl_3N_3O_3S$) C, H, N.

*4.1.6.3. 2,4-Dichloro-5-[5-(4-methylphenyl)-1,3,4-oxadiazol-2-yl] benzenesulfonamide (**7i**).* Starting from **6d** (1.0 g), the title compound **7i** was obtained (0.851 g, 42%) and crystallized from methanol: m.p. 194–196 °C; IR (KBr) 3385, 3288 (NH_2), 3088, 2923 (C–H sp^2, sp^3), 1585, 1556, 1496 (C=C, C=N), 1357, 1172 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 2.41 (s, 3H, CH_3), 7.45 (d, $J = 8.06$ Hz, 2H, H arom.), 7.96–7.99 (m, 4H, H arom., NH_2), 8.17 (s, 1H, H-3), 8.65 (s, 1H, H-6) ppm; MALDI-TOF *m/z* obsd: 385.9, $[M + H]^+$, 407.8, $[M + Na]^+$ calcd: 384.9; Anal. ($C_{15}H_{11}Cl_2N_3O_3S$) C, H, N.

*4.1.7. 2,4-Dichloro-5-(5-phenyl-1,3,4-thiadiazol-2-yl) benzenesulfonamide (**7j**)*

The **6b** (0.505 g, 1.3 mmol) was dissolved in dry tetrahydrofuran (15 ml), Lawesson's reagent (LR, 0.526 g) was added and stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure, residue was washed with water and petroleum ether, treated with tetrahydrofuran and evaporated again. Ethanol (20 ml) was added and heated at reflux for 5 min, after cooling to room temperature the precipitate of **7j** (0.273 g, 54%) was filtered off and recrystallized from methanol/DMF (4/1): m.p. 231–233 °C; IR (KBr) 3294, 3171 (NH_2), 3091, 3071, 2925 (C–H sp^2, sp^3), 1573, 1536, 1458 (C=C, C=N), 1362, 1174 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.58–7.62 (m, 3H, H arom.), 7.96 (s, 2H, NH_2), 8.08 (d, $J = 7.32$ Hz, 2H, H arom.), 8.19 (s, 1H, H-3), 8.84 (s, 1H, H-6) ppm; MALDI-TOF *m/z* obsd: 387.8, $[M + H]^+$, 409.8, $[M + Na]^+$ calcd: 386.9; Anal. ($C_{14}H_9Cl_2N_3O_2S_2$) C, H, N.

*4.1.8. 2,4-Dichloro-5-sulfamoylbenzoyl azide (**8**)*

The **4** (2.0 g, 7.04 mmol) was dissolved in 2% hydrochloric acid (26 ml). The reaction mixture was cooled and 3.8 M aqueous solution of sodium nitrite (2 ml) was added dropwise. The precipitation of 2,4-dichloro-5-sulfamoylbenzoyl azide **8** was filtered off and washed with icy water (1.53 g, 74%): m.p. 127–130 °C; IR (KBr) 3385, 3276 (NH_2), 2145 (N_3), 1704 (C=O), 1337, 1170 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.91 (s, 2H, NH_2), 8.08 (s, 1H, H-3), 8.86 (s, 1H, H-6) ppm. Anal. ($C_7H_4Cl_2N_4O_3S$) C, H, N.

*4.1.9. 2,4-Dichloro-5-isocyanatobenzenesulfonamide (**9**)*

The **8** (1.0 g, 3.4 mmol) was dissolved in dry toluene (40 ml) and the reaction mixture was heated at reflux until the nitrogen bubbles had disappeared (about 1 h). After cooling the precipitation of 2,4-dichloro-5-isocyanatobenzenesulfonamide **9** was filtered off (0.786 g, 87%): m.p. 143–145 °C; IR (KBr) 3350, 3274 (NH_2), 2267 (NCO), 1349, 1177 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.72 (s, 2H, NH_2), 7.88 (s, 1H, H-3), 8.88 (s, 1H, H-6) ppm. Anal. ($C_7H_4Cl_2N_2O_3S$) C, H, N.

*4.1.10. Preparation of 2,4-dichloro-5-ureidobenzenesulfonamides (**10a–i**)*

To the stirring solution of isocyanate **9** (0.75 mmol) in dry toluene (10 ml) the adequate amine (0.75 mmol) was added. The reaction mixture was heated at reflux for 0.5–6 h. After cooling, the precipitation of desired 2,4-dichloro-5-ureidobenzenesulfonamide was filtered off and crystallized, with the exception of compounds **10b** and **10e**,**f** which were isolated as described below.

*4.1.10.1. 2,4-Dichloro-5-(3-phenylureido)benzenesulfonamide (**10a**).* Starting from **9** (0.2 g) and aniline (0.071 g) the title compound **10a** was obtained (0.2 g, 73%): m.p. 232–235 °C; IR (KBr) 3377 (NH, NH_2), 1699 (C=O), 1373, 1162 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.1 (m, 1H, H arom.), 7.27–7.35 (m, 2H, H arom.), 7.16–7.2 (m, 2H, H arom.), 7.71 (s, 2H, NH_2), 7.81 (s, 1H, H-3), 8.55 (s, 1H, NH), 8.98 (s, 1H, H-6), 9.53 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6) δ 118.63, 120.75, 122.77, 123.09, 125.16, 129.22, 131.51, 135.65, 139.29, 140.4, 152.07 ppm; MALDI-TOF *m/z* obsd: 391.9, $[M + H]^+$, 413.8, $[M + Na]^+$ calcd: 391.0; Anal. ($C_{13}H_{11}Cl_2N_3O_3S$) C, H, N.

*4.1.10.2. 2,4-Dichloro-5-[3-(4-chlorophenyl)ureido]benzenesulfonamide (**10b**).* Starting from **9** (0.2 g) and *p*-chloroaniline (0.1 g) the title compound **10b** was obtained by evaporating of solvents, heating the residue with 50% methanol and filtration the precipitation of desired compound (0.21 g, 71%): m.p. 242–245 °C; IR (KBr) 3337, 3259 (NH, NH_2), 1700 (C=O), 1371, 1160 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.35 (d, $J = 8.79$ Hz, 2H, H arom.), 7.49 (d, $J = 8.79$ Hz, 2H, H arom.), 7.7 (s, 2H, NH_2), 7.84 (s, 1H, H-3), 8.6 (s, 1H, NH), 8.94 (s, 1H, H-6), 9.63 (s, 1H, NH) ppm; MALDI-TOF *m/z* obsd: 495.8, $[M + H]^+$, 417.8, $[M + Na]^+$ calcd: 394.9; Anal. ($C_{13}H_{10}Cl_3N_3O_3S$) C, H, N.

*4.1.10.3. 2,4-Dichloro-5-[3-(4-sulfamoylphenyl)ureido]benzenesulfonamide (**10c**).* Starting from **9** (0.2 g) and sulfanilamide (0.13 g) the title compound **10c** was obtained and crystallized from 70% DMF (0.15 g, 48%): m.p. 261–264 °C; IR (KBr) 3363, 3279 (NH, NH_2), 1703 (C=O), 1319, 1161 (SO_2) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6) δ 7.25 (s, 2H, NH_2), 7.61–7.66 (m, 2H, H arom.), 7.71 (s, 2H, NH_2), 7.75–7.79 (m, 2H, H arom.), 7.87 (s, 1H, H-3), 8.7 (s, 1H, NH), 8.97 (s, 1H, H-6), 9.88 (s, 1H, NH) ppm. Anal. ($C_{13}H_{12}Cl_2N_4O_5S_2$) C, H, N.

*4.1.10.4. 5-(3-Benzylureido)-2,4-dichlorobenzenesulfonamide (**10d**).* Starting from **9** (0.2 g) and benzylamine (0.078 g) the title compound **10d** was obtained (0.11 g, 40%): m.p. 178–180 °C; IR (KBr) 3398 (NH, NH_2), 1684 (C=O), 1371, 1161 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.31 (d, $J = 5.37$ Hz 2H, CH_2), 7.24–7.27 (m, 1H, H arom.), 7.31–7.36 (m, 4H, H arom.), 7.59 (t, $J = 5.37$ Hz, 1H, NH), 7.64 (s, 2H, NH_2), 7.77 (s, 1H, H-3), 8.4 (s, 1H, NH), 8.98 (s, 1H, H-6) ppm; ^{13}C NMR (DMSO-d_6) δ 43.16, 120.31, 122.27, 124.39, 127.24, 127.58, 128.70, 131.35, 136.28, 139.80, 140.32, 154.64. Anal. ($C_{14}H_{13}Cl_2N_3O_3S$) C, H, N.

*4.1.10.5. 5-[3-(Benzyloxy)ureido]-2,4-dichlorobenzenesulfonamide (**10e**).* Starting from **9** (0.2 g) and O-benzylhydroxylamine hydrochloride (0.12 g) with addition of dry pyridine (0.06 ml) the title compound **10e** was obtained by evaporating the solvent and precipitating with water (0.14 g, 48%): m.p. 150–153 °C; IR (KBr) 3382, 3280 (NH, NH_2), 1680 (C=O), 1374, 1163 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 4.88 (s, 2H, OCH_2), 7.36–7.49 (m, 5H, H arom.), 7.73 (s, 2H, NH_2), 7.88 (s, 1H, H-3), 8.4 (s, 1H, NH), 8.71 (s, 1H, H-6), 10.15 (s, 1H, ONH) ppm. Anal. ($C_{14}H_{13}Cl_2N_3O_4S$) C, H, N.

*4.1.10.6. 5-(2-(Benzoylhydrazinecarbonyl)-2,4-dichlorobenzenesulfonamide (**10f**).* Starting from **9** (0.2 g) and benzohydrazide (0.1 g) the title compound **10f** was obtained by evaporating the solvent under reduced pressure, heating the residue with 50% methanol and filtration the desired compound (0.15 g, 50%): m.p. 231–233 °C; IR (KBr) 3357, 3251 (NH, NH_2), 1708 (C=O), 1310, 1160 (SO_2) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) δ 7.49–7.52

(m, 2H, H arom.), 7.57–7.6 (m, 1H, H arom.), 7.69 (s, 2H, NH_2), 7.85 (s, H, H-3), 7.89 (d, $J = 7.81$ Hz, 2H, H arom.), 8.65 (s, 1H, NH), 8.83 (s, 1H, NH), 9.0 (s, 1H, H-6), 10.45 (s, 1H, NH) ppm, ^{13}C NMR (DMSO-d_6) δ 121.23, 123.59, 127.76, 128.76, 131.61, 132.23, 132.54, 135.45, 140.42, 155.06, 166.65 ppm. Anal. ($C_{14}H_{12}Cl_2N_4O_4S$) C, H, N.

*4.1.10.7. 5-[3-(1H-Indol-1-yl)ureido]-2,4-dichlorobenzenesulfonamide (**10g**).* Starting from **9** (0.15 g) and 1-aminoindole (0.074 g) the title compound **10g** was obtained and crystallized from ethanol (0.075 g, 33%): m.p. 243–244 °C; IR (KBr) 3368, 3317, 3270 (NH, NH_2), 1662 (C=O), 1372, 1165 (SO_2) cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6) δ 6.49 (d, 1H, $J = 3.24$ Hz, H-3 indole), 7.05–7.23 (m, 2H, H arom.), 7.33 (d, 1H, $J = 7.76$ Hz, H arom.), 7.41 (d, 1H, $J = 3.24$ Hz, H-2 indole), 7.58 (d, 1H, $J = 7.76$ Hz), 7.68 (s, 2H, NH_2), 7.88 (s, 1H, H-3), 8.7 (s, 1H, H-6), 8.84 (s, 1H, NH), 10.24 (s, 1H, NH) ppm. Anal. ($C_{15}H_{12}Cl_2N_4O_3S$) C, H, N.

*4.1.10.8. 2,4-Dichloro-5-[3-(3-morpholinopropyl)ureido]benzenesulfonamide (**10h**).* Starting from **9** (0.2 g) and 3-morpholinopropan-1-amine (0.11 g) the title compound **10h** was obtained and crystallized from water (0.27 g, 88%): m.p. 230–232 °C; IR (KBr) 3386 (NH, NH_2), 1692 (C=O), 1371, 1161 (SO_2) cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.56–1.61 (m, 2H, propyl), 2.27–2.32 (m, 6H, morpholine, propyl), 3.1–3.14 (m, 2H, propyl), 3.55–3.56 (m, 4H, morpholine), 7.12 (s, 1H, NH), 7.6 (s, 2H, NH_2), 7.75 (s, 1H, H-3), 8.28 (s, 1H, NH), 8.94 (s, 1H, H-6) ppm; ^{13}C NMR (DMSO-d_6) 18.83, 26.67, 37.64, 53.63, 55.98, 56.30, 66.46, 120.30, 122.07, 124.27, 131.30, 136.37, 140.28, 154.58; MALDI-TOF *m/z* obsd: 412.9, $[M + H]^+$ calcd: 412.0; Anal. ($C_{14}H_{20}Cl_2N_4O_4S$) C, H, N.

*4.1.10.9. 4-(Benzodioxol-5-ylmethyl)-N-(2,4-dichloro-5-sulfamoylphenyl)piperazine-1-carboxamide (**10i**).* Starting from **9** (0.267 g) and 1-piperonylpiperazine (0.22 g) the title compound **10i** was obtained and crystallized from ethanol (0.268 g, 55%): m.p. 183–185 °C; IR (KBr) 3466, 3248 (NH, NH_2), 1646 (C=O), 1362, 1165 (SO_2) cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.34–2.35 (m, 4H, $2CH_2$), 3.41 (s, 2H, CH_2N), 3.43–3.44 (m, 4H, $2CH_2$), 5.98 (s, 2H, OCH_2O), 6.75–6.76 (m, 1H, H arom.), 6.84–6.85 (m, 1H, H arom.), 6.87 (s, 1H, H arom.), 7.7 (s, 2H, NH_2), 7.81 (s, 1H, H-3), 8.1 (s, 1H, H-6), 8.48 (s, 1H, NH) ppm. Anal. ($C_{19}H_{20}Cl_2N_4O_5S$) C, H, N.

4.2. CA inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity [46]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of E–I complex. The inhibition constants were obtained by non-linear last-squares methods using PRISM 3, as reported earlier [47,48] and represent the mean from at least three different determinations.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.039.

References

[1] C.M. Booth, A.H. Calvert, G. Giaccone, M.W. Lobbezoo, L.K. Seymour, E.A. Eisenhauer, Endpoints and other considerations in phase I studies of targeted anticancer therapy: recommendations from the task force on Methodology for the Development of Innovative Cancer Therapies (MDICT), EJC 44 (2008) 19–24.
[2] D. Neri, C.T. Supuran, Interfering with pH regulation in tumours as a therapeutic strategy, Nat. Rev. Drug. Discov. 10 (2011) 767–777.
[3] C.T. Supuran, Carbonic anhydrases: novel therapeutic applications for inhibitors and activators, Nat. Rev. Drug. Discov. 7 (2008) 168–181.
[4] D. Vullo, M. Franchi, E. Gallori, J. Antel, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of mitochondrial isozyme V with aromatic and heterocyclic sulfonamides, J. Med. Chem. 47 (2004) 1272–1279.
[5] I. Nishimori, D. Vullo, A. Innocenti, A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Carbonic anhydrase inhibitors. The mitochondrial isozyme VB as a new target for sulfonamide and sulfamate inhibitors, J. Med. Chem. 48 (2005) 7860–7866.
[6] I. Nishimori, T. Minakuchi, S. Onishi, D. Vullo, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors. DNA cloning, characterization and inhibition studies of the human secretory isoform VI, a new target for sulfonamide and sulfamate inhibitors, J. Med. Chem. 50 (2007) 381–388.
[7] D. Vullo, J. Voipio, A. Innocenti, C. Rivera, H. Ranki, A. Scozzafava, K. Kaila, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isozyme VII with aromatic and heterocyclic sulfonamides, Bioorg. Med. Chem. Lett. 15 (2005) 971–976.
[8] I. Nishimori, Acatalytic CAs: carbonic anhydrase-related proteins, in: C.T. Supuran, A. Scozzafava, J. Conway (Eds.), Carbonic Anhydrase – its Inhibitors and Activators, CRC Press, Boca Raton, 2004, pp. 25–43.
[9] D. Vullo, M. Franchi, E. Gallori, J. Pastorek, A. Scozzafava, S. Pastorekova, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides, Bioorg. Med. Chem. Lett. 13 (2003) 1005–1009.
[10] D. Vullo, A. Innocenti, I. Nishimori, J. Pastorek, A. Scozzafava, S. Pastoreková, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides – a new target for the design of antitumor and antiglaucoma drugs? Bioorg. Med. Chem. Lett. 15 (2005) 963–969.
[11] J. Lehtonen, B. Shen, M. Vihinen, A. Casini, A. Scozzafava, C.T. Supuran, A.K. Parkkila, J. Saarnio, A.J. Kivelä, A. Waheed, W.S. Sly, S. Parkkila, Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family, J. Biol. Chem. 279 (2004) 2719–2727.
[12] V. Alterio, R.M. Vitale, S.M. Monti, C. Pedone, A. Scozzafava, A. Cecchi, G. De Simone, C.T. Supuran, Carbonic anhydrase inhibitors: X-ray and molecular modeling study for the interaction of a fluorescent antitumor sulfonamide with isozyme II and IX, J. Am. Chem. Soc. 128 (2006) 8329–8335.
[13] J. Pastorek, S. Pastorekova, I. Callebaut, J.P. Mornon, V. Zelnik, R. Opavsky, M. Zatovicova, S. Liao, D. Portetelle, E.J. Stanbridge, J. Zavada, A. Burny, R. Kettmann, Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and putative helix-loop-helix DNA binding segment, Oncogene 9 (1994) 2788–2888.
[14] R. Opavsky, S. Pastorekova, V. Zelnik, A. Gibadulinova, E.J. Stanbridge, J. Zavada, R. Kettmann, J. Pastorek, Human MN/CA9 gene, a novel member of the carbonic anhydrase family: structure and exon to protein domain relationships, Genomics 33 (1996) 480–487.
[15] O. Türeci, U. Sahin, E. Vollmar, S. Siemer, E. Gottert, G. Seitz, A.K. Parkkila, G.N. Shah, J.H. Grubb, M. Pfreundschuh, W.S. Sly, Human carbonic anhydrase XII: cDNA cloning, expression and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cancers, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7608–7613.
[16] A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Modulation of carbonic anhydrase activity and its applications in therapy, Expert Opin. Ther. Pat. 14 (2004) 667–702.
[17] C.T. Supuran, A. Scozzafava, A. Casini, Carbonic anhydrase inhibitors, Med. Res. Rev. 23 (2003) 146–189.
[18] E. Svastova, N. Zilka, M. Zatovicova, M. Gibadulinova, F. Ciampor, J. Pastorek, S. Pastorekova, Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with β-catenin, Exp. Cell. Res. 290 (2003) 332–345.
[19] S. Pastorekova, S. Parkkila, J. Zavada, Tumor-associated carbonic anhydrases and their clinical significance, Adv. Clin. Chem. 42 (2006) 167–216.
[20] P. Swietach, R.D. Vaughen-Jones, A.L. Harris, Regulation of tumor pH and the role of carbonic anhydrase 9, Cancer Metastasis Rev. 26 (2007) 299–310.
[21] E. Svastova, A. Hulikova, M. Rafajova, M. Zatovicova, A. Gibadulinova, A. Casini, A. Cecchi, A. Scozzafava, C.T. Supuran, J. Pastorek, S. Pastorekova, Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH, FEBS Lett. 577 (2004) 439–445.
[22] P.C. McDonald, J.Y. Winum, C.T. Supuran, S. Dedhar, Recent developments in targeting carbonic anhydrase IX for cancer therapeutics, Oncotarget 3 (2012) 84–97.

[23] N. Robertson, C. Potter, A.L. Harris, Role of carbonic anhydrase IX in human tumor cell growth, survival, and invasion, Cancer Res. 64 (2004) 6160–6165.
[24] J.Y. Winum, M. Rami, A. Scozzafava, J.L. Montero, C.T. Supuran, Carbonic anhydrase IX: a new drugable target for the design of antitumor agents, Med. Res. Rev. 28 (2008) 445–463.
[25] T. Mann, D. Keilin, Sulphanilamide as a specific carbonic anhydrase inhibitor, Nature 146 (1940) 164–165.
[26] T.H. Maren, Relations between structure and biological activity of sulfonamides, Annu. Rev. Pharmacol. Toxicol. 16 (1976) 309–327.
[27] T.H. Maren, Carbonic anhydrase: chemistry, physiology and inhibition, Physiol. Rev. 47 (1967) 595–781.
[28] C.T. Supuran, A. Scozzafava, Carbonic anhydrase inhibitors and their therapeutic potential, Exp. Opin. Ther. Pat. 10 (2000) 575–600.
[29] J. Drew, Drug discovery: a historical perspective, Science 287 (2000) 1960–1964.
[30] S.M. Monti, C.T. Supuran, G. De Simone, Anticancer carbonic anhydrase inhibitors: a patent review (2008–2013), Expert Opin. Ther. Pat. 23 (2013) 737–749.
[31] C.T. Supuran, F. Briganti, S. Tilli, W.R. Chegwidden, A. Scozzafava, Carbonic anhydrase inhibitors: sulfonamides as antitumor agents? Bioorg. Med. Chem. 9 (2001) 703–714.
[32] C.T. Supuran, Indisulam: an anticancer sulfonamide in clinical development, Expert Opin. Investig. Drugs 12 (2003) 283–287.
[33] F. Abbate, A. Casini, T. Owa, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors: E7070, a sulfonamide anticancer agent, potently inhibits cytosolic isozymes I and II, and transmembrane, tumor-associated isozyme IX, Bioorg. Med. Chem. Lett. 14 (2004) 217–223.
[34] Ö. Özensoy, L. Puccetti, G. Fasolis, O. Arslan, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors: inhibition of the tumor-associated isozymes IX and XII with a library of aromatic and heteroaromatic sulfonamides, Bioorg. Med. Chem. Lett. 15 (2005) 4862–4866.
[35] J.Y. Winum, J.M. Dogne, A. Casini, X. de Leval, J.L. Montero, A. Scozzafava, D. Vullo, A. Innocenti, C.T. Supuran, Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic membrane-associated carbonic anhydrase isozymes I, II, and IX with sulfonamides incorporating hydrazino moieties, J. Med. Chem. 48 (2005) 2121–2125.
[36] F. Pacchiano, F. Carta, P.C. McDonald, Y. Lou, D. Vullo, A. Scozzafava, S. Dedhar, C.T. Supuran, Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IC and show antimetastatic activity in a model of breast cancer metastasis, J. Med. Chem. 54 (2011) 1896–1902.
[37] M.A. Ilies, D. Vullo, J. Pastorek, A. Scozzafava, M. Ilies, M.T. Caproiu, S. Pastorekova, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of tumor-associated isozyme IX by halogenosulfanilamide and halogenophenylaminobenzolamide derivatives, J. Med. Chem. 46 (2003) 2187–2196.
[38] Y. Lou, P.C. McDonald, A. Oloumi, S. Chia, C. Ostlund, A. Ahmadi, A. Kyle, U. auf dem Keller, S. Leung, D. Huntsman, B. Clarke, B.W. Sutherland, D. Waterhouse, M. Bally, C. Roskelley, C.M. Overall, A. Minchinton, F. Pacchiano, F. Carta, A. Scozzafava, N. Touisni, J.Y. Winum, C.T. Supuran, S. Dedhar, Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors, Cancer Res. 17 (2011) 3364–3376.
[39] F.E. Lock, P.C. McDonald, Y. Lou1, I. Serrano, S.C. Chafe, C. Ostlund, S. Aparicio, J.Y. Winum, C.T. Supuran, S. Dedhar, Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche, Oncogene (2013), http://dx.doi.org/10.1038/onc.2012.550.
[40] F. Sączewski, J. Sławiński, A. Kornicka, Z. Brzozowski, E. Pomarnacka, A. Innocenti, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the cytosolic human isozymes I and II, and the transmembrane, tumor-associated isozymes IX and XII with substituted aromatic sulfonamides activatable in hypoxic tumors, Bioorg. Med. Chem. Lett. 16 (2006) 4846–4851.
[41] F. Sączewski, A. Innocenti, Z. Brzozowski, J. Sławiński, E. Pomarnacka, A. Kornicka, A. Scozzafava, C.T. Supuran, Carbonic anhydrase inhibitors. Selective inhibition of human tumor-associated isozymes IX and XII and cytosolic isozymes I and II with some substituted-2-mercaptobenzenesulfonamides, J. Enzyme Inhib. Med. Chem. 21 (2006) 563–568.
[42] F. Sączewski, A. Innocenti, J. Sławiński, A. Kornicka, Z. Brzozowski, E. Pomarnacka, A. Scozzafava, C. Temperini, C.T. Supuran, Carbonic anhydrase inhibitors: inhibition of human cytosolic isozymes I and II and tumor-associated isozymes IX and XII with S-substituted 4-chloro-2-mercapto-5-methyl-benzenesulfonamides, Bioorg. Med. Chem. 16 (2008) 3933–3940.
[43] M.L. Hoefle, L.T. Blouin, H.A. De Wald, A. Holmes, D. Williams, Diuretics. 4-Substituted 3-sulfamoylbenzoic acid hydrazides, J. Med. Chem. 11 (1968) 970–973.
[44] E. Pomarnacka, S. Angielski, A. Hoppe, Pochodne kwasu 4-chloro-5-sulfoamoilobenzoesowego. 8. Synteza i właściwości diuretyczne pochodnych pirazolo [3,2-b] chinazoliny i 1-benzoilopirazolu, Acta Pol. Pharm. 41 (1984) 141–151.
[45] K. Brożewicz, J. Sławiński, Synthesis and in vitro activity of novel 2-(benzylthio)-4-chloro-5-(1,3,4-oxadiazol-2-yl)benzenesulfonamide derivatives, Monatsh. Chem. 143 (2012) 975–984.
[46] R.G. Khalifah, The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C, J. Biol. Med. 246 (1971) 2561–2573.
[47] J.R. Casey, P.E. Morgan, D. Vullo, A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Carbonic anhydrase inhibitors. Design of selective, membrane-impermeant inhibitors targeting the human tumor-associated isozyme IX, J. Med. Chem. 47 (2004) 2337–2347.
[48] M.C. Alley, D.A. Scudiero, P.A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, M.R. Boyd, Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay, Cancer Res. 48 (1988) 589–601.