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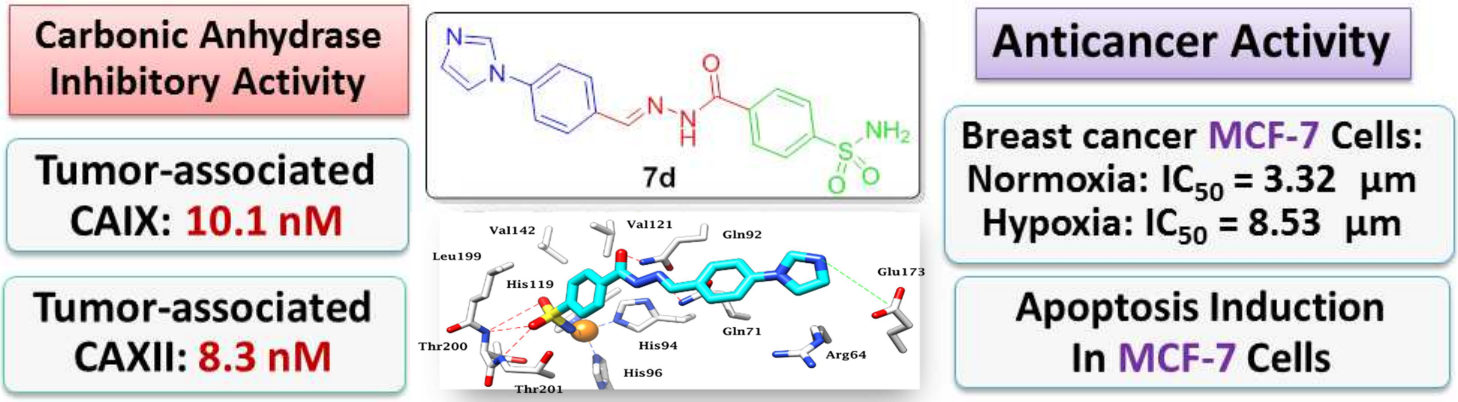
Graphical Abstract



Application of hydrazino and hydrazido linkers to connect benzenesulfonamides with hydrophilic/phobic tails for targeting the middle region of human carbonic anhydrases active site: selective inhibitors of hCA IX.

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ABSTRACT

Herein we report the design and synthesis of three different sets of novel benzenesulfonamides (**5a-e**, **7a-e** and **10a-d**) incorporating hydrophilic/hydrophobic tails by hydrazido or hydrazino linkers. The newly synthesized benzenesulfonamides were examined *in vitro* for their inhibitory activity towards four human (h) carbonic anhydrase (hCA, EC 4.2.1.1) isoforms, hCA I, II, IX and XII using a stopped-flow CO_2 hydrase assay. All these isoforms were inhibited by the sulfonamides (**5a-e**, **7a-e** and **10a-d**) with variable degrees in the following K_I ranges: 76.8–357.4 nM for hCA I, 8.2–94.6 nM for hCA II, 2.0–46.3 nM for hCA XI, and 8.3–88.3 nM for hCA XII. The sulfonamide **7d** exhibited potent anti-proliferative activity against breast MCF-7 cancer cell line under both normoxic and hypoxic conditions with IC_{50} values equal 3.32 ± 0.06 and 8.53 ± 0.32μM, respectively, which are comparable to the reference drug doxorubicin (IC_{50} = 2.36 ± 0.04 and 8.39 ± 0.25 μM, respectively). Furthermore, **7d** was screened for cell cycle disturbance and apoptosis induction in MCF-7 cells. It was found to persuade cell cycle arrest at G2-M stage as well as to alter the Sub-G1 phase, also, **7d** resulted in a significant increase in the percent of annexinV-FITC positive apoptotic cells from 1.03 to 18.54%. Molecular docking study was carried out for **7d** within the hCA IX and hCA XII active sites to rationalize the obtained inhibition results.

Keywords: Benzenesulfonamides; Selective hCA IX inhibitors; Molecular modeling; Synthesis; Anticancer activity.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) catalyze the hydration of CO_2 to bicarbonate and proton as well as other hydrolytic reactions [1]. Up to now, seven genetic families have been discovered, the α-, β, γ-, δ-, ζ-, η-, and θ-CAs [1]. Fifteen different α-CA isoforms have been identified and characterized in human (h) so far, of which twelve are catalytically active and three are inactive (CA-related proteins) [2]. hCAs are involved in a plethora of physiological and pathological processes, such as gas exchange, transport of CO_2 and HCO_3^- across membranes, biosynthetic reactions, acid-base tuning, calcification and tumorigenesis/metastatization [2,3]. The latter processes see isoforms hCA IX and XII implicated in the tumor cells pH regulatory machinery, with their inhibition having been validated as a winning anti-tumor strategy [3]. Notably, hCA IX expression is strictly related to cancer and poor prognosis in tumors, while hCA XII is present in many normal tissues and organs, such as endometrium, colon, kidney and eye. hCA I and II are cytosolic ubiquitous isoforms to whom most CA activity in human body is due. Their inhibition, unless desired, should be avoided to overcome the unpleasant side effects due to promiscuous inhibition [3].
Literature survey regarding several hCA inhibitors (hCAIs) revealed that the main skeleton of these compounds consists of a certain linker between a benzene sulfonamide group on one side and another aryl or heteroaryl moiety on the other side. The previous finding was concluded from studying the structure of different potent hCAs inhibitors as shown in **Fig. 1**. For example, a ureido linker was incorporated between benzene sulfonamide group and 4-flourophenyl group in **SLC-0111** (**I**); a synthetic compound in phase I/II clinical trials in combination therapy for the treatment of metastatic solid tumors, with *Ki* (hCA IX) = 45 nM and *Ki* (hCA XII) = 4.5 nM [4-8]. The ureido linker was again involved in other hCA inhibitors with a heteroaryl oxindole moiety attached in the other side as in compounds **IIa** (hCA IX; *Ki* = 5.3 nM, hCA XII; *Ki* = 6.3 nM) and **IIb** (hCA IX; *Ki* = 14.8 nM, hCA XII; *Ki* = 0.64 nM (**Fig. 1**) [9,10]. In other hCA inhibitors, the ureido linker was replaced by a hydrazido linker as represented in compound **III** (*Ki* (hCA IX) = 8.9 nM; *Ki* (hCA XII) = 9.2 nM), compound **IV** (*Ki* (hCA IX) = 17.3 nM) and compound **V** (*Ki* (hCA IX) = 30.4 nM) [11-13]. The approach of using a linker between a benzene sulfonamide moiety and aryl or heteroaryl moiety was applied again but through using a hydrazine linker as shown in compound **VI** (hCA IX; *Ki* = 5.3 nM) (**Fig. 1**) [14].

In this study, our target is to synthesize three series of hCA inhibitors using the aforementioned approach. A hydrazido group was used as a linker between a benzene

sulfonamide moiety from one side and a para substituted phenyl group at the other side as in the target compounds **7a-e**. The choice of the other side moiety was based upon the same rational of compound **VII** (hCA; II *Ki* = 0.5 nM, hCA VII; *Ki* = 0.45 nM) (**Fig. 1**) [15] which assumes that mixing hydrophilic/hydrophobic rings in this region will allow the synthesized compounds to target both the hydrophilic and the hydrophobic regions in the middle of hCA enzyme. Consequently, *p*-substituted phenyl ring with aliphatic secondary amines were employed in this region to achieve the previous rational. Two other series **5a-e** and **10a-d** containing a hydrazine linker, again coupled with mixed hydrophilic/hydrophobic moieties were synthesized (**Fig. 1**). The target compounds were biologically evaluated against different isoforms of hCA and were tested for their cytotoxicity against cancer cells under hypoxic conditions. Moreover, molecular docking studies were performed to rationalize their biological activities.

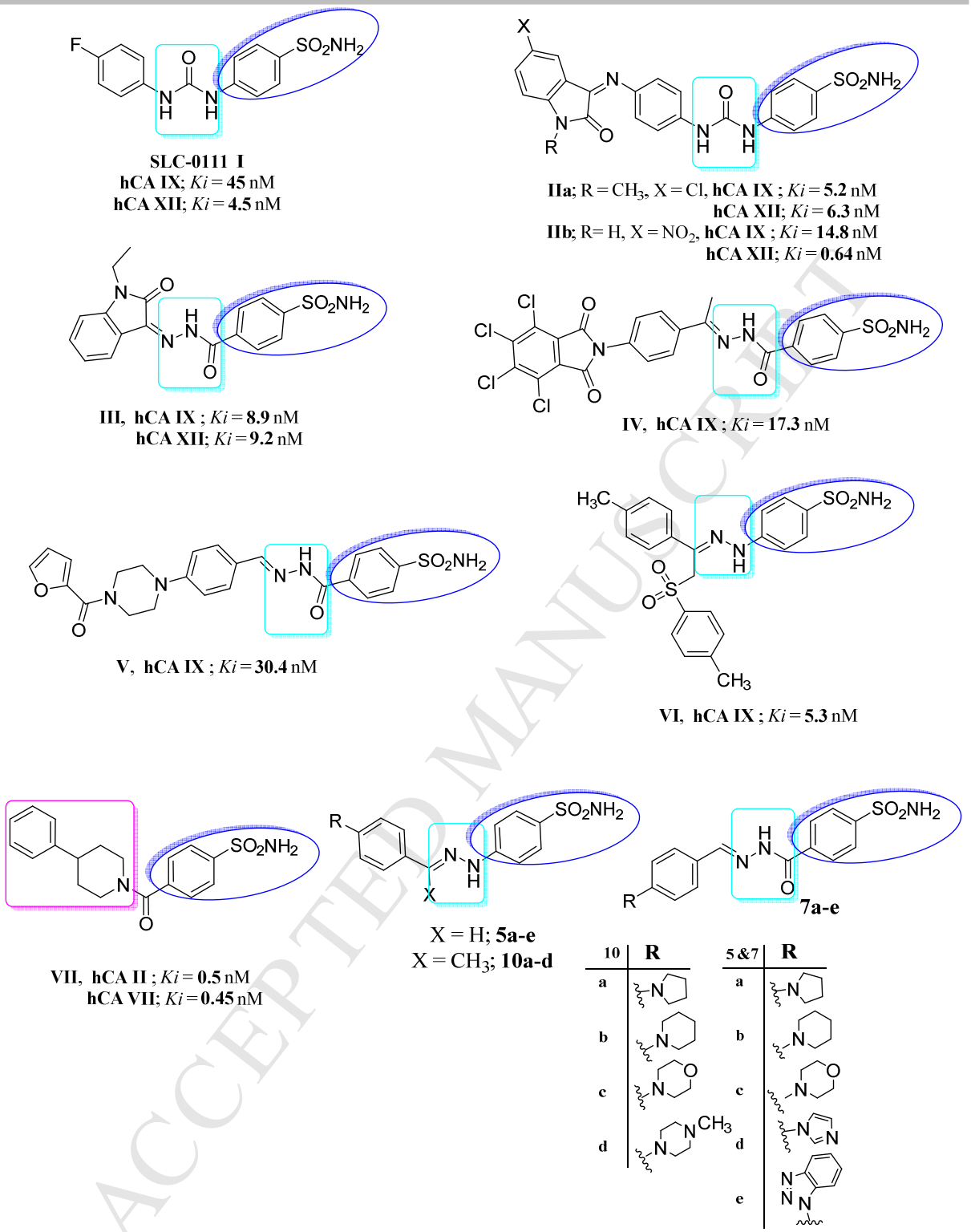
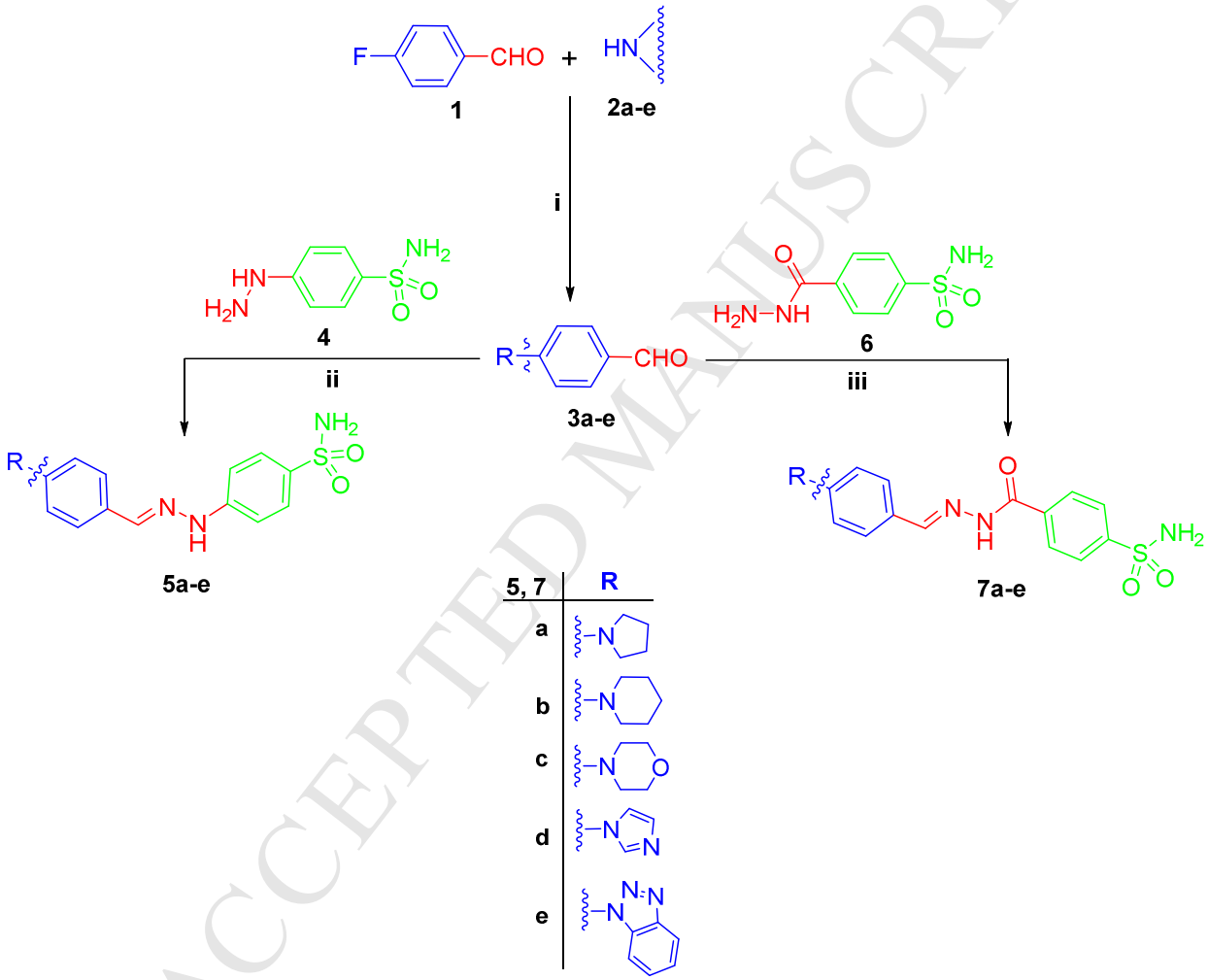


Fig. 1. Structures of some reported CA inhibitors **I-VII**, and the targeted sulfonamides **5a-e**, **7a-e** and **10a-d**.

2. Results and Discussion

2.1. Chemistry

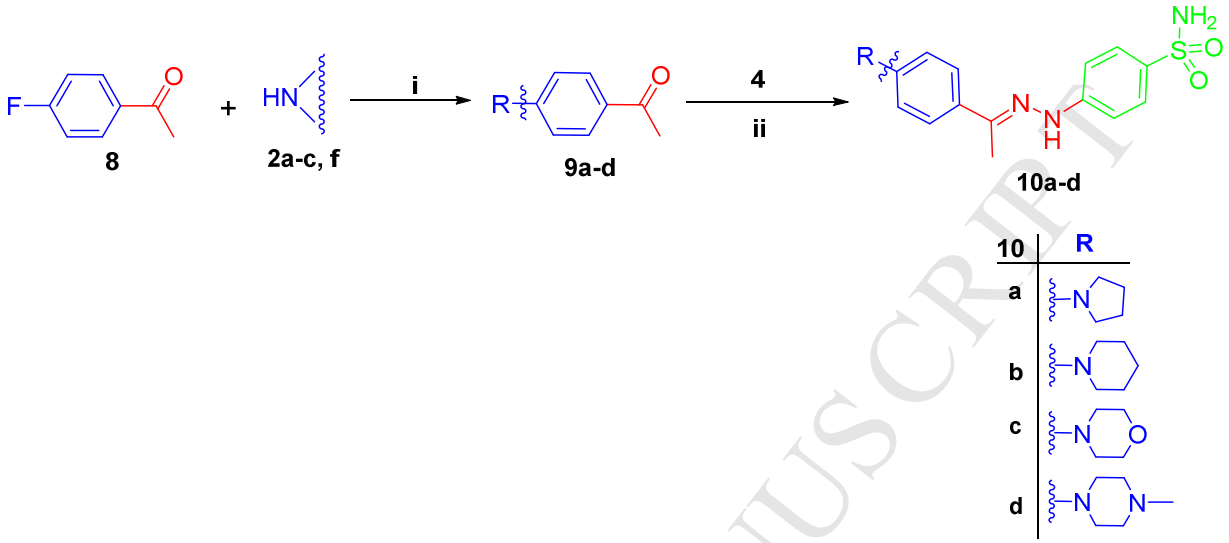
As a starting point for preparation of the targeted compounds **5a-e**, **7a-e** and **10a-d**, *p*-substituted aldehydes (**3a-e**) (**Scheme 1**) and *p*-substituted acetophenone derivatives (**9a-d**) (**Scheme 2**) were synthesized from *p*-fluorobenzaldehyde and *p*-fluoroacetophenone through nucleophilic substitution reactions *via* different secondary amines where the amines acted as nucleophiles in exchange of the fluorine atom as reported [16, 17]. Consequently, the aforementioned *p*-substituted aldehydes (**3a-e**) were reacted with both 4-hydrazinobenzene sulfonamide (**4**) and 4-sulfamidobenzoic acid hydrazide (**6**) to give the corresponding hydrazones **5a-e** and hydrazides **7a-e**, respectively (**Scheme 1**).



Scheme 1. Synthetic scheme for compounds **5a-e** and **7a-e**. Reagents and conditions: **(i)** K_2CO_3/ DMSO/ reflux for 3-5 h; **(ii)** EtOH/ AcOH / reflux for 3 h; **(iii**) AcOH / reflux for 2 h.

On the other hand, the previously synthesized *p*-substituted acetophenone derivatives (**9a-d**) were subjected to the reaction with both 4-hydrazinobenzene sulfonamide (**4**) and 4-sulfamidobenzoic acid hydrazide (**6**) using the same conditions adopted in the reaction with aldehydes (**3a-e**) as in scheme 1. However, we managed to obtain the corresponding

hydrazones **10a-d** only and not the hydrazides (scheme 2). Further trials were performed to get the corresponding hydrazides using conditions that are more drastic. But unfortunately we failed to obtain the desired hydrazides even in the presence of strong dehydrating agents as conc. H_2SO_4 or $ZnCl_2$.



Scheme 2. Synthetic scheme for compounds **10a-d**. Reagents and conditions: **(i)** K_2CO_3 / DMSO / reflux for 6 h; **(ii)** EtOH/AcOH / reflux for 3 h.

2.2. Biological Evaluation.

2.2.1. Carbonic anhydrase inhibition

All the newly prepared sulfonamides (**5a–e**, **7a–e** and **10a–d**) were evaluated for their ability to inhibit hCA isoforms, hCA I and II (cytosolic) as well as hCA IX and XII (transmembrane, tumor associated isoforms) *via* a stopped flow CO_2 hydrase assay [18] using acetazolamide (AAZ) as a standard inhibitor. The following SAR can be gathered from the data shown in Table 1:

Table 1: Inhibition data of human CA isoforms hCA I, II, IX and XII with compounds reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO_2 hydrase assay.

Compound	K_I (nM)*			
	hCA I	**hCA II**	**hCA IX**	**hCA XII**
5a	96.2 ± 5.8	29.9 ± 2.0	19.4 ± 1.2	50.2 ± 4.2
5b	100.8 ± 6.9	24.0 ± 1.8	20.9 ± 1.3	29.6 ± 1.5
5c	87.4 ± 5.1	34.5 ± 2.6	16.5 ± 1.1	13.1 ± 0.8
5d	76.8 ± 4.3	17.9 ± 1.2	14.6 ± 0.9	31.6 ± 2.4
5e	240.4 ± 16.7	94.6 ± 5.3	46.3 ± 2.7	88.3 ± 6.2
7a	347.3 ± 27.0	12.5 ± 1.1	3.1 ± 0.2	10.1 ± 0.7
7b	152.9 ± 14.2	9.3 ± 0.7	3.3 ± 0.2	10.1 ± 1.2
7c	175.4 ± 11.3	15.6 ± 1.2	8.6 ± 0.5	9.2 ± 0.7
7d	129.8 ± 10.5	8.2 ± 0.6	10.1 ± 0.8	8.3 ± 0.6
7e	357.4 ± 24.8	62.3 ± 4.3	23.7 ± 0.1	51.4 ± 4.5
10a	95.4 ± 7.1	57.7 ± 5.0	18.3 ± 1.2	36.4 ± 2.2
10b	110.6 ± 9.5	69.2 ± 4.7	7.2 ± 0.5	8.7 ± 0.6
10c	149.5 ± 13.2	65.6 ± 3.9	8.9 ± 0.6	9.7 ± 0.4
10d	266.1 ± 19.4	89.5 ± 5.8	2.0 ± 0.1	9.2 ± 0.5
AAZ	250 ± 15	12 ± 0.8	25 ± 1.4	5.7 ± 0.4

* Inhibition data are expressed as means ± SEM of 3 different assays.

(i) The ubiquitous hCA I is quite effectively inhibited by all reported sulfonamides. The inhibition constants (K_Is) span in the range 76.8-357.3 nM. Notably, unsubstituted hydrazones **5a-e** are slightly more effective (K_Is 76.8-100.8 nM, except **5e**) than hydrazides **7a-e** and methylhydrazones **10a-d**. Within subsets **5a-e** and **7a-e**, compounds **e** bearing a benzotriazole tail feel the effect of the steric hindrance of the pendant and show K_Is of 240.4 and 357.4 nM. All other heterocycles incorporating compounds show similar inhibition to hCA I.

(ii) The other ubiquitous and physiologically relevant isoform, hCA II, is potently inhibited by most of the reported compounds (K_Is 8.2-94.6 nM). In particular, hydrazides **7a-e** act as the most potent hCA II inhibitors in this study, showing K_Is in the low nanomolar range (8.2-15.6 nM) with the exception of **7e** whose K_I rises to 62.3 nM likely because of steric hindrance. As a general trend, hydrazones **5a-e** and **10a-d** show a partially lowered inhibitory efficacy compared to hydrazide compounds. One could speculate about a H-bond in the ligand-target adduct where the carbonyl group of hydrazides acts as acceptor with an active site residue of hCA II enhancing the binding with respect to hydrazone compounds. The addition of a methyl group to the hydrazone linker (**10a-d**) further decreases the binding with respect to unsubstituted derivatives (**5a-d**) probably arousing unfavorable clashes to the

binding of **5a-d**. The benzotriazole group of compound **5e** elicits a worsening of the inhibitory activity to 94.6 nM.

(iii) The tumor-associated isoforms hCA IX and XII are potently inhibited in a low nanomolar range by the screened sulfonamides. Against hCA IX, K_Is reach one-digit nanomolar values with hydrazides **7a-c** (3.1-8.6 nM) and methylhydrazones **10b-d** (2.0-8.9 nM). The unsubstituted hydrazones **5a-e** show the worst hCA IX inhibition though remaining very potent inhibitors (K_Is 14.6-46.3 nM). The rather flat inhibition reported with different substituents disables to devise more accurate SAR.

(iv) hCA XII is also inhibited with single-digit K_Is by hydrazides **7c** and **7d** (8.3-9.2 nM) and methylhydrazones **10b-d** (8.7-9.7 nM). Increasing the size of the tail such as in the benzotriazole derivatives **5e** and **7e** decreases the inhibition efficacy to 88.3 and 51.4 nM, respectively.

(v) the selectivity index (SI) data reported in Table 2 show that while actually all compounds are selective CAIs for the tumor-associated isoforms over hCA I (SI in the range 4.8-133.1 for hCA IX and 1.9-34.4 for hCA XII), significant selectivity for the target CAs over hCA II exist only within the subset of methylhydrazones **10a-d**. In detail, the four compounds exhibit a II/IX selectivity ranging between 3.2 and 44.8, whereas **10b-d** are 7- to 10-fold II/XII selective CAIs. The latter compounds could represent interesting candidates for further anti-tumor assessments.

Table 2: Selectivity index (SI) of sulfonamides reported here for hCA IX and XII over hCA I and II calculated as ratio $K_{I\ off\text{-}target\ CA}$ / $K_{I\ target\ CA}$.

Compound	***SI*** **($K_{I\ off\text{-}target\ CA}$ / $K_{I\ target\ CA}$)**			
	I / IX	**I / XII**	**II / IX**	**II/ XII**
5a	5.0	1.9	1.5	0.6
5b	4.8	3.4	1.1	0.8
5c	5.3	6.7	2.1	2.6
5d	5.3	2.4	1.2	0.6
5e	5.2	2.7	2.0	1.1
7a	112.0	34.4	4.0	1.2
7b	46.3	15.1	2.8	0.9
7c	20.4	19.1	1.8	1.7
7d	12.9	15.6	0.8	1.0
7e	15.1	7.0	2.6	1.2
10a	5.2	2.6	3.2	1.6
10b	15.4	12.7	9.6	8.0

10c	16.8	15.4	7.4	6.8
10d	133.1	28.9	44.8	9.7
AAZ	10.0	43.9	0.5	2.1

2.2.2. In vitro anti-proliferative activity towards breast cancer MCF-7 cell line

On account of hCA IX high expression in different breast malignancies, hCA IX has been correlated with the prognosis and therapy outcomes in breast cancer patients [19].

Herein, the sulfonamides **7d** and **10d** were evaluated for their anti-proliferative activities towards breast cancer MCF-7 cell line using doxorubicin as positive control, under normoxic and hypoxixc conditions adopting the MTT colorimetric assay as reported by T. Mosmann [20]. Induction of hypoxia was chemically established *via* the usage of cobalt (II) chloride hexahydrate as a chemical inducer of HIF-1α. The results were expressed as median growth inhibitory concentration (IC_{50}) values that represented the concentration of compound required to produce a 50% inhibition of cell growth after 48 h of incubation, compared to the untreated controls (**Table 3**).

Table 3. *In vitro* anti-proliferative activity of sulfonamides **7d** and **10d** against breast MCF-7 cancer cell line.

Comp.	**IC_{50} (μM)**[a]	
	Normoxia	**Hypoxia**
7d	3.32 ± 0.06	8.53 ± 0.32
10d	7.72 ± 0.22	17.34 ± 0.66
Dox.	2.36 ± 0.04	8.39 ± 0.25

[a] IC_{50} values are the mean ± S.D. of three separate experiments.

As displayed in **Table 3**, sulfonamide **7d** displayed potent anti-proliferative activities under both normoxic and hypoxic conditions with IC_{50} values equal 3.32 ± 0.06 and 8.53 ± 0.32μM, respectively, which are comparable to the reference drug doxorubicin (IC_{50} = 2.36 ± 0.04 and 8.39 ± 0.25μM, respectively). Whereas, the sulfonamide **10d** exhibited moderate activity against MCF-7 cell line with IC_{50} values equal 7.72 ± 0.22 and 17.34 ± 0.66 μM under normoxic and hypoxic conditions, respectively.

2.2.3. Cell Cycle Analysis

In the current study, the sulfonamide **7d** was examined for its effect on the cell cycle progression in MCF-7 cells (**Fig. 2**). This impact was illustrated by DNA flow cytometric

analysis; where MCF-7 cells were treated with compound **7d** at its IC_{50} concentration (IC_{50} = 3.32 µM).

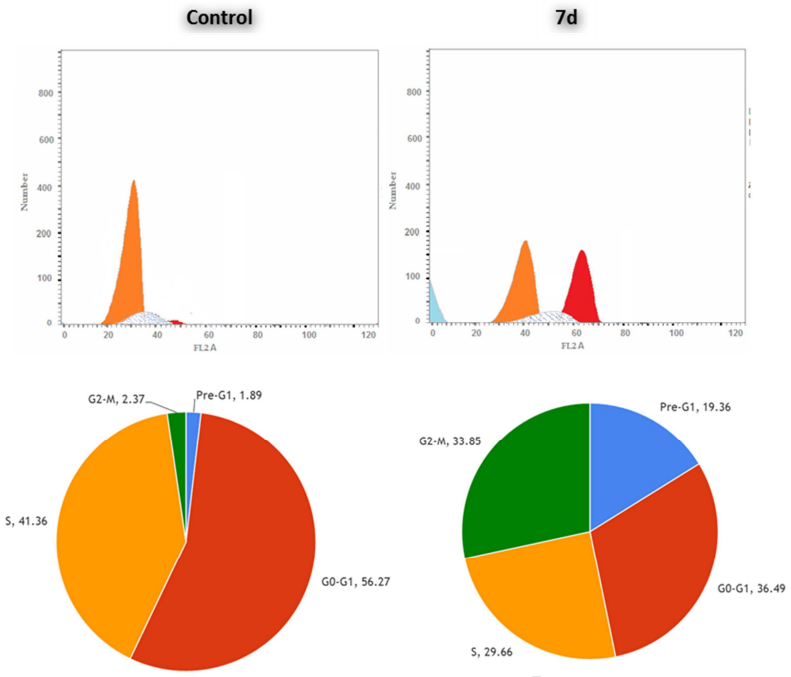


Fig. 2. Effect of sulfonamide **7d** on the phases of cell cycle of MCF-7 cells.

As displayed in **Fig. 2**, the assay outcomes highlighted that exposure of MCF-7 cells to sulfonamide **7d** resulted in a significant increase in the percentage of cells at Sub-G_1 by more than 10-folds, with concurrent significant arrest in the G_2-M phase by 14-folds in comparison to the control.

2.2.4. AnnexinV-FITC/Propidium Iodide Analysis of Apoptosis.

AnnexinV-FITC/PI dual staining assay was utilized to evaluate the impact of sulfonamide **7d** on both early and late apoptosis percentages in MCF-7 cells (**Fig. 3**, **Table 4**).

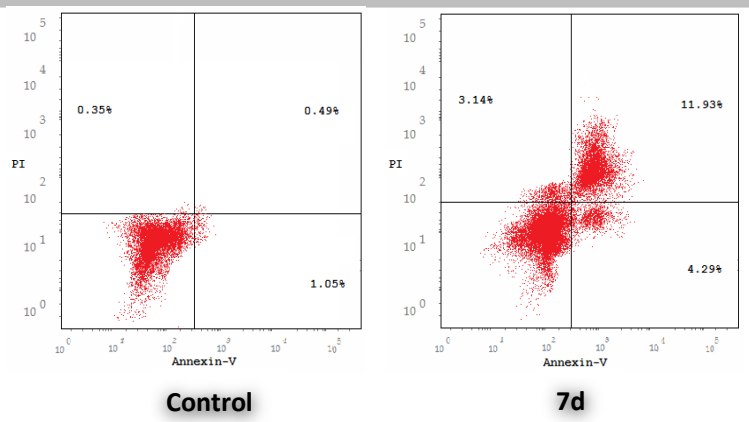


Fig. 3. Effect of sulfonamide **7d** on the percentage of annexin V-FITC-positive staining in MCF-7 cells. The experiments were done in triplicates. The four quadrants identified as: **LL**, viable; **LR**, early apoptotic; **UR**, late apoptotic; **UL**, necrotic.

As shown in **Fig. 3**, the AnnexinV assay results revealed that treatment of MCF-7 cells with sulfonamide **7d** led to a significant increase in the percentage of annexinV-FITC-positive apoptotic cells for both the early (LR; from 1.05% to 4.29%) and late apoptotic (LR; from 1.05% to 4.29%) phases, which represents more than tenfold total increase compared to the control (**Table 4**).

Table 4. Distribution of apoptotic cells in the AnnexinV-FITC/PI dual staining assay in MCF-7 cells.

Comp.	**Early Apoptosis** (Lower Right %)	**Late Apoptosis** (Upper Right %)	**Total** (L.R % + U.R %)
7d	4.29	11.93	16.22
Control	1.05	0.49	1.54

3. Molecular docking studies

Regarding the inhibitory activity of the sulfonamide **7d** against both hCA IX and hCA XII, docking simulations were performed in order to obtain a detailed explanation about the method of its interaction with the active site of both isoforms. Molecular docking simulations of the sulfonamide **7d** inside the active site of hCA IX (PDB code 5FL4) confirmed the role of the different parts of this compound in its binding with the receptor as discussed in the introduction section. At first, the sulfonamide group played an important role as ZBG by interacting with the catalytic zinc atom through a metallic bond, beside formation of H-bond with Thr200. In addition, the carbonyl group within the hydrazide moiety accepted a H-bond

from GLN92. The terminal imidazole moiety in its protonated state was able to form ionic bond with carboxylate ion of Glu 137 (**Fig. 4**).

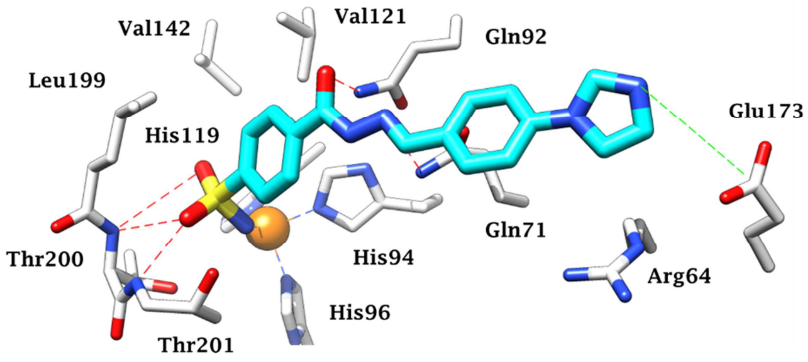


Fig. 4. Predicted binding orientations of compound **7d** within the active site of CA IX (PDB code: 5FL4). Hydrogen bonds and salt bridged are represented by red and green dashed lines, respectively.

Concerning docking simulations of compound **7d** within hCA XII active site (PDB code 1JD0), the sulfonamide group played the same role as in hCA IX. Here, we can find the hydrazide linker did not show any interaction inside the active site, but the terminal imidazole moiety stabilized the binding orientation by forming an anchoring π- π interaction with Tyr20 as illustrated in **Fig. 5**.

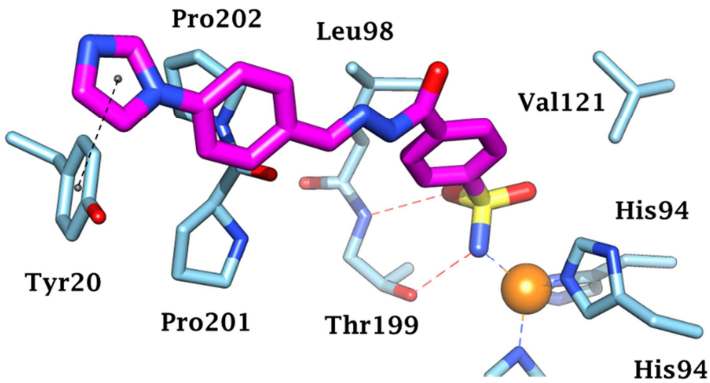


Fig. 5. Predicted binding orientations of compound **7d** within the active site of CA XII (PDB code: 1JD0). Hydrogen bonds and π-π interactions are represented by red and black dashed lines, respectively.

4. Conclusions

In conclusion, we report here the design and synthesis of three series of novel sulfonamides (**5a-e**, **7a-e** and **10a-d**) incorporating mixed hydrophilic/hydrophobic tails linked to benzenesulfonamide (as zinc anchoring moieties) through hydrazide and hydrazine linkers. The structures of the novel derivatives were confirmed by different

spectral and elemental analyses methods. Biological evaluation of the newly prepared sulfonamides was performed against hCA I, II, IX and XII. All the tested isoforms were inhibited by the synthesized sulfonamides **5a-e**, **7a-e** and **10a-d**, in variable degrees with the following K_Is ranges: 76.8–357.4 nM for hCA I, 8.2–94.6 nM for hCA II, 2.0–46.3 nM for hCA XI, and 8.3–88.3 nM for hCA XII. Best activity was observed towards the tumor associated isoform hCA IX, where compounds **7a-c** and **10b-d** emerged as single-digit nanomolar hCA IX inhibitors (K_Is in the range of 2.0–8.9 nM). Besides, all the remaining compounds, except **5e**, were more potent than AAZ with K_Is in a range of 10.1–23.7 nM. On the other hand, the sulfonamide **7d** displayed potent anti-proliferative activity against breast MCF-7 cancer cell line under both normoxic and hypoxic conditions (IC_{50} = 3.32 ± 0.06 and 8.53 ± 0.32 μM, respectively), which is comparable to the reference drug doxorubicin (IC_{50} = 2.36 ± 0.04 and 8.39 ± 0.25μM, respectively). Furthermore, **7d** was screened for cell cycle disturbance and apoptosis induction in MCF-7 cells. It was found to persuade cell cycle arrest at G2-M stage as well as to alter the Sub-G1 phase, also, **7d** resulted in a significant increase in the percentage of annexinV-FITC positive apoptotic cells from 1.03 to 18.54%. Finally, a molecular docking study for **7d** was carried out within the hCA IX and hCA XII active sites to rationalize the obtained inhibition results.

5. Experimental

5.1. Chemistry

5.1.1. General

Melting points were determined using Stuart SMP10 digital melting point apparatus and were uncorrected. Infrared (IR) Spectra were recorded as KBr disks using a Shimadzu FT-IR 8400S infrared spectrophotometer. Mass spectra were measured as m/z (Intensity %) using Thermo Scientific ISOLT mass spectroscopy at the Regional Center for Mycology and Biotechnology.NMR spectra were recorded on a Bruker Ascend 400/ R (^{1}H: 400, ^{13}C: 100 MHz) spectrometer. ^{1}H NMR spectra were run at 400 MHz and ^{13}C spectra were run at 100 MHz in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts are expressed in δ values (ppm) using the solvent peak as internal standard. All coupling constant (*J*) values are given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Elemental analyses were performed at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. Analytical thin layer chromatography (TLC) on silica gel pre-coated F254

Merck plates containing UV indicator was employed routinely to follow the course of reactions. Unless otherwise noted, all solvents and reagents were commercially available and used without further purification. Synthesis and analytical data of compounds **3a-e** [16], **4** [21], **6** [11] and **9a-d** [16, 17] were found as reported.

*5.1.2. General procedure for the preparation of compounds **5a-e**.*

To a solution of the appropriate substituted aldehydes **3a-e** (1 mmol) in absolute ethanol in the presence of two drops of glacial acetic acid, 4-hydrazinobenzenesulfonamide hydrochloride **4** (0.223 g, 1 mmol) was added. The reaction mixture was heated under reflux for 3-6 hours. The obtained solid was collected by filtration, dried and recrystallized from ethanol/DMF to afford compounds **5a-e**.

*5.1.2.1. 4-(2-(4-(pyrrolidin-1-yl)benzylidene)hydrazinyl)benzenesulfonamide (**5a**).* Purple crystals, (yield 61%), m.p. 236-239°C; IR (KBr, ν_{max} / cm^{-1}): 3448, 3325, 3248 (NH_2 & NH), 1330 and 1153 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.96-1.97 (m, 4H, pyrrolidine Hs), 3.28-3.34 (m, 4H, pyrrolidine Hs), 6.57 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.02 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.07 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.50 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.63 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.84 (s, 1H, = C-H), 10.44 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 25.40, 47.73 (pyrrolidine carbons), 111.07, 111.74, 112.09, 122.47, 127.90, 132.63, 141.11, 148.54, 148.71; ESI MS *m/z*: 344 $[M]^+$; Anal. Calcd. for $C_{17}H_{20}N_4O_2S$ (344.43): C, 59.28; H, 5.85; N, 16.27; Found C, 59.14; H, 6.01; N, 16.43.

*5.1.2.2. 4-(2-(4-(piperidin-1-yl)benzylidene)hydrazinyl)benzenesulfonamide (**5b**).* Brick red powder, (yield 55%), m.p. 246-248°C; IR (KBr, ν_{max} / cm^{-1}): 3402, 3325, 3236 (NH_2 & NH), 1276 and 1141 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.63-1.66 (m, 2H, piperidine Hs), 1.85-1.88 (m, 4H, piperidine Hs), 3.44-3.46 (m, 4H, piperidine Hs), 7.08 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.16 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.59-7.75 (m, 6H, Ar-H), 7.95 (s, 1H, = C-H), 10.90 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 22.00, 24.05, 54.50 (piperidine carbons) 111.74, 120.53, 120.80, 127.70, 127.93, 133.97, 138.28, 144.50, 148.12; ESI MS *m/z*: 358 $[M]^+$; Anal. Calcd. for $C_{18}H_{22}N_4O_2S$ (358.46): C, 60.31; H, 6.19; N, 15.63; Found C, 60.48; H, 6.34; N, 15.46.

*5.1.2.3. 4-(2-(4-morpholinobenzylidene)hydrazinyl)benzenesulfonamide (**5c**).* Grey powder, (yield 62%), m.p. > 300°C; IR (KBr, ν_{max} / cm^{-1}): 3348, 3263, 3205 (NH_2 & NH), 1327 and 1161 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 3.18 (t, *J* = 4.7 Hz, 4H, morpholine Hs), 3.75 (t, *J* = 4.7 Hz, 4H, morpholine Hs), 6.98 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.04 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.10 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.56 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.64 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.96 (s, 1H, = C-H), 10.57 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 48.28, 66.45 (morpholine carbons), 111.28, 115.14, 126.31, 127.61, 127.92, 133.13, 140.10, 148.54, 151.74; ESI MS *m/z*: 360 $[M]^+$; Anal. Calcd. for $C_{17}H_{20}N_4O_3S$ (360.43): C, 56.65; H, 5.59; N, 15.54; Found C, 56.79; H, 5.67; N, 15.70.

*5.1.2.4. 4-(2-(4-(1H-imidazol-1-yl)benzylidene)hydrazinyl)benzenesulfonamide (**5d**).* Yellow powder, (yield 61%), m.p. 270-272°C; IR (KBr, ν_{max} / cm^{-1}): 3394, 3305, 3217 (NH_2 & NH), 1323 and 1141 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 7.13 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.24 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.69 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.87 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.91-7.93 (m, 3H, Ar-H), 8.10 (s, 1H, = C-H), 8.34 (s, 1H, Ar-H), 9.79 (s, 1H, Ar-H), 11.37 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 111.92, 121.06, 121.62, 122.66, 127.66, 127.91, 134.29, 134.84, 136.91, 137.52, 148.02; ESI MS *m/z*: 341 $[M]^+$; Anal. Calcd. for $C_{16}H_{15}N_5O_2S$ (341.39): C, 56.29; H, 4.43; N, 20.51; Found C, 56.45; H, 4.61; N, 20.79.

*5.1.2.5. 4-(2-(4-(1H-benzo[d][1,2,3]triazol-1-yl)benzylidene)hydrazinyl)benzenesulfonamide (**5e**).* Canary yellow powder, (yield 45%), m.p. 230-232°C; IR (KBr, ν_{max} / cm^{-1}): 3309, 3282, 3232 (NH_2 & NH), 1327 and 1153 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 7.11 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.22 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.53-7.57 (m, 1H, Ar-H), 7.71 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.95 (d, *J* = 8.5 Hz, 2H, Ar-H), 8.01 (d, *J* = 8.4 Hz, 2H, Ar-H), 8.05 (m, 1H, Ar-H), 8.08 (s, 1H, =C-H), 8.22 (d, *J* = 8.4 Hz, 1H, Ar-H), 8.37 (d, *J* = 8.5 Hz, 1H, Ar-H), 10.99 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 111.54, 111.93, 118.63, 120.23, 121.02, 123.38, 125.33, 127.99, 129.31, 132.14, 134.38, 138.04, 144.99, 146.27, 148.03; ESI MS *m/z*: 392 $[M]^+$; Anal. Calcd. for $C_{19}H_{16}N_6O_2S$ (392.44): C, 58.15; H, 4.11; N, 21.42; Found C, 58.32; H, 4.27; N, 21.68.

*5.1.3. General procedure for the preparation of compounds **7a-e**.*

To a solution of the appropriate substituted aldehydes **3a-e** (1 mmol) in glacial acetic acid, 4-(hydrazinecarbonyl)benzenesulfonamide **6** (0.215 g, 1 mmol) was added. The reaction

mixture was heated under reflux for 2-4 hours.. The solid formed was collected by filtration, dried and recrystallized from ethanol/DMF to afford compounds **7a-e**.

*5.1.3.1. 4-(2-(4-(Pyrrolidin-1-yl)benzylidene)hydrazine-1-carbonyl)benzenesulfonamide (**7a**).* Yellowish brown powder, (yield 70%), m.p. > 300°C; IR (KBr, ν_{max} / cm^{-1}): 3305, 3194, 3113 (NH_2 & NH), 1342 and 1161 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.96-1.98 (m, 4H, pyrrolidine Hs), 3.28-3.31 (m, 4H, pyrrolidine Hs), 6.61 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.51 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.55 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.95 (d, *J* = 8.3 Hz, 2H, Ar-H), 8.05 (d, *J* = 8.3 Hz, 2H, Ar-H), 8.31 (s, 1H, = C-H), 11.67 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 25.43, 47.71 (pyrrolidine carbons), 112.06, 121.09, 126.17, 128.64, 129.24, 137.19, 146.81, 149.54, 150.11, 162.08 (C=O); ESI MS *m/z*: 372 $[M]^+$; Anal. Calcd. for $C_{18}H_{20}N_4O_3S$ (372.44): C, 58.05; H, 5.41; N, 15.04; Found C, 58.23; H, 5.66; N, 15.28.

*5.1.3.2. 4-(2-(4-(Piperidin-1-yl)benzylidene)hydrazine-1-carbonyl)benzenesulfonamide (**7b**).* Green powder, (yield 61%), m.p. 269-271°C; IR (KBr, ν_{max} / cm^{-1}): 3379, 3305, 3267 (NH_2 & NH), 1338 and 1161 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.54-1.62 (m, 6H, piperidine Hs), 3.17-3.32 (m, 4H, piperidine Hs), 6.99 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.52 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.57 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.95 (d, *J* = 8.4 Hz, 2H, Ar-H), 8.06 (d, *J* = 8.3 Hz, 2H, Ar-H), 8.33 (s, 1H, = C-H), 11.75 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 24.39, 25.42, 48.81 (piperidine carbons), 115.01, 123.50, 126.19, 128.68, 129.03, 137.07, 146.89, 149.50, 152.97, 162.22 (C=O); ESI MS *m/z*: 386 $[M]^+$; Anal. Calcd. for $C_{19}H_{22}N_4O_3S$ (386.47): C, 59.05; H, 5.74; N, 14.50; Found C, 58.89; H, 5.87; N, 14.71.

*5.1.3.3. 4-(2-(4-Morpholinobenzylidene)hydrazine-1-carbonyl)benzenesulfonamide (**7c**).* Buff powder, (yield 85%), m.p. 290-292°C; IR (KBr, ν_{max} / cm^{-1}): 3417, 3317, 3205 (NH_2 & NH), 1342 and 1165 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 3.22-3.25 (m, 4H, morpholine Hs), 3.73-3.76 (m, 4H, morpholine Hs), 7.03 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.52 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.61 (d, *J* = 8.5 Hz, 2H, Ar-H), 7.95 (d, *J* = 8.2 Hz, 2H, Ar-H), 8.06 (d, *J* = 8.3 Hz, 2H, Ar-H), 8.35 (s, 1H, = C-H), 11.79 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 47.87, 66.41 (morpholine carbons), 114.81, 124.70, 126.20, 128.70, 128.94, 137.02, 146.92, 149.33, 152.81, 162.31 (C=O); ESI MS *m/z*: 388 $[M]^+$; Anal. Calcd. for $C_{18}H_{20}N_4O_4S$ (388.44): C, 55.66; H, 5.19; N, 14.42; Found C, 55.87; H, 5.31; N, 14.70.

*5.1.3.4. 4-(2-(4-(1H-Imidazol-1-yl)benzylidene)hydrazine-1-carbonyl)benzenesulfonamide (**7d**).* White powder, (yield 56%), m.p. > 300°C; IR (KBr, ν_{max} / cm^{-1}): 3280, 3221, 3136 (NH_2 &

NH), 1307 and 1165 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 7.15 (s, 1H, Ar-H), 7.54 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.80 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.85 (s, 1H, Ar-H), 7.90 (d, *J* = 8.4 Hz, 2H, Ar-H), 7.98 (d, *J* = 8.2 Hz, 2H, Ar-H), 8.10 (d, *J* = 8.2 Hz, 2H, Ar-H), 8.38 (s, 1H, Ar-H), 8.51 (s, 1H, = C-H), 12.08 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 118.30, 120.89, 126.26, 128.84, 129.15, 130.60, 133.02, 136.02, 136.72, 138.48, 147.17, 147.92, 162.70 (C=O); ESI MS *m/z*: 369 $[M]^+$; Anal. Calcd. for $C_{17}H_{15}N_5O_3S$ (369.40): C, 55.28; H, 4.09; N, 18.96; Found C, 55.46; H, 4.32; N, 19.21.

*5.1.3.5. 4-(2-(4-(1H-Benzo[d][1,2,3]triazol-1-yl)benzylidene)hydrazine-1-carbonyl)benzene sulfonamide (**7e**).* Yellowish white powder, (yield 87%), m.p. > 300°C; IR (KBr, ν_{max} / cm^{-1}): 3417, 3344, 3244 (NH_2 & NH), 1334 and 1161 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 7.56-7.58 (m, 4H, Ar-H and D_2O exchangable, -SO_2NH_2), 7.72 (t, *J* = 7.6 Hz, 1H, Ar-H), 7.99 (d, *J* = 8.0 Hz, 2H, Ar-H), 8.03-8.13 (m, 3H, Ar-H), 8.12 (d, *J* = 8.1 Hz, 2H, Ar-H), 8.22 (d, *J* = 8.3 Hz, 1H, Ar-H), 8.44 (d, *J* = 8.4 Hz, 1H, Ar-H), 8.60 (s, 1H, = CH), 12.18 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 111.60, 118.71, 120.29, 121.10, 123.28, 125.42, 126.29, 128.47, 128.89, 129.27, 132.03, 134.80, 136.66, 137.91, 145.07, 146.33, 147.68, 162.78 (C=O); ESI MS *m/z*: 420 $[M]^+$; Anal. Calcd. for $C_{20}H_{16}N_6O_3S$ (420.45): C, 57.13; H, 3.84; N, 19.99; Found C, 57.40; H, 4.02; N, 20.16.

*5.1.4. General procedure for the preparation of compounds **10a-d**.*

To a solution of the appropriate substituted acetophenones **9a-d** (1 mmol) in absolute ethanol in the presence of catalytic amount of glacial acetic acid, 4-hydrazinobenzenesulfonamide hydrochloride (0.223 g, 1 mmol) was added. The reaction mixture was heated under reflux for 6-8 hours.. The solid formed was collected by filtration, dried and recrystallized from ethanol/DMF to afford compounds **10a-d**.

*5.1.4.1. 4-(2-(1-(4-(Pyrrolidin-1-yl)phenyl)ethylidene)hydrazinyl)benzenesulfonamide (**10a**).* Yellowish brown powder, (yield 45%), m.p. 291-292°C; IR (KBr, ν_{max} / cm^{-1}): 3479, 3336, 3259 (NH_2 & NH), 1315 and 1145 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.97-2.00 (m, 4H, pyrrolidine Hs), 2.24 (s, 3H, C<u>H</u>$_3$), 3.29 (m, 4H, pyrrolidine Hs), 6.63 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.05 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.26 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.65 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.69 (d, *J* = 8.6 Hz, 2H, Ar-H), 9.53 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 13.48 (-<u>C</u>H$_3$), 25.35, 26.32, 47.71, 47.99 (pyrrolidinecarbons),111.14, 112.01, 113.72, 124.57, 126.30, 127.49, 130.84, 133.09, 136.76, 145.25, 147.97, 149.43,

151.18; ESI MS *m/z*: 358 $[M]^+$; Anal. Calcd. for $C_{18}H_{22}N_4O_2S$ (358.46): C, 60.31; H, 6.19; N, 15.63; Found C, 60.58; H, 6.22; N, 15.80.

*5.1.4.2. 4-(2-(1-(4-(Piperidin-1-yl)phenyl)ethylidene)hydrazinyl) benzenesulfonamide (**10b**).* Black crystals, (yield 57%), m.p. 240-242°C; IR (KBr, ν_{max} / cm^{-1}): 3421, 3197, 3132 (NH_2 & NH), 1330 and 1157 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 1.59-1.71 (m, 4H, piperidine Hs), 2.43 (s, 3H, C<u>H</u>$_3$), 3.29-3.44 (m, 6H, piperidine Hs), 7.18 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.47 (d, *J* = 8.8 Hz, 2H, Ar-H), 7.58 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.79 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.85 (d, *J* = 8.3 Hz, 2H, Ar-H) , 8.86 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 24.32 (-<u>C</u>H$_3$), 25.20, 26.52, 48.54 (piperidinecarbons), 113.72, 125.50, 126.04, 127.15, 129.39, 130.03, 130.67, 132.25, 141.49, 142.67, 143.10, 144.60; ESI MS *m/z*: 372 $[M]^+$; Anal. Calcd. for $C_{19}H_{24}N_4O_2S$ (372.49): C, 61.27; H, 6.49; N, 15.04; Found C, 61.51; H, 6.37; N, 15.29.

*5.1.4.3. 4-(2-(1-(4-Morpholinophenyl)ethylidene)hydrazinyl)benzenesulfonamide (**10c**).* Brown powder, (yield 50%), m.p. 255-257°C; IR (KBr, ν_{max} / cm^{-1}): 3394, 3336, 3275 (NH_2 & NH), 1334 and 1149 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 2.25 (s, 3H, C<u>H</u>$_3$), 3.18 (t, *J* = 4.3 Hz, 4H, morpholine Hs), 3.77 (t, *J* = 4.4 Hz, 4H, morpholine Hs), 7.00 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.05 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.29 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.66 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.72 (d, *J* = 8.7 Hz, 2H, Ar-H), 9.60 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 13.58 (-<u>C</u>H$_3$), 49.87, 65.89 (morpholine carbons), 112.30, 113.51, 116.53, 126.99, 127.68, 130.50, 132.35, 133.68, 143.74, 149.18; ESI MS *m/z*: 374 $[M]^+$; Anal. Calcd. for $C_{18}H_{22}N_4O_3S$ (374.46): C, 57.74; H, 5.92; N, 14.96; Found C, 58.01; H, 5.87; N, 15.12.

*5.1.4.4. 4-(2-(1-(4-(4-Methylpiperazin-1-yl)phenyl)ethylidene)hydrazinyl)benzenesulfonamide (**10d**).* Buff powder, (yield 46%), m.p. 281-283°C; IR (KBr, ν_{max} / cm^{-1}): 3421, 3282, 3205 (NH_2 & NH), 1330 and 1138 (SO_2); ^{1}H NMR (DMSO-d_6) δ *ppm*: 2.26 (s, 3H, C<u>H</u>$_3$), 2.77 (s, 3H, C<u>H</u>$_3$), 3.34-3.46 (m, 8H, piperazine Hs), 7.02 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.06 (s, 2H, D_2O exchangable, -SO_2NH_2), 7.30 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.66 (d, *J* = 8.6 Hz, 2H, Ar-H), 7.73 (d, *J* = 8.5 Hz, 2H, Ar-H), 9.64 (s, 1H, D_2O exchangable, -NH); ^{13}C NMR (DMSO-d_6) δ ppm: 13.60, 19.02 (2 -<u>C</u>H$_3$), 42.54, 45.63, 52.49, 56.49 (piperazine carbons), 112.21, 115.78, 126.96, 127.68, 130.77, 133.58, 143.85, 149.25, 149.82; ESI MS *m/z*: 387 $[M]^+$; Anal. Calcd. for $C_{19}H_{25}N_5O_2S$ (387.50): C, 58.89; H, 6.50; N, 18.07; Found C, 59.12; H, 6.37; N, 18.40.

5.2. Biological Evaluation

5.2.1. CA inhibitory assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity, as reported earlier [18]. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation as reported earlier [22,23], and represent the mean from at least three different determinations. The four tested CA isofoms were recombinant ones obtained in-house as reported earlier [24-26].

5.2.2. Anti-proliferative activity towards MCF-7 breast cancer cell line

Breast cancer MCF-7 cell line was obtained from American Type Culture Collection (ATCC). The cells were propagated in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine (2.5 mM), HEPES buffer (10 mM), 50 μg/mL gentamycin. The hypoxia inducer $CoCl_2$ (100 μM) was in case of the hypoxic condition. All cells were maintained at 37 C in a humidified atmosphere with 5% CO_2. Cytotoxicity was determined following the MTT assay, as reported earlier [20, 27].

5.2.3. Cell cycle analysis

MCF-7 cells were treated with sulfonamide **7d** for 24 h at its IC_{50} concentration then cells were washed with ice-cold phosphate buffered saline (PBS). The treated cells were collected by centrifugation, fixed in ice-cold 70% (*v*/*v*) ethanol, washed with PBS, re-suspended with 100 μg/mL RNase, stained with 40 μg/mL PI, and analyzed by flow cytometry using FACS Calibur (Becton Dickinson, BD, USA). The cell cycle distributions were calculated using CellQuest software 5.1 (Becton Dickinson) [28, 29].

5.2.4. Annexin V-FITC apoptosis assay

Phosphatidylserine externalization was assayed using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, USA) according to the manufacturer's instructions, as reported earlier [28, 29].

5.3. Computational studies

The crystal structures of hCA IX (PDB 5FL4) [30] and hCA XII (PDB 1JD0) [31] were obtained from protein databank and were prepared according to the Protein Preparation wizard in Maestro - Schrödinger suite 2017-1 [32] in order to adding missing atoms by Prime, assining the protonation states, optimization of the hydrogen bonds and refinement process by running a restrained minimization (OPLS3 force field). 3D ligand structures were drawn and prepared

minimized using LigPrep [33] in order to predict ionization states for the ligands using a pH of 7.0 ± 0.5. Before docking process, the grid box was prepared at size 20 Å. Zn atom was chosen as a metal constrained. In docking simulations were done using Glide, the protein was treated as rigid, compounds were flexibly docked, and scoring was assigned according to the xtra precision (XP) mode.

References

[1] C.T. Supuran. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat. Rev. Drug Discov. 7 (2008) 168–81.

[2] V. Alterio, A. Di Fiore, K. D'Ambrosio , C.T. Supuran, G. De Simone. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem. Rev. 112 (2012) 4421–68.

[3] A. Nocentini, C.T. Supuran CT. Carbonic anhydrase inhibitors as antitumor/antimetastatic agents: a patent review (2008-2018). Expert Opin. Ther. Pat. 28 (2018) 729-740.

[4] C.L. Lomelino, B.P. Mahon, R. McKenna, F. Carta, C.T. Supuran, Kinetic and X-ray crystallographic investigations on carbonic anhydrase isoforms I, II, IX and XII of a thioureido analog of SLC-0111, Bioorg. Med. Chem., 24 (2016) 976-981.

[5] Safety Study of SLCe0111 in Subjects with advanced solid tumours. Available from, https://clinicaltrials.gov/ct2/show/study/NCT02215850?term-SLC_0111&rank-1.

[6] F. Carta, D. Vullo, S.M. Osman, Z. AlOthman, C.T. Supuran, Synthesis and carbonic anhydrase inhibition of a series of SLC-0111 analogs, Bioorg. Med. Chem., 25 (2017) 2569-2576.

[7] M. Bozdag, F. Carta, M. Ceruso, M. Ferraroni, P.C. McDonald, S. Dedhar, C.T. Supuran, Discovery of 4-hydroxy-3-(3-(phenylureido) benzenesulfonamides as SLC-0111 analogues for the treatment of hypoxic tumors overexpressing carbonic anhydrase IX, J. Med. Chem., 61 (2018) 6328-6338.

[8] W.M. Eldehna, M.F. Abo-Ashour, E. Berrino, D. Vullo, H.A. Ghabbour, S.T. Al-Rashood, G.S. Hassan, H.M. Alkahtani, A.A. Almehizia, A. Alharbi, SLC-0111 enaminone analogs, 3/4-(3-aryl-3-oxopropenyl) aminobenzenesulfonamides, as novel selective subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform IX, Bioorganic chemistry, (2018).

[9] W.M. Eldehna, M. Fares, M. Ceruso, H.A. Ghabbour, S.M. Abou-Seri, H.A. Abdel-Aziz, D.A.A. El Ella, C.T. Supuran, Amido/ureidosubstituted benzenesulfonamides-isatin conjugates

as low nanomolar/subnanomolar inhibitors of the tumor-associated carbonic anhydrase isoform XII, Eur. J. Med. Chem., (2016).

[10] W.M. Eldehna, M.F. Abo-Ashour, A. Nocentini, R.S. El-Haggar, S. Bua, A. Bonardi, S.T. Al-Rashood, G.S. Hassan, P. Gratteri, H.A. Abdel-Aziz, Enhancement of the tail hydrophobic interactions within the carbonic anhydrase IX active site via structural extension: Synthesis of novel N-substituted isatins-SLC-0111 hybrids as carbonic anhydrase inhibitors and antitumor agents, Eur. J. Med. Chem., (2018).

[11] M.F. Abo-Ashour, W.M. Eldehna, A. Nocentini, H.S. Ibrahim, S. Bua, S.M. Abou-Seri, C.T. Supuran, Novel hydrazido benzenesulfonamides-isatin conjugates: Synthesis, carbonic anhydrase inhibitory activity and molecular modeling studies, Eur. J. Med. Chem., 157 (2018) 28-36.

[12] A.-M. Alaa, A.S. El-Azab, M.A.A. El-Enin, A.A. Almehizia, C.T. Supuran, A. Nocentini, Synthesis of novel isoindoline-1, 3-dione-based oximes and benzenesulfonamide hydrazones as selective inhibitors of the tumor-associated carbonic anhydrase IX, Bioorganic chemistry, 80 (2018) 706-713.

[13] C.B. Mishra, S. Kumari, A. Angeli, S. Bua, M. Buonanno, S.M. Monti, M. Tiwari, C.T. Supuran, Discovery of potent anti-convulsant carbonic anhydrase inhibitors: Design, synthesis, in vitro and in vivo appraisal, Eur. J. Med. Chem., 156 (2018) 430-443.

[14] H.S. Ibrahim, H.A. Allam, W.R. Mahmoud, A. Bonardi, A. Nocentini, P. Gratteri, E.S. Ibrahim, H.A. Abdel-Aziz, C.T. Supuran, Dual-tail arylsulfone-based benzenesulfonamides differently match the hydrophobic and hydrophilic halves of human carbonic anhydrases active sites: Selective inhibitors for the tumor-associated hCA IX isoform, Eur. J. Med. Chem., 152 (2018) 1-9.

[15] M.R. Buemi, A. Di Fiore, L. De Luca, A. Angeli, F. Mancuso, S. Ferro, S.M. Monti, M. Buonanno, E. Russo, G. De Sarro, Exploring structural properties of potent human carbonic anhydrase inhibitors bearing a 4-(cycloalkylamino-1-carbonyl) benzenesulfonamide moiety, Eur. J. Med. Chem., (2018).

[16] P. Magdolen, M. Mečiarová, Š. Toma, Ultrasound effect on the synthesis of 4-alkyl-(aryl) aminobenzaldehydes, Tetrahedron, 57 (2001) 4781-4785.

[17] H.S. Ibrahim, M. E. Albakri, W. R. Mahmoud, H. A. Allam, A. M. Reda, and H. A. Abdel-Aziz. Synthesis and biological evaluation of some novel thiobenzimidazole derivatives as anti-renal cancer agents through inhibition of c-MET kinase. Bioorg. Chem. 85 (2019) 337-348.

[18] 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. Chem. 246 (1971) 2561-2573.
[19] N.K. Tafreshi, M.C. Lloyd, J.B. Proemsey, M.M. Bui, J. Kim, R.J. Gillies, D.L. Morse, Evaluation of CAIX and CAXII expression in breast cancer at varied O2 levels: CAIX is the superior surrogate imaging biomarker of tumor hypoxia, Mol. Imag. Biol. 18 (2016) 219-231.
[20] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55-63.
[21] Soliman, Preparation and antidiabetic activity of some sulfonylurea derivatives of 3, 5-disubstituted pyrazoles, J. Med. Chem., 22 (1979) 321-325.
[22] W.M. Eldehna, G.H. Al-Ansary, S. Bua, A. Nocentini, P. Gratteri, A. Altoukhy, H. Ghabbour, H.Y. Ahmed, C.T. Supuran, Novel indolin-2-one-based sulfonamides as carbonic anhydrase inhibitors: synthesis, in vitro biological evaluation against carbonic anhydrases isoforms I, II, IV and VII and molecular docking studies, Eur. J. Med. Chem. 127 (2017) 521-530.
[23] W.M. Eldehna, A. Nocentini, S.T. Al-Rashood, G.S. Hassan, H.M. Alkahtani, A.A. Almehizia, A.M. Reda, H.A. Abdel-Aziz, C.T. Supuran, Tumor-associated carbonic anhydrase isoform IX and XII inhibitory properties of certain isatin bearing sulfonamides endowed with in vitro anticancer activity towards colon cancer, Bioorg. Chem. 81 (2018) 425-432.
[24] H. S. Ibrahim, S. M. Abou-Seri, M. Tanc, M. M. Elaasser, H. A. Abdel-Aziz, and C. T. Supuran. Isatin-pyrazole benzenesulfonamide hybrids potently inhibit tumor-associated carbonic anhydrase isoforms IX and XII. Eur. J. Med. Chem. 103 (2015) 583-593.
[25] A. Nocentini, P. Gratteri, C.T. Supuran. Phosphorus versus Sulfur: Discovery of Benzenephosphonamidates as Versatile Sulfonamide-Mimic Chemotypes Acting as Carbonic Anhydrase Inhibitors. Chemistry. 25 (2019) 1188-1192.
[26] A. Nocentini, F. Carta, M. Tanc, S. Selleri, C.T. Supuran, C. Bazzicalupi, P. Gratteri. Deciphering the Mechanism of Human Carbonic Anhydrases Inhibition with Sulfocoumarins: Computational and Experimental Studies. Chemistry. 24 (2018) 7840-7844
[27] M. El-Naggar, H. Almahli, H.S. Ibrahim, W.M. Eldehna, H.A. Abdel-Aziz, Pyridine-Ureas as Potential Anticancer Agents: Synthesis and In Vitro Biological Evaluation, Molecules 23 (2018) 1459.
[28] H.S. Ibrahim, W.M. Eldehna, A.L. Fallacara, E.R. Ahmed, H.A. Ghabbour, M.M. Elaasser, M. Botta, Botta, S.M. Abou-seri, and H.A. Abdel-Aziz, 2018. One-pot synthesis of spiro (indoline-3, 4′-pyrazolo [3, 4-b] pyridine)-5′-carbonitriles as p53-MDM2 interaction inhibitors. Future Med Chem, 24 (2018) 2771-2789.

[29] W.M. Eldehna, M.F. Abo-Ashour, H.S. Ibrahim, G.H. Al-Ansary, H.A. Ghabbour, M.M. Elaasser, H. YA Ahmed, N.A. Safwat, Novel [(3-indolylmethylene) hydrazono] indolin-2-ones as apoptotic anti-proliferative agents: design, synthesis and in vitro biological evaluation, J. Enzyme Inhib. Med. Chem. 33, (2018), 686-700.

[30] J. Leitans, A. Kazaks, A. Balode, J. Ivanova, R. Zalubovskis, C.T. Supuran, K. Tars, Efficient expression and crystallization system of cancer-associated carbonic anhydrase isoform IX, J. Med. Chem., 58 (2015) 9004-9009.

[31] D.A. Whittington, A. Waheed, B. Ulmasov, G.N. Shah, J.H. Grubb, W.S. Sly, D.W. Christianson, Crystal structure of the dimeric extracellular domain of human carbonic anhydrase XII, a bitopic membrane protein overexpressed in certain cancer tumor cells, Proc. Natl. Acad. Sci., 98 (2001) 9545-9550.

[32] L. Schrödinger, Schrödinger, LLC; New York, NY: 2015, PyMOL Molecular Graphics System, Version, 1 (2016).

[33] Schrödinger Release 2018-1: LigPrep, Schrödinger, LLC, New York, NY, 2018.

Highlights

- Three sets of novel benzenesulfonamides (**5a-e**, **7a-e** and **10a-d**) were designed and synthesized.

- Inhibitory activity of the prepared sulfonamides was evaluated toward hCA I, II, IX and XII.

- hCA IX was efficiently inhibited by all sulfonamides with K_Is in the range of 2.0–46.3 nM.

- Antiproliferative and proapoptotic activities towards breast cancer MCF-7 cells were examined.

- Docking was done to provide insights in the binding interactions of the reported sulfonamides.