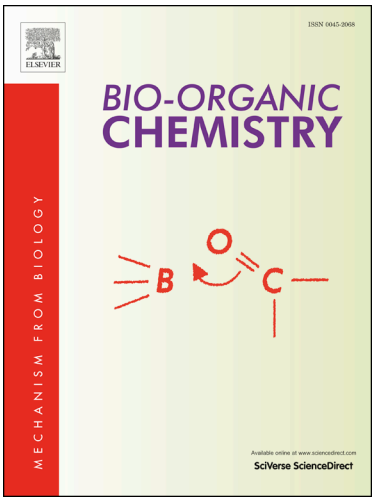

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Halise Inci Gul[a*], Cem Yamali[a], Merve Bulbuller[a], Petek Ballar Kirmizibayrak[b], Mustafa Gul[c], Andrea Angeli[d], Silvia Bua[d], Claudiu T. Supuran[d*]

[a]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey

[b]Department of Biochemistry, Faculty of Pharmacy, Ege University, Bornova, Izmir, Turkey

[c]Department of Physiology, Faculty of Medicine, Ataturk University, Erzurum, Turkey.

[d]Neurofarba Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, Università degli Studi di Firenze, Via U. Schiff 6, 50019 Sesto Fiorentino (Florence), Italy.

*Corresponding authors:

Prof. Halise Inci Gul (H.I. Gul)

Ataturk University

Faculty of Pharmacy

Department of Pharmaceutical Chemistry

25240, Erzurum, TURKEY

Tel: +90 4422315219

Fax: +90 4422315201

E-mail: incigul1967@yahoo.com

Prof. Claudiu T Supuran (C.T. Supuran)

University of Florence

Neurofarba Department

Via Ugo Schiff 6,

Polo Scientifico, 50019-Sesto Fiorentino (Firenze), Italy

Phone: +39-055-4573729/3005

Mobile: +39-349-6639626

E-mail: claudiu.supuran@unifi.it

Abstract

In this study, new dibenzensulfonamides, **7-9**, having the chemical structure 4,4'-(5'-chloro-3'-methyl-5-aryl-3,4-dihydro-1'*H*,*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamide were synthesized in five steps to develop new anticancer drug candidates. Their chemical structures were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectra. Cytotoxicities of the dibenzensulfonamides were investigated towards HCC1937, MCF7, HeLa, A549 as tumor cell lines and towards MRC5 and Vero as non-tumor cells. Carbonic anhydrase (CAs, EC 4.2.1.1) inhibitory effects of the dibenzensulfonamides **7-9** were also evaluated on the cytosolic human (h) hCA I and II and the tumor-associated hCA IX and XII isoenzymes. Results indicate that both **7** and **8** induced cleavage of poly (ADP ribose) polymerase (PARP), activation of caspases -3, -7 and -9 which are the hallmarks of apoptosis. Meanwhile both compounds induced autophagy in HCC1937 cells which is shown by enhanced expression of LC3 and decreased level of p62 protein. The compounds tested were also effectively inhibited tumor-associated hCA IX and hCA XII isoenzymes in the range of 20.7-28.1 nM and 4.5-9.3 nM, respectively.

Keywords: Sulfonamide; pyrazole; carbonic anhydrase; anticancer; apoptosis; autophagy

1. Introduction

Cancer is the second leading cause of death in developed countries. According to the National Cancer Institue's data, more than 200 drugs have been using for the treatment of cancer nevertheless there is no cancer treatment that is 100% effective against cancer.[1] The chemotherapy is the most common method for the treatment of different types of cancer but it fails to cure most cancer patients with advanced disease due to the occurrence of drug resistance and several side effects such as anemia, appetite loss, diarrhea, hair loss, pain, sexual and sleep problems.[2] Therefore, there is an urgent need to give much attention to modify drug leads from the point of view of medicinal chemistry and drug design to fulfill more potent and effective therapies of cancer.

Cancer pathophysiology is characterized by many changes in a cell's genetic and metabolic composition that lead to abnormal cell growth and malignancy.[3] Apoptosis, the intrinsic death program in cells, plays a crucial role in regulating tissue homeostasis, balancing cell death and survival, and regulating cell proliferation. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly or intracellularly, and is consequently implicated in multiple biochemical events.[3] The extracellular signals that induce apoptosis include toxins, growth factors, hormones, nitric oxide, or cytokines, and these must either cross the plasma membrane or transduce effective responses from the mitochondria by inducing apoptotic pathways such as caspase-3 and caspase-8.[4] Many anticancer drugs induce apoptosis by causing DNA damage that enhances cell death signals. Therefore, the induction of apoptotic cell death is an important mechanism underlying the therapeutic properties of many anticancer drugs.[5]

Caspases are well known executioners of apoptosis. Initiator caspases; caspases -8 and -9, are activated by dimerization and autocatalytic cleavage in presence of apoptotic signals.[6] Initiator caspases (caspase 2, 8, 9 and 10) initiate the apoptosis signal by activating the executioner caspases (caspase 3, 6 and 7) that leads to apoptosis via mass proteolysis.[7] Among several substrates of the activated caspases, the DNA damage sensor poly(adenosine 5′-diphosphate-ribose) polymerase 1 *(*PARP1) is a critical protein, cleavage of which is widely used apoptotic hallmark. Caspases -3 and -7 are reported to cleave PARP1 into 89-and 24-kDa fragments during drug induced apoptosis.[8-10]

Like apoptosis, autophagy has been suggested as an important mechanism in maintaining cellular homeostasis and during development of multicellular organisms.[11] Autophagy is a homeostatic, catabolic degradation process by which the cargo is delivered to

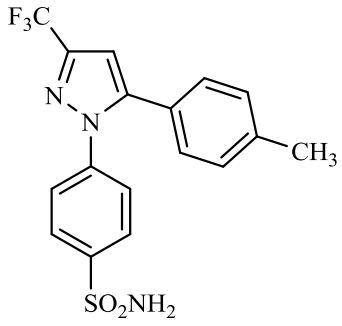
the lysosome for degradation.[11] Autophagy is essential for the removal of damaged or unwanted organelles and protein aggregates and allows recycling of cellular components to sustain proper function and metabolism of the cell. Modulating autophagy has recently emerged as a promising therapeutic approach for certain cancer types.[12]

Microtubule-associated protein light chain 3 (LC3) widely used for determination of the status of autophagy. LC3-I is conjugated with the phospholipid phosphatidylethanolamine (PE) to form LC3-II during autophagosomes formation. Although LC3-II formation is commonly used to demonstrate autophagy activation, this result may also refer to the inhibition of autophagic flux.[13] Thus, another marker namely p62, a selective substrate of autophagy, is being used in autophagy studies as the autophagy flux marker.[14,15]

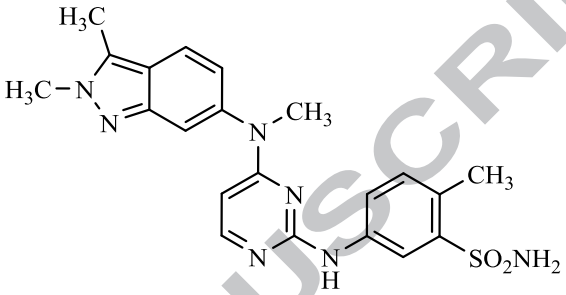
Carbonic anhydrase (CAs, EC 4.2.1.1), a zinc-containing enzyme, catalyzes the reversible reaction between CO_2 and HCO_3^-.[16] CAs are present in many organisms and have an essential role in pathological and physiological events including pH regulation, bone resorption, osteoporosis, glaucoma, cancer, and neurological disorders.[17] CA IX and XII are the two CA isoenzymes mainly found in hypoxic tumors, and are atractive targets for cancer therapy.[17,18] Among the 16 CA isoforms known in vertebrates, CA IX, a hypoxia induced, membrane-tethered enzyme, has been demonstrated to play a role in pH balance, regulation of cell proliferation, adhesion, malignant cell invasion and cancer stem cell population regulation.[19] Overexpression of CA IX specifically in hypoxic cancer cells makes it a good therapeutic target as well as a biomarker for cancer.[20] CA XII is also a membrane-bonded cancer-associated CA isoenzyme. It has been suggested that CA XII expression is upregulated under hypoxic conditions similar to CA IX, but not in all tumor types.[21] Sulfonamides bearing heterocylic and aromatic rings have been reported as attractive scaffolds for the development of new selective CA inhibitors.[18, 22-28]

Pyrazole and its derivatives have drawn considerable attention of the medicinal chemists because of their high therapeutic values.[29] Several drugs carring pyrazole ring are already in the market such as celecoxib (Figure 1), deracoxib, etoricoxib, phenazone, metamizole, phenylbutazone, sulfinpyrazone, oxyphenbutazone for the treatment of several diseases.[29] Several pyrazole/pyrazoline derivatives have been tested in terms of their antitumor[30,31], cytotoxic[18,23,24,27,28], antiangiogenic[32], proapoptotic[33] properties. They may exert their anticancer effects through inhibition of different types of enzymes, proteins, and receptors, which play important roles in cell division such as cyclin dependent kinase (CDK)[34], aurora kinase A=B=C[35], heat shock protein (HSP) 90[36], epidermal growth factor receptor (EGFR)[37], and tumor growth factor (TGF)-b type.[38]

Several sulfonamide derivatives have recently been reported with antitumor/cytotoxic activities *in vitro* and/or *in vivo.*[18,23,24,27,28,39] Therefore, there is much optimism that they may lead to novel alternative anticancer drugs, devoid of the side effects of the presently available agents.[22,39] Recently several sulfonamide derivatives such as Pazopanib[40] (Figure 1), E7010[41], and E7070[42] have been reported as potent anticancer agents and are in advanced clinical trials.



Celecoxib



Pazopanib

Figure 1. Representative compounds having pyrazole and/or sulfonamide pharmacophore/s which are in clinical use or clinical trial for the treatment of several diseases

Recently, pyrazole and its derivatives have drawn attention of our research group because of their impressive cytotoxicities and CA inhibitory effects.[18,23-28] In the light of these informations it was aimed to synthesize sulfonamides having the chemical structure of 4,4'-(5'-chloro-3'-methyl-5-aryl-3,4-dihydro-1'*H*,*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamides, the compounds **7-9** as possible drug candidates and investigate their cytotoxic/anticancer effects on several cancer and normal cell lines and CA inhibitory effects on several CA isoenzymes. Finally, it was also aimed to investigate their possible mechanism of action for their anticancer properties if they are found as cytotoxic.

2. Results and Discussion

2.1 Chemistry

The synthetic pathway which was presented at Scheme 1 was used for the synthesis of target compounds **7-9** based on the literature with some modifications.[43] The compound **1** was synthesized by the condensation of 4-hydrazinobenzenesulfonamide hydrochloride with ethylaceto acetate in ethanol. The peak which was observed at δ 2.11 ppm belonging -CH_3 group in ^{1}H NMR confirmed the chemical structure of the compound **1** while -CH_2 protons of the compound **1** hid under DMSO solvent peak. The compound **1** was reactad with DMF/$POCl_3$ under Vilsmeier-Haack condition to obtain the compound **2**. In ^{1}H NMR, peaks seen at δ 9.89 ppm (aldehyde proton), δ 8.25 ppm (-N=CH- proton), δ 3.14, 2.92, 2.43 ppm (-CH_3 protons) confirmed the chemical structure of **2**. By the Vilsmeier-Haack reaction, the

amine group on sulfonamide group was reacted with DMF. Dimethylaminomethyl group on sulfonamide group of the compound **2** was removed by reacting compound **2** with hydrochloric acid in methanol to obtain the compound **3**. At the ^{1}H NMR spectrum of the compound **3**, methyl peaks that belong to DMF moiety were not seen. Then, the compound **3** was reacted with a suitable acetophenone (4-methoxyacetophenone for **4**, 2,4-dimethoxyacetophenone for **5** and 2,4,5-trimethoxyacetophenone for **6**) under Claisen-Schmidt condensation condition. Chemical structure of the chalcones were confirmed by ^{1}H NMR spectra. Additional methoxy and aryl proton signals in the spectrum confirmed the chemical structure of the compounds **4-6**. The chalcones **4-6** were used to synthesize the target compounds **7-9** without further purification. At the final step, 4,4'-(5'-chloro-3'-methyl-5-aryl-3,4-dihydro-1'*H*,*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamides, **7-9**, were obtained by the condensation of chalcones **4-6** with 4-hydrazinobenzenesulfonamide hydrochloride in ethanol including glacial acetic acid. The chemical structures of the newly synthesized compounds **7-9** were eluciated by ^{1}H NMR, ^{13}C NMR and HRMS spectra. Results obtained by NMRs confirmed pyrazoline ring formation. The peak of the methine proton on fifth position of pyrazoline ring were seen at δ 5.45 ppm as doublet of doublet with coupling constant of 12.5 Hz and 5.5 Hz in ^{1}H NMR. On the other hand, peaks of the methylene protons on fourth position of pyrazoline ring were not seen completely since peaks at issue hidden under the signals of methoxy protons and used NMR solvent signals. ^{13}C NMR and HRMS results were also confirmed the chemical structure of the compounds **7-9**.

2.2 Cytotoxic / Anticancer Activity

The primary goal of this study was to investigate whether the compounds **7-9** had cytotoxic or anticancer properties. For this aim, cytotoxicities of the compounds **7-9** were tested on four tumor cell lines as HCC1937, MCF7, HeLa and A549 and on two non-tumor cells as MRC5 and Vero. Cytotoxicity results presented at Table 1 indicated that the compound **7** showed selectivity toward HCC1937 with an IC_{50} value of 22.39 ± 1.76 µM (Table 1). On the other hand, the compound **8** showed higher potency against breast and lung cancer cell lines. Both compounds did not affect the viability of Vero cells and had little cytotoxicity on MRC5 fibroblasts. Interestingly the compound **9** did not show any cytotoxicity at the same conditions although it has similar chemical structure.

To understand the mechanism underlying the cytotoxic effects of the compounds **7** and **8**, we investigated the critical molecular events known to regulate the apoptotic and autophagic machinery. For this purpose, HCC1937 cell line was used to investigate the mechanism of actions of the compounds **7** and **8**. In order to evaluate the levels of proteins

associated with apoptosis and autophagy in HCC1937, the cells were cultured with ½xIC_{50} and 1xIC_{50} doses of compounds for 24 h. Then the total protein levels from each treatment for autophagy and apoptotic associated protein expressions were assayed by Western blotting. Results are shown in Figure 2A and 2B. Our results indicate that both **7** and **8** induced cleavage of poly (ADP ribose) polymerase (PARP), activation of caspases -3, -7 and -9 which are the hallmarks of apoptosis. Moreover these effects found to be in a dose-dependent manner. Meanwhile both compounds induced autophagy in HCC1937 cells which is shown by enhanced expression of LC3 and decreased level of p62 protein suggesting augmentation of autophagic flux.

It is noteworthy that while the compound **7** has very specific cytotoxicity toward HCC1937, the compound **8** was cytotoxic toward HCC1937, MCF7 and A549. In addition, both compounds found to have lower cytotoxicity towards nontumoral cell lines. HCC1937 breast cancer cell line has a defective BRCA1 gene (Breast Cancer gene 1). It is defective on Homologous Recombination, a type of DNA repair mechanism. PARP inhibitors are well known for their high efficacy in cancer cells with HR deficiency, such as those with BRCA gene mutations. These cancer cells are very dependent on PARP-mediated repair for survival. Therefore, the accumulation of single strand breaks in DNA by PARP inhibitor treatment eventually leads to double strand breaks after encountering by replication forks, which would not be repaired in the absence of BRCA, resulting in cell death.[44,45] On the other hand compounds **7** and **8** induced apoptosis by the activation of predominant apoptosis executor caspases at 24-hour treatment at IC_{50} concentrations of the compounds in question. Similarly, it was demonstrated that cleaving of apoptotic marker PARP at 89 kDa increased in a concentration dependent manner in HCC1937 cell line. It was also found that both **7** and **8** increased LC3-II levels but decreased p62 levels; demonstrating their autophagy inducing effect. In conclusion these findings suggests that compounds **7** and **8** induced cell death via the activation of both autophagy and apoptosis in HCC1937 cells in a dose-dependent manner.

The solubility of the compound should be optimum between lipid and water phases for optimum bioactivity. The indicator of solubility is partition coefficient (P) or its logarithmic value logP. LogP values of the target compounds (3.24 for **7**, 3.11 for **8** and 2.98 for **9**) were calculated by ChemDraw Professional 16.0 Software (PerkinElmer Informatics, Waltham, MA, USA). When logP values of the compounds considered, it can be said there are relationship between logP and cytotoxicity. However, it can not be decide exactly whether cytotoxicity is related to logP value since the number of the compounds is limited.

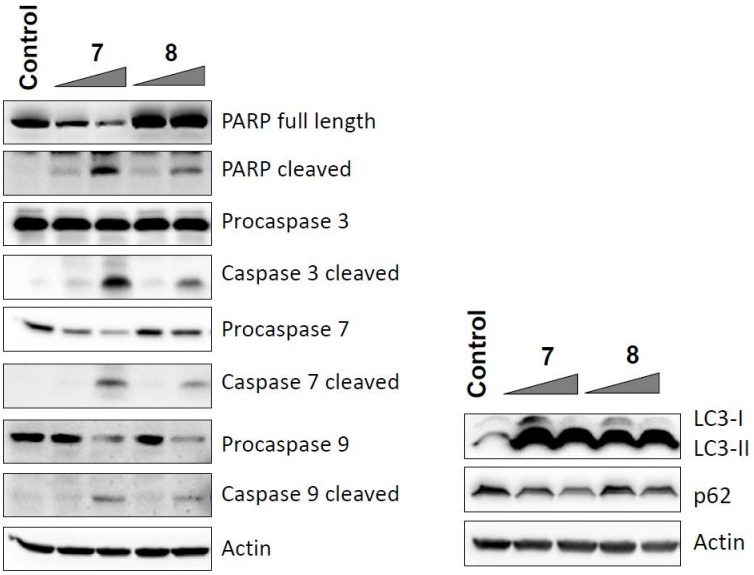


Figure 2A and 2B. The compounds **7** and **8** induced the levels of apoptosis and autophagy associated proteins. HCC1937 cells were incubated with the compounds at ½xIC_{50} and 1xIC_{50} concentrations for 24 hours. The expression levels of apoptosis related proteins (full length and cleaved PARP1; procaspases 3, 7, 9 and their active cleaved fragments (A) and autophagy related proteins (LC3 and p62) (B) were determined by immunoblotting. Actin was used as the loading control.

Table 1: IC_{50} Values (µM) for compounds **7**-**9** against different cell lines following 48 h exposure

	IC_{50} (µM)					
	Cancer cell lines				**Normal cells**	
Compound	HeLa	HCC1937	MCF7	A549	MRC5	Vero
7	79.79 ± 5.43	22.39 ± 1.76	47.25 ± 3.23	50.36 ± 3.69	45.13 ± 3.53	-
8	60.6 ± 4.36	16.9 ± 1.53	17.65 ± 2.13	23.1 ± 2.43	48.35 ± 3.08	-
9	-	-	-	-	-	-

- : Compound was not cytotoxic at the condition studied.

2.3 CA Inhibitory Effects

The compounds **7-9** were tested for their CA inhibitory properties by stopped-flow, CO_2 hydrase method against four isoforms, the cytosolic human (h) hCA I and II and the tumor-associated hCA IX and XII. The standard sulfonamide inhibitor acetazolamide (AAZ) was used as a reference drug. The following structures-activity relationship can be observed from the inhibition data of Table 2.

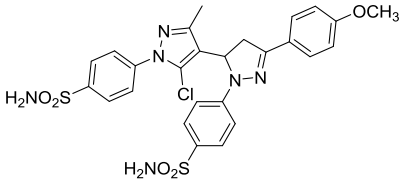
The ubiquitous cytosolic hCA I was the least inhibited among the enzymatic isoforms herein considered, in fact the compounds **7-9** showed K_Is spanning between 47.9 and 170.4 nM. It is evidence that the compound **8** which has di-methoxy group showed the best activity (K_I 47.9 nM). Concerning the inhibitory potency of the compound **8**, it was found that positions 2 and 4 on phenyl moiety prove to be crucial for the efficacy against hCA I, in fact the disubstituted compound **8** (K_I 47.9 nM) resulted 3 time more potent, when compared to the monosubstituted compound **7 (**K_I 170.4 nM**)**.

The physiologically dominant isoform hCA II was potently inhibited by dibenzenesulfonamides (**7-9**) preparated in this study. Substantial differences between the three derivatives can not be found, considering that the compounds showed similar affinities. However it could be stressed that the presence of two sulfonamide groups is indisputably the most responsible for a very strong activity reported here with their nanomolar activity of the compound **7**, **8** and **9** with K_I 4.3, 5.5 and 6.4 nM, respectively, for this isoform.

The transmembrane, tumor-associated hCA IX was effectively inhibited by all compounds herein reported, with K_I values comprised between 20.7 and 28.1 nM. As mentionated above in the other isoform hCA II, the addition of substituents, such as -OCH_3 groups, in position 2 and 5 of the phenyl moiety (compared to compound **7** which possessed only one), improved, even if only slightly, the inhibitory properties of dimethoxy-substituted compound **8** (K_I 25.4 nM) and of trimethoxy-substituted compound **9** (K_I 20.7 nM).

The other tumor-associated isoform, hCA XII, was potently inhibited by **7-9** here synthesized, with K_I that spanning between 4.5 and 9.3 nM. It is interesting the case of the trend a bit different for the compound **9,** which was the least active, with a K_I 9.3 nM, and this result is probably attributable to the presence of the three methoxy groups in 2, 4 and 5 positions of the aromatic ring. Contrary to the trend of compounds observed against hCA II and IX, this last aspect supports the hypothesis, in this case, that substitutions in *para* and *ortho* positions are favored to increase the potency against this isoform.

Table 2: Inhibition data of human CA isoforms hCA I, II, IX and XII with the compounds **7-9** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay.



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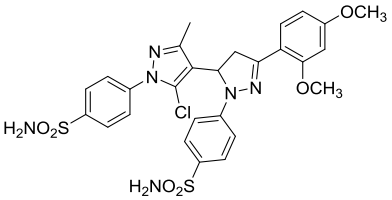

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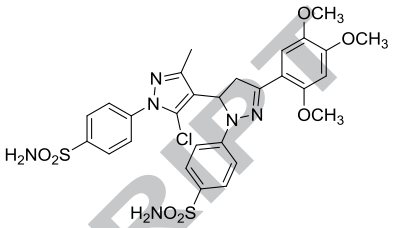

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	K_I * (nM)			
Compound	hCA I	hCA II	hCA IX	hCA XII
7	170.4	6.4	28.1	5.1
8	47.9	5.5	25.4	4.5
9	110.8	4.3	20.7	9.3
AAZ	250	12.1	25.8	5.7

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of the reported values).

3. Conclusions

Newly synthesized dibenzenesulfonamides **7-9** were reported here for the first time with their cytotoxic/anticancer properties and inhibitory effects on hCA I, II, IX and XII isoenzymes. The possible mechanism of cytotoxic action of the compounds **7** and **8** may result from their effects on critical moleculer events known to regulate the apoptosis and autophagy machinery according to results obtained. The compounds were also effectively inhibited hCA I, hCA II, hCA IX and hCA XII isoenzymes in nanomolar concentration.

4. Methods and Materials

4.1 Chemistry

The chemical structures of the final compounds were confirmed by the Nuclear Magnetic Resonance (NMR) spectra ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz) (Varian Mercury Plus spectrometer, Varian inc., Palo Alto, California, U.S.) and HRMS (Shimadzu, Kyoto, Japan). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are expressed in hertz (Hz). Mass spectra (HRMS) for the compounds were taken using a liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization modes. Shimadzu's LCMS Solution software was used for data analysis. Melting

points were determined using an Electrothermal 9100/IA9100 instrument (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. Reactions were monitored by Thin Layer Chromatography (TLC) using silicagel 60 HF254 (Merck KGaA). Dichloromethane:methanol (4.5:0.5), petrol ether:ethylacetate (1:4), ethylacetate:methanol (4.8:0.2), dichloromethane:methanol (4.8:0.2) solvent mixtures were used as TLC solvent systems. DMSO-*d6* (Merck) was used as a NMR solvent.

4.1.1 Synthesis of 4-(3-methyl-5-oxo-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide, Compound 1[43], Scheme 1

Ethylacetoacetate (17.5 g, 134 mmol) was added onto 4-hydrazinobenzenesulfonamide hydrochloride (30 g, 134 mmol) in ethanol (100 ml). The mixture was refluxed for 6 hours. The reaction process was monitored using TLC [Dichloromethane:methanol (4.5:0.5)]. After stopped the reaction, mixture was kept at room temparature for a day. The solid obtained was filtered and then washed with cold ethanol for **1**. It was crystallized from ethanol. Cream colour solid. Yield 64 %. Mp= 255-257 °C; 237-238 °C[43]. ^{1}H NMR (DMSO-d6) δ, ppm: 7.90 (d, *J*=9 Hz, 2H, ArH), 7.83 (d, *J*= 9 Hz, 2H, ArH), 7.33 (s, 2H, SO_2NH_2), 2.11(s, 3H , -CH_3) (-CH_2 protons hidden under DMSO peak).

4.1.2 Synthesis of N'-((4-(5-chloro-4-formyl-3-methyl-1*H*-pyrazol-1-yl)phenyl)sulfonyl)-N,N-dimethylformimidamide, Compound 2[43], Scheme 1

4-(3-Methyl-5-oxo-4,5-dihydro-1*H*-pyrazol-1-yl)benzenesulfonamide (20 g, 79 mmol, compound **1**) was dissolved in dimethylformamide (DMF) (200 ml) at room temparature and then phosphoryl chloride ($POCl_3$) was added slowly into the reaction flask and the mixture was heated for 23 hours at 60-70 °C. The reaction process was followed by TLC [Dichloromethane:methanol (4.5:0.5)]. Resulting mixture was poured into the cold water and the mixture was neutralized with a saturated sodium hydrogen carbonate solution to pH=7. The mixture was kept at room temparature for several hours. The solid obtained was filtered, dried, and crystallized from ethanol for **2**. Orange colour solid. 44 % Yield. Mp= 172-174 °C; 162-164 °C[43]. ^{1}H NMR (DMSO-d6) δ, ppm:9.89 (s,1H, aldehyde proton), 8,25 (s,1H, -N=CH), 7.95 (d, *J*=8,4 Hz, 2H, ArH), 7.78 (d, *J*=8,4 Hz, 2H, ArH), 3.14(s, 3H, -CH_3), 2.92 (s, 3H , -CH_3), 2,43 (s, 3H, -CH_3).

4.1.3 Synthesis of 4-(5-chloro-4-formyl-3-methyl-1*H*-pyrazol-1-yl)benzenesulfonamide, Compound 3[43], Scheme 1

The compound N'-((4-(5-chloro-4-formyl-3-methyl-1*H*-pyrazol-1-yl)phenyl)sulfonyl)-N,N-dimethylformimidamide (18.7 gr, 57.2 mmol, compound **2**) was heated in methanolic HCl solution (25% HCl in methanol) for 6 hours. The reaction process was followed by TLC [Dichloromethane:methanol (4.5:0.5)]. After kept at room temperature for a day, the precipitate formed was filtered, washed with cold water and ethanol for three times for **3**. Light brown colour solid. Yield 63%. Mp=199-202 °C; 220-222 °C[43]. ^{1}H NMR (DMSO-d6) δ, ppm: 9,90 (s, 1H, aldehyde proton), 8.00 (d, *J*=8,8 Hz, 2H, ArH), 7.84 (d, *J*=8.8 Hz, 2H, ArH), 7.55 (s, 2H, SO_2NH_2), 2.44(s, 3H, -CH_3).

4.1.4 General synthesis method of 4-(5-chloro-4-(3-(aryl)-3-oxoprop-1-en-1-yl)-3-methyl-1*H*-pyrazol-1-yl)benzenesulfonamides, Compound 4-6[43], Scheme 1

The compound 4-(5-chloro-4-formyl-3-methyl-1*H*-pyrazol-1-yl)benzenesulfonamide (2 g, 6.7 mmol, compound **3**) and suitable acetophenone [4-methoxyacetophenone (1 g, 6.7 mmol) for **4**, 2,4-dimethoxyacetophenone (1.2 gr, 6.7 mmol) for **5**, 2,4,5-trimethoxyacetophenone (1.5 g, 6.7 mmol) for **6**] were added into the cold etanolic solution of KOH (3.7 g, 66.7 mmol, in ethanol 35 ml) and the mixture was stirred for 30 min at ice cold condition. Then, the reaction flask was stirred at room temperature for additional 30 hours. The reaction process was followed by TLC [Petrol ether (40-60°C):ethylacetate (1:4) for **4**, ethylacetate: methanol (4.8:0.2) for **5**, dichloromethane:methanol (4.8:0.2) for **6**]. After the reaction was stopped, the contents of the flask was poured into ice cold water and neutralizated with HCl solution (37 %) to pH= 3. The solids obtained were filtered, washed with water, and dried. Chemical structures of the chalcone analogues **4-6** were confirmed according to ^{1}H NMR spectra results. The chalcone analogues were used to synthesize the final compounds **7-9** without further purification.

4.1.5 General synthesis method of 4,4'-(5'-chloro-3'-methyl-5-aryl-3,4-dihydro-1'*H*,*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamide, Compound 7-9[43], Scheme 1

An acidic ethanolic solution [ethanol:glacial acetic acid (15:15)] of a suitable chalcone analogue **4-6** (1 mmol) and 4-hydrazinobenzenesulfonamide hydrochloride (2.5 mmol) was refluxed for 25-45 hours. The reaction was monitored using TLC [ethyl acetate:methanol (4.8:0.2)]. After the reaction was stopped, the mixture was poured onto the cold water (100 ml). The crude compounds were filtered, and crystallized from chloroform-methanol. Spectral and phycisal data of the dibenzenesulfonamides (the compounds **7**, **8**, and **9**) were presented below.

4.1.5.1 4,4'-(5'-Chloro-5-(4-methoxyphenyl)-3'-methyl-3,4-dihydro-1'*H*,2*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamide, Compound 7[43], Scheme 1

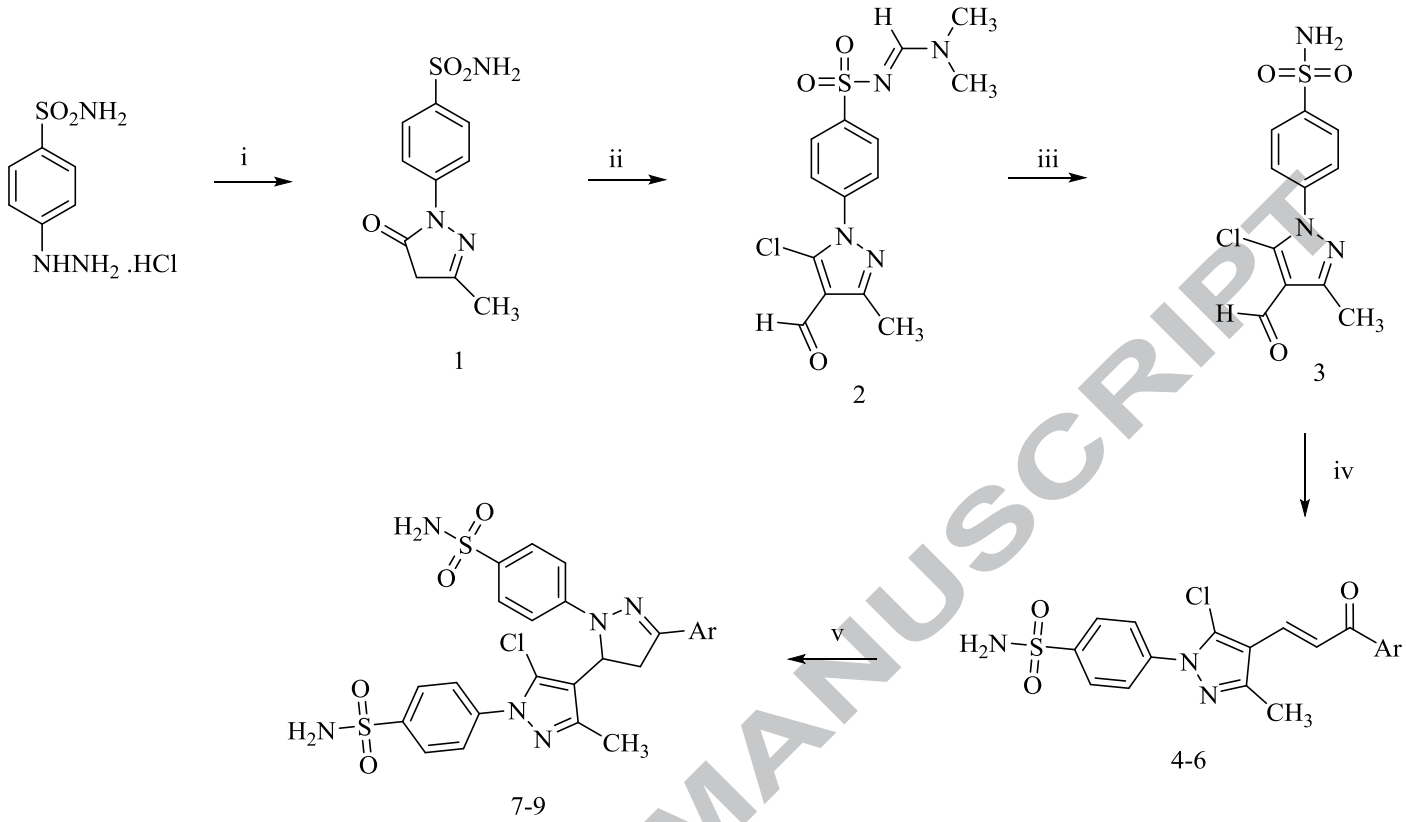
Yellow colour solid. Yield 57 %. Mp= 191-193 °C[43]. ^{1}H NMR (DMSO-d6, 400 MHz, δ, ppm) 7.94 (d, *J*= 8.8 Hz, 2H), 7.75 (d, *J*= 6.8 Hz, 2H), 7.72 (d, *J*= 6.8 Hz, 2H), 7.61 (d, *J*= 8.8 Hz, 2H), 7.51 (s, 2H), 7.04-6.99 (m, 6H), 5. 45 (dd, *J*= 12.5, 5.5 Hz, 1H, pyrazoline), 3.77 (s, 3H, -OCH_3), 2.05 (s, 3H, -CH_3). Another 2 peaks of protons on pyrazoline ring was hidden under DMSO-d6 peak. ^{13}C NMR (DMSO-d6, 100 MHz, δ, ppm) 160.1, 150.7, 148.7, 146.6, 143.9, 140.4, 133.4, 128.4, 127.9, 127.6, 125.6, 125.4, 124.7, 118.4, 114.9, 112.2, 55.9, 54.0, 40.5, 13.4. HRMS Calculated $[M+H]^+$ 601.1089; found $[M+H]^+$ 601.1063

4.1.5.2 4,4'-(5'-Chloro-5-(2,4-dimethoxyphenyl)-3'-methyl-3,4-dihydro-1'*H*,2*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamide, Compound 8, Scheme 1

Light green colour solid. Yield 9%. Mp=178-180 °C. ^{1}H NMR (DMSO-d6, 400 MHz, δ, ppm) 7.92 (d, *J*= 8.4 Hz, 2H), 7.82 (d, *J*= 8.3 Hz, 1H), 7.54 (d, *J*=8.4 Hz, 2H), 7.58 (d, *J*=8.8 Hz, 2H), 7.50 (s, 2H), 7.01 (s, 2H), 6.97 (d, *J*= 8.8 Hz, 2H), 6.61 (d, *J*= 8.3, 1.8 Hz, 2H), 5.45 (dd, *J*= 12.5, 5.5 Hz, 1H), 3.79 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3), 2.06 (s, 3H, -CH_3). Another 2 peaks of protons on pyrazoline ring was hidden under DMSO-d6 peak. ^{13}C NMR (DMSO-d6, 100 MHz, δ, ppm) 153.6, 152.0, 149.8, 148.7, 146.6, 143.9, 143.6, 140.4, 133.3, 127.9, 127.5, 125.4, 118.7, 112.2, 111.8, 99.2, 56.4, 56.1, 53.7, 40.5, 13.5. HRMS Calculated $[M+H]^+$ 631.1195; found $[M+H]^+$ 631.1199.

4.1.5.3 4,4'-(5'-Chloro-3'-methyl-5-(2,4,5-trimethoxyphenyl)-3,4-dihydro-1'*H*,2*H*-[3,4'-bipyrazole]-1',2-diyl)dibenzenesulfonamide, Compound 9 , Scheme 1

Yellow colour solid. Yield 12 %. Mp=184-185 °C. ^{1}H NMR (DMSO-d6, 400 MHz, δ, ppm) 7.93 (d, *J*= 8.8 Hz, 2H), 7.74 (d, *J*= 8.8 Hz, 2H), 7.58 (d, *J*= 9.1 Hz, 2H), 7.50 (s, 2H), 7.44 (s, 1H), 7.03- 6.98 (m, 4H), 6.72 (s, 1H), 5.45 (dd, *J*= 12.4, 5.9 Hz, 1H), 3.81 (s, 3H, -OCH_3), 3.79 (s, 3H, -OCH_3), 3.76 (s, 3H, - OCH_3), 2.06 (s, 3H, -CH_3). Another 2 peaks of protons on pyrazoline ring was hidden under DMSO-d6 peak. ^{13}C NMR (DMSO-d6, 100 MHz, δ, ppm) 162.5, 159.6, 149.9, 148.7, 146.6, 143.9, 140.4, 133.2, 130.2, 127.9, 127.5, 125.4, 118.6, 113.7, 112.1, 106.9, 99.4, 57.3, 56.9, 56.4, 53.9, 40.5, 13.5. HRMS Calculated $[M+H]^+$ 661.1300; found $[M+H]^+$ 661.1324.



Reagent and conditions. (i) Ethylacetoacetate, ethanol, reflux (ii) Dimethylformamide (DMF), phosphorus oxychloride ($POCl_3$), heat (iii) HCl solution (25% in methanol), reflux (iv) KOH solution in ethanol, a suitable acetophenone (4-methoxyacetophenone for **4**, 2,4-dimethoxyacetophenone for **5**, 2,4,5-trimethoxyacetophenone for **6**) (v) 4-Hydrazinobenzenesulfonamide.HCl, ethanol, glacial acetic acid.

Scheme 1. Synthetic pathway for the compounds **7-9**

4.2 Biological Activity

4.2.1 Cell Culture

Human breast cancer cell lines HCC1937 and MCF7, human endometrial carcinoma HeLa, human lung carcinoma A549, human lung fibroblasts MRC5 and African green monkey kidney cell line Vero were obtained from American Type Culture Collection and maintained as exponentially growing monolayers by culturing according to the supplier's instructions.

4.2.2 Cytotoxicity Test

The cytotoxic potencies of test compounds were determined by using WST1 Cell Protliferation Assay (Roche) according to the manufacturer's instructions. All tested compounds were dissolved in DMSO. Cells were seeded into 96-well plate at a density of 5000 cells/well for HeLa, MCF7, Vero, A549, HCC1937 and 10000 cells per well for MRC5. Next day, cells were treated with compounds with the final concentrations of 1, 2, 5, 10, 25 μM and incubated for 48 hours at conventional cell culture conditions. DMSO was used negative solvent control. The ratio of surviving cells after compound treatment was determined by the colorimetric WST-1 assay (Roche) as indicated in the protocol provided by manufacturer. The absorbance was measured by using Varioscan microplate reader (Thermo) at at 450 nm and a 620-nm reference filter. To determine the IC_{50} values, a sigmoid-dose response curve was fitted to the data using nonlinear regression in GraphPad Prism 5 software.

4.2.3 Immunoblotting

HCC1937 cells, incubated with the respective conditions, were harvested and lysed. An equal amount of protein was separated by SDS-PAGE (5–15%) and transferred to PVDF membranes. The blotted membranes were blocked with 5% skim milk for 1 h and then incubated with the desired primary antibodies at 37°C for 1 h. Mouse monoclonal antibodies used in this study included anti-actin (Sigma), anti-caspase 9 (Cell Signaling) and anti-cleaved caspase 9 (Cell Signaling). The rabbit polyclonal antibodies used were anti-PARP-1, anti caspase 3, anti-caspase 7, anti-LC3 and anti-p62 (Cell Signaling). Then the immunoreactive bands were visualised by enhanced chemiluminescence using horseradish peroxidase-conjugated IgG secondary antibodies (Thermo). To quantify equal loading, membrane was reprobed with β-actin antibody. Chemiluminescence signals were detected using Clarity ECL substrate solution (BIORAD) by Fusion-FX7 (Vilber Lourmat).

4.2.4 Carbonic Anhydrase I, II, IX, and XII Inhibition Studies

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration reaction. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH=7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100s. 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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E –I complex. The inhibition percantage were obtained by using PRISM 3, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier. [18,23,27,46-50]

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Declaration of Interest

The authors declare that they have no competing interests.

Figure Legends and Tables

Figure 1. Representative compounds having pyrazole and/or sulfonamide pharmacophore/s which are in clinical use or clinical trial for the treatment of several diseases

Figure 2A and 2B. The compounds **7** and **8** induced the levels of apoptosis and autophagy associated proteins. HCC1937 cells were incubated with the compounds at ½xIC_{50} and 1xIC_{50} concentrations for 24 hours. The expression levels of apoptosis related proteins (full length and cleaved PARP1; procaspases 3, 7, 9 and their active cleaved fragments (A) and autophagy related proteins (LC3 and p62) (B) were determined by immunoblotting. Actin was used as the loading control.

Scheme 1. Synthetic pathway for the compounds **7-9**

Table 1: IC_{50} Values (μM) for compounds **7-9** against different cell lines following 48 h exposure

Table 2: Inhibition data of human CA isoforms hCA I, II, IX and XII with the compounds **7-9** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay.

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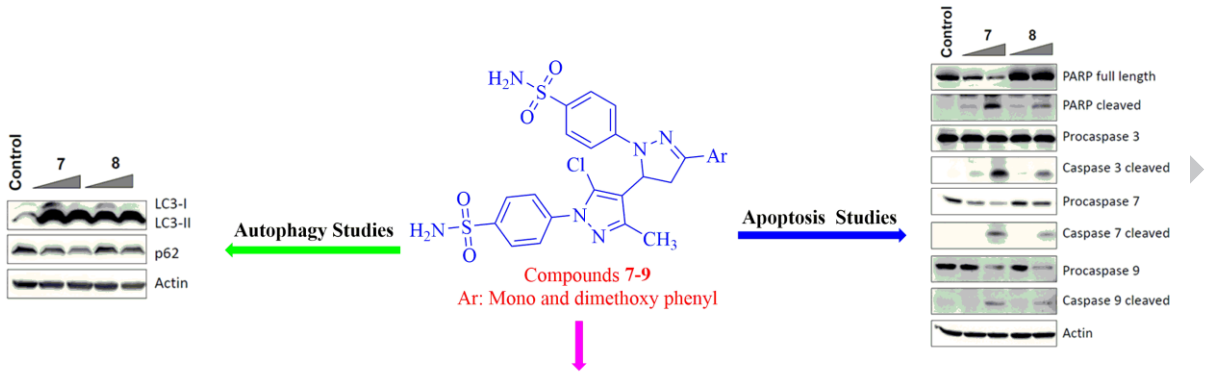
Graphical abstract



Tumor-associated hCA IX and XII isoenzymes inhibition studies

	K_I (nM)	
	hCA IX	hCA XII
7	28.1	5.1
8	25.4	4.5
9	20.7	9.3
AAZ	25.8	5.7

Highlights

The compounds **7** and **8** induced cleavage of poly (ADP ribose) polymerase (PARP).

The compounds **7** and **8** induced autophagy in HCC1937 cells.

The compounds were effectively inhibited hCA IX and hCA XII isoenzymes.