European Journal of Medicinal Chemistry 96 (2015) 425–435

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Synthesis, carbonic anhydrase inhibition and cytotoxic activity of novel chromone-based sulfonamide derivatives

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ARTICLE INFO

Article history:
Received 2 January 2015
Received in revised form
31 March 2015
Accepted 14 April 2015
Available online 15 April 2015

Keywords:
Chromone
Sulfonamide
Breast cancer
Lung cancer
Carbonic anhydrase

ABSTRACT

Four series of sulfonamides incorporating chromone moieties were synthesized and assessed for their cytotoxic activity against MCF-7 and A-549 cell lines, considering the fact that some of these tumors overexpress isoforms of carbonic anhydrase (CA, EC 4.2.1.1) which is inhibited by sulfonamides. Most new sulfonamides showed weak inhibitory activity against the offtarget, cytosolic isoforms hCA I, II but effectively inhibited the tumor-associated hCA IX and XII. The most active compounds featured a primary SO_2NH_2 group and were active in the low micromolar range against MCF-7 and A-549 cell lines. Compound **4a** showed IC_{50} of 0.72 and 0.50 μM against MCF-7 and A-549 cell lines, respectively, and was further evaluated for its proapoptotic activity which proved enhanced in both tumor types.

1. Introduction

Chromones are naturally occurring oxygen containing heterocyclic compounds that are widely distributed in plant kingdom and form the basic nucleus of important compounds such as anthocyanin and flavonoids. Chromones are also well known for their antimicrobial [1,2], antitumor [3,4], and antiviral [5,6] activities. Chromone-3-carboxaldehyde are important synthons in the synthesis of chromone-based bioactive molecules [7,8]. They are widely used due to their ease of preparation and modification via Vilsmeier Haack reaction [9,10].

The clinical and medicinal importance of sulfonamides is well documented. The sulfonamide moiety (−SO_2NH−R) is an active pharmacophore, exhibiting a wide variety of pharmacological activities such as antimicrobial, antimalarial, insulin-releasing antidiabetic, anti-HIV, high ceiling diuretic, antithyroid, and antitumor activities [11–15]. Among the broad spectrum of activities elicited by sulfonamides is their role as inhibitors of the zinc containing metalloenzyme carbonic anhydrase (CA). Many sulfonamide CA inhibitors have been used as therapeutic agents against various diseases including glaucoma, gastro-duodenal ulcers, acid–base disequilibria, and various neurological disorders [16–19].

Carbonic anhydrases (CAs, EC 4.2.1.1) are a group of metalloenzymes that are involved in pH buffering of extra- and intracellular spaces by catalyzing the reversible hydration of carbon dioxide (cellular waste product) to bicarbonate and a proton [20–25]. This family of enzymes comprises six genetic families, i.e., α, β, γ, δ, ζ and η-classes. Among the 16 known human (h) CA isoforms belonging to class α, the human cytosolic isoforms hCA I and hCA II are ubiquitous in the body and are targets for anticonvulsant, diuretic and anti-glaucoma drugs. On the other hand, the transmembrane isoforms hCA IX and hCA XII, possessing an extracellular active site, are associated with some types of cancers being induced by tumor hypoxia. This being the case, these two isoforms justifiably represent good candidates for antitumor drug design, with one sulfonamide CA IX/XII inhibitor currently in Phase I clinical trials for the treatment of primary tumors/metastases overexpressing these enzymes [26–28].

In addition to the aforementioned facts, literature revealed combined chromone-sulfonamides hybrids as potent antitumor and CA inhibitors as exemplified by compound **3a** and **I** which featured a chromone nucleus linked to a sulfonamide moiety either

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http://dx.doi.org/10.1016/j.ejmech.2015.04.033
0223-5234/

through a methine (=CH–) group [28]; or through a heterocyclic pyrimidine spacer [29], respectively (Fig. 1). Accordingly, with the goal of producing new antitumor agents, a hybrid pharmacophoric approach was adopted in the design of the target compounds in which the chromone and an appropriately *N*-(un)substitutedsulfonamide moieties were combined via a methine spacer (General formula A) in one molecule. Further investigation was performed by including the reduced methyleneamino (–CH_2NH–) linker instead of the methineamino one (=CH–NH–) in order to impart structure flexibility (General formula B). Another group of target compounds were designed in such a way to separate the chromone and the sulfonamide pharmacophores through incorporation of a heterocyclic spacer. Searching for suitable heterocyclic rings of antitumor importance unveiled the potential activity of the thiazolinone [30–32] and its isosteric imidazolinone moieties [33,34]. Consequently, and motivated by the positive biological activities of many derivatives incorporating these heterocyclic rings, compounds of general formulas C and D were synthesized (Fig. 2). The new compounds were screened in vitro against two cell lines: breast MCF-7 and lung A-549. Furthermore, the derivatives that showed remarkable activity profile were tested for their CA inhibitory activity.

2. Discussion

2.1. Chemistry

The key starting compound, chromone-3-carboxaldehyde **2**, was prepared from 2-hydroxyacetophenone **1**, dimethylformamide (DMF) and phosphorous oxychloride according to the Vilsmeier–Haack reaction [35,36]. The first series of target compounds **3a**–**f** was synthesized via reacting **2** with the appropriate sulfonamide in ethanol in the presence of catalytic amount of glacial acetic acid. The expected Schiff's base derivatives were not obtained, but rather the enamine adducts **3a**–**f** were afforded. This could be explained on the basis of the presence of a very reactive electron deficient center at C2 of the chromone nucleus which facilitated the nucleophilic attack of an ethanol molecule (solvent) that underwent addition on the C2–C3 double bond. In addition, it was observed that performing the reaction in different solvents such as methanol or n-butanol resulted in the 2-methoxy and 2-n-butyloxy derivatives, respectively, as confirmed by spectral data. Moreover, literature provided similar explanation for this unexpected reaction [37–39]. Trials to carry out the reaction using acetic acid as a solvent or aprotic solvents such as toluene or benzene gave a mixture of two products that were difficult to be separated. The major product consisted of an enamine adduct in which a second molecule of the sulfonamide acted as nucleophile that underwent addition to the C2–C3 olefinic bond of the chromone ring. The minor product was the expected Schiff's base. Similar unexpected reactions were also reported in literature [1,40] (Scheme 1).

The second series of target compounds was obtained by reducing the enamine adducts **3a**–**f** with sodium borohydride in ethanol which furnished the methylene-amino derivatives **4a**–**f** together with the elimination of the ethoxy group at C2 of the chromone ring (Scheme 1).

The chromone-sulfonamide hybrids incorporating a thiazolinone spacer **7a**–**f** were obtained by reacting the chromone-3-carboxaldehyde **2** with the appropriate thiazolinone-bearing sulfonamide derivatives **6a**–**f**. This was achieved by reaction of the appropriate sulfonamide with chloroacetyl chloride in DMF according to the literature procedures to give the N^4-chloroacetamides **5a**–**f** [41–44]. The latter were reacted with ammonium thiocyanate to undergo intramolecular cyclization with elimination of ammonium chloride to produce the corresponding thiazolinone intermediates **6a**–**f**. Knoevenagel condensation of the aldehyde **2** with the active methylene protons of the intermediates **6a**–**f** was performed in glacial acetic acid in the presence of anhydrous sodium acetate to produce the third group of target compounds **7a**–**f** (Scheme 2).

Finally, the last series of compounds comprising the 5-oxo-imidazolyl-benzenesulfonamides **10a**–**f** and **11a**–**f** were prepared via reaction of the appropriate oxazolone **9a**,**b** with the appropriate sulfonamide as illustrated in Scheme 3. The intermediates **9a**,**b** were obtained by benzoylation of glycine with the appropriate benzoyl chloride in sodium hydroxide solution (10%) to afford **8a**,**b** [45,46], followed by Erlenmeyer cyclocondensation reaction with the aldehyde **2** in acetic anhydride in the presence of anhydrous sodium acetate [47]. The target imidazolinones **10a**–**f** and **11a**–**f** were obtained by the reaction of the corresponding **9a**,**b** with the appropriate sulfonamide in glacial acetic acid and anhydrous sodium acetate.

2.2. In vitro cytotoxic activity

All target compounds were screened in vitro for their cytotoxic activity against breast (MCF-7) and lung (A-549) cells using the sulforhodamine B (SRB) assay as described by Skehan et al. [48]. IC_{50} values (μM) were calculated from the mean value of three separate experiments. Doxorubicin (DOX) was used as a positive control. Results were listed in Table 1. Concerning the activity against the breast cell line, most compounds of the first series, **3a**–**f**, showed higher activity than doxorubicin (IC_{50} = 22.19–30.06 μM) except compounds **3d** and **3e**. The reduced derivatives **4a**–**f** demonstrated a decrease in activity compared to compounds **3a**–**f**, except for compound **4a** that exhibited a highly potent activity in a submicromolar value (IC_{50} = 0.72 μM). Substitution of the aminomethine or methylamino spacer by thiazolinone or imidazolinone moieties as in compounds **7a**–**f**, **10a**–**f** and **11a**–**f**, respectively, was not in favor of activity except for the N^4-unsubstituted-sulfonamide derivatives **7a**, **10a** and **11a** which showed increase in activity (IC_{50} = 8.91, 11.50 and 24.31 μM, respectively).

As per the activity of the tested compounds against the lung A-549 cell line, all compounds were less potent than doxorubicin except for compounds **4a**, **7a**, **10a** and **11a** (IC_{50} = 0.50, 2.23, 1.73 and 13.77 μM, respectively). Compound **4e** was almost equipotent to doxorubicin (IC_{50} = 25.59 μM).

Regarding the effect of the N^4-substitution on the sulfonamide

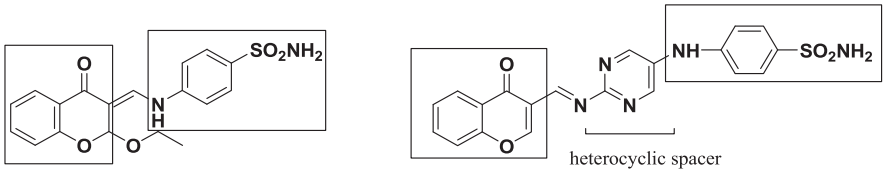


Fig. 1. Examples of chromone-sulfonamide hybrids as antitumor and CA inhibitors.

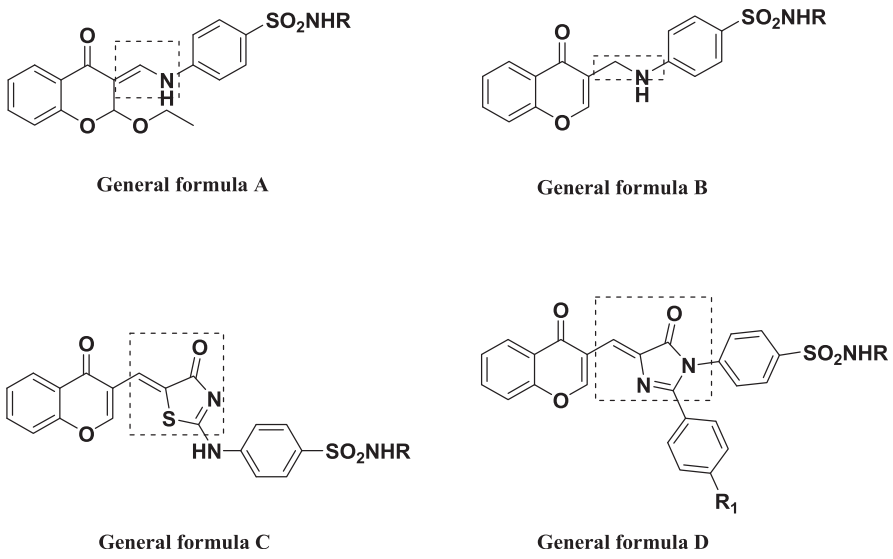
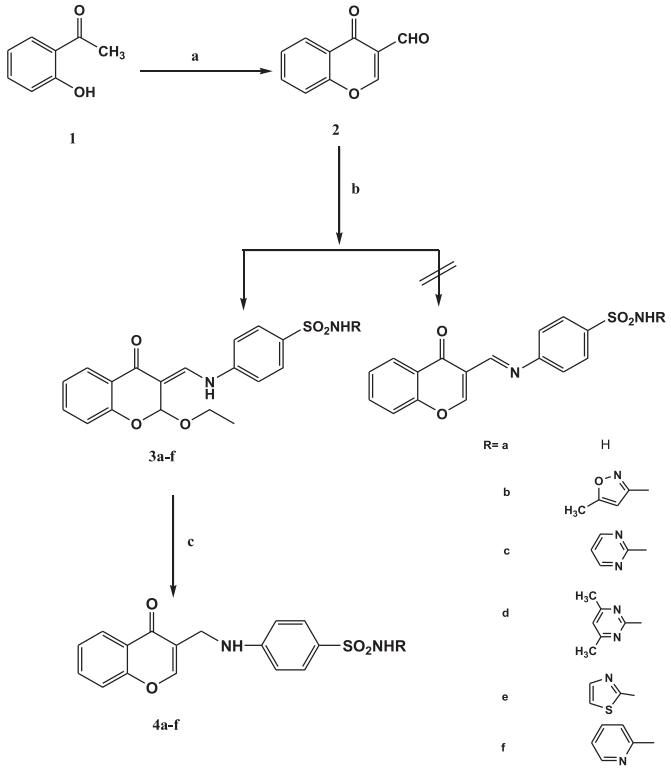


Fig. 2. General formulas of target compounds.

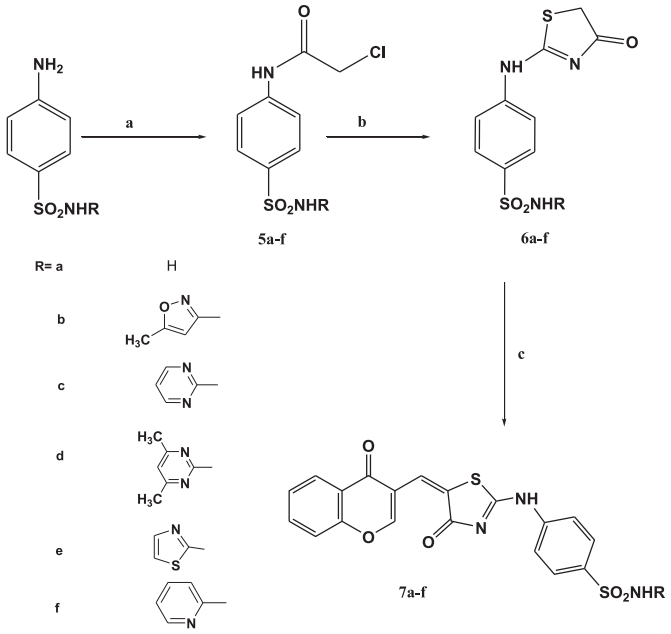
moiety, it could be clearly observed that the unsubstituted derivatives were the most potent within all series. Compound **4a** represented a promising candidate molecule being active against both cell lines in the submicromolar range.

2.3. Carbonic anhydrase inhibition

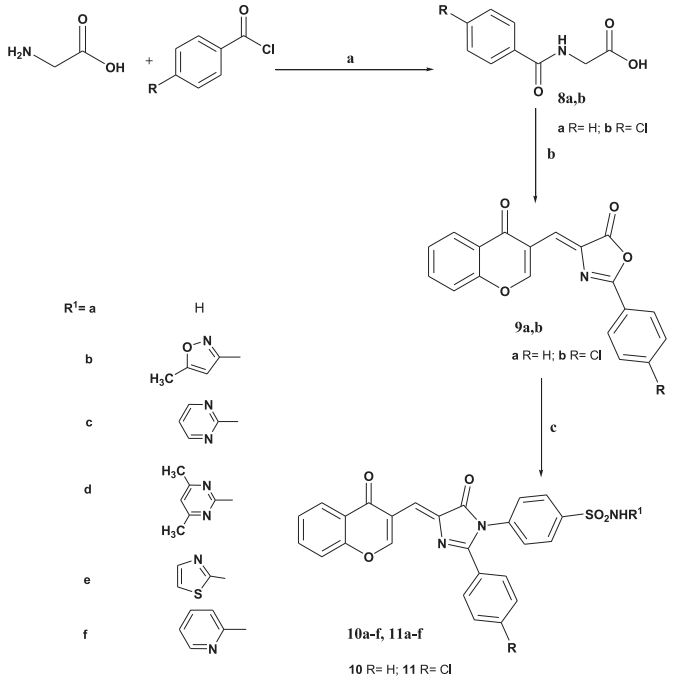
The CA inhibitory ability of some selected sulfonamides that demonstrated good activity profile in the in vitro antitumor assay was assessed against the cytosolic isoforms hCA I and II as well as the membrane associated isoforms hCA IX and XII employing a stopped flow assay method [49] and the results are shown in Table 2. The well known CA inhibitor acetazolamide (AZA) was used as the reference drug for this assay. Data obtained from the CA inhibition assay revealed that the tested compounds were selective to the tumor associated isoforms hCA IX and hCA XII with activity in the nanomolar range. Very weak or no activity at all was observed against hCA I and II, except for compound **7a** (hCA II $K_i = 12.95$ nM). This selectivity could be considered a desirable attribute for cytotoxic agents to guard against the occurrence of untoward side effects. The hCA IX isoform was highly inhibited by compounds **3b**, **3e**, **3f**, **7a**, **10a** and **11a** ($K_i = 14.26–23.84$ nM) surpassing the activity of AZA. The rest of the compounds were either equipotent or



Scheme 1. Reagents and reaction conditions. a: Dry DMF, $POCl_3$, overnight; b: appropriate sulfonamide, abs. ethanol, drops of gl.acetic acid, reflux, 3.5 h; c: $NaBH_4$, abs. ethanol, RT, 24 h.



Scheme 2. Reagents and reaction conditions. a: chloroacetyl chloride, DMF, RT, 2–4 h; b: NH_4SCN, ethanol, reflux 3–12 h; c: compound **2**, gl.acetic acid, CH_3COONa anh., boiling water bath 3–12 h.



Scheme 3. Reagents and reaction conditions. a: NaOH solution (10%), stir, 2 h; b: compound **2**, acetic anhydride, CH_3COONa anh., boiling water bath, 5 h; c: appropriate sulfonamide, gl.acetic acid, CH_3COONa anh., boiling water bath, 8–24 h.

slightly less potent than AZA. With regards to the profiling assay against hCA XII, only two compounds, **4a** and **10a**, displayed slightly higher activity than AZA (K_i = 4.54 and 5.23 nM, respectively).

Table 1
IC_{50} values ± SEM of the new compounds against MCF-7 and A-549 cell lines.

Compound	IC_{50} (μM) ± SEM MCF-7	IC_{50} (μM) ± SEM A-549
3a	22.19 ± 1.01	33.39 ± 6.74
3b	25.80 ± 0.58	57.23 ± 0.63
3c	30.06 ± 1.13	>100
3d	45.62 ± 1.56	36.35 ± 1.70
3e	48.61 ± 4.10	>100
3f	28.81 ± 1.57	39.05 ± 1.13
4a	0.72 ± 0.03	0.50 ± 0.02
4b	34.50 ± 1.64	59.66 ± 0.29
4c	>100	85.25 ± 4.24
4d	93.57 ± 5.71	55.83 ± 2.73
4e	58.61 ± 4.33	25.59 ± 2.70
4f	>100	>100
7a	8.91 ± 1.08	2.23 ± 55.91
7b	>100	>100
7c	82.60 ± 7.20	>100
7d	>100	>100
7e	>100	>100
7f	47.10 ± 3.17	>100
10a	11.50 ± 1.19	1.73 ± 40.36
10b	30.01 ± 1.40	>100
10c	>100	40.47 ± 1.84
10d	99.71 ± 5.90	63.21 ± 2.78
10e	88.48 ± 8.25	88.40 ± 3.02
10f	>100	>100
11a	24.31 ± 2.83	13.77 ± 2.08
11b	>100	>100
11c	>100	59.03 ± 7.14
11d	93.92 ± 5.49	52.61 ± 2.47
11e	>100	>100
11f	>100	>100
DOX	33.13 ± 2.90	26.81 ± 2.50

Table 2
K_i values of tested compounds on hCA I, II, IX and XII.

Compound	K_i (nM)[a]			
	hCA I	hCA II	hCA IX	hCA XII
3b	>10,000	>10,000	23.84	424.04
3d	>10,000	>10,000	37.62	79.35
3e	>10,000	>10,000	19.91	22.88
3f	>10,000	>10,000	20.93	62.02
4a	965.03	370.02	29.75	4.54
4b	>10,000	>10,000	32.09	10.76
7a	398.78	12.95	14.26	9.91
7c	>10,000	>10,000	24.74	85.83
7f	>10,000	1064.56	25.58	546.55
10a	8003.15	75.22	23.04	5.23
10b	>10,000	1112.0	25.24	9.71
11a	>10,000	722.05	18.39	6.24
AZA	250.00	12.00	25.00	5.70

AZA (Acetazolamide), a well-known CAI, was used as a standard for comparison.
[a] Mean from 3 different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).

Compounds of moderate potency against hCA XII were **4b**, **7a**, **10b** and **11a** (K_i = 6.24–10.76 nM). Also, it could be noted that the most active compounds on hCA IX (**7a** and **11a**) and hCA XII (**4a** and **10a**) were those derivatives whose sulfonamide pharmacophore possessed a free sulfamoyl group.

2.4. Apoptosis study

To examine the involvement of apoptosis in the cytotoxic activity of the new sulfonamide derivatives, MCF-7 and A-549 cells were incubated with the most potent compound **4a** at its IC_{50} concentration for 24 h. The percentage of apoptotic cells were determined by Annexin V-FITC/PI staining and the stained cells were detected by flow cytometry (Fig. 3). The results showed that **4a** induced apoptosis in MCF-7 cells where the percentage of early apoptotic cells reached 94.12%; whereas, the percentage of early apoptotic cells attained 74.77% in A-549 cells.

3. Conclusion

Four series of new chromone-based sulfonamide derivatives were synthesized and tested for their in vitro cytotoxic activity against breast (MCF-7) and lung (A-549) cell lines. Compared to doxorubicin, the tested against breast cancer cell lines than lung cancer cell lines. Compounds in which the chromone and sulfonamide phrmacophores were separated by a small spacer group such as = CH–N– or $-CH_2NH-$, **3a**–**f** and **4a**–**f**, respectively, were more potent than those more widely separated by a heterocyclic thiazolinone or imidazolinone rings, **7a**–**f**, **10a**–**f** and **11a**–f, respectively. Also, it was noted that derivatives possessing a free sulfamoyl group, **4a**, **7a**, **10a** and **11a** were the most potent. Compounds showing high activity profile in the in vitro cell line screening were further evaluated for their carbonic anhydrase inhibitory activity against hCA I, II, IX and XII isoforms. The profiling enzyme assay revealed that the tested compounds were selective to the tumor associated isoforms hCA IX and XII in the nanomolar range, and that the compounds that exhibited activity higher than that expressed by acetazolamide were those having a free sulfamoyl group. Finally, compound **4a**, which possessed the highest activity against the tested cell lines, was further investigated for its proapoptotic activity where induction of apoptosis was markedly observed in both cancer cell types. In conclusion, it could be claimed that compound **4a**, denoting IC_{50} of 0.72 and 0.50 μM against MCF-7 and A-549 cells; and a K_i of 29.75 and 4.54 nM on

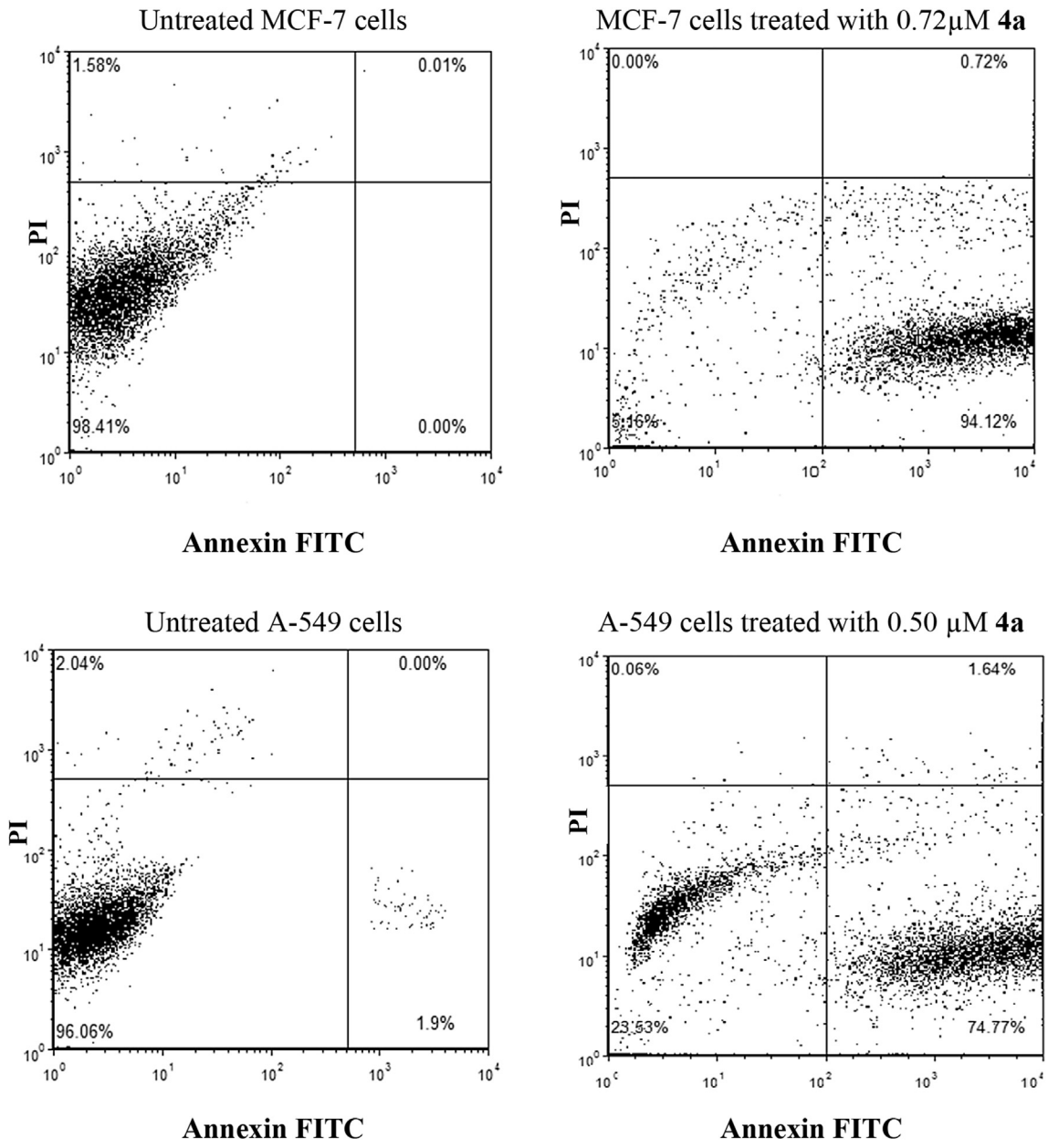


Fig. 3. Flow cytometry histograms of MCF-7 and A-549 cells after 24 h treatment with **4a**.

hCA IX and XII; and which raised the % of early apoptotic cells to 94.12 and 74.77% in breast and lung cells, respectively, could be a promising lead for future investigation.

4. Experimental

4.1. Chemistry

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. TLC was monitored on FLUKA silica gel TLC aluminum cards (0.2 mm thickness) with fluorescent indicator 254 nm using chloroform: methanol (9.5:0.5) as eluent. Melting points were performed on Stuart SMP3 version 5 digital melting point apparatus and were uncorrected. Elemental microanalyses were performed at the Microanalytical Center, at the Regional Center for Mycology and Biotechnology, Al-Azhar University. ^{1}H NMR spectra were measured in dimethylsulphoxide (DMSO-d_6) on a Varian Mercury VX-300 MHz spectrophotometer or Bruker AVANCE III Nano Bay 400 MHz FT-NMR spectrophotometer. Chemical Shifts were reported in δ as parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. ^{13}C NMR spectra were recorded on Bruker AVANCE III Nano Bay 400 MHz FT-NMR spectrophotometer at 100 MHz. Mass spectra were performed on Fennigan MAT, SSQ 7000 mass spectrophotometer at 70 eV. Infrared Spectra were recorded on Schimadzu FT-IR 8400S spectrophotometer (Shimadzu, Kyoto, Japan), and expressed in wave number (cm^{-1}), using potassium bromide discs.

Compounds **2** [35,36], **3a** [28], **3c** [39], **5a**–**f** [40–43], **6a** [50], **6f** [51], **8a**,**b** [44,45], **9a**,**b** [46] were prepared according to the reported procedures.

4.1.1. General method for the synthesis of compounds (*3a–f*)

A solution of chromone-3-carboxaldehyde **2** (1.74 g, 10 mmol), the appropriate sulfonamide (10 mmol) and glacial acetic acid (0.5 mL) in absolute ethanol (10 mL) was refluxed for 3.5 h and kept

overnight at room temperature. The separated solid was filtered off and crystallized from a mixture of ethanol:acetone mixture (1:1).

*4.1.1.1. 4-(((2-Ethoxy-4-oxochroman-3-ylidene)methyl)amino)benzenesulfonamide (**3a**).* (Yield: 85%, m.p. 158–160 °C as reported) [28].

*4.1.1.2. 4-(((2-Ethoxy-4-oxochroman-3-ylidene)methyl)amino)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (**3b**).* Yield: 88%; m.p. 208–211 °C. IR (cm^{-1}): 3365 (NH), 3061 (aromatic CH), 2974 (aliphatic CH), 1649 (C=O), 1284(SO_2^{asym}), 1165 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 1.07 (t, 3H, J = 7.0 Hz, $CH_2\underline{CH_3}$), 2.30 (s, 3H, CH_3), 3.81 (q, 2H, J = 7.0 Hz, $\underline{CH_2}CH_3$), 5.94 (s, 1H, CH-2 chromanone), 6.15 (s, 1H, CH, isoxazole), 6.69 (d, 2H, J = 8.7 Hz, CH-3, CH-5 of phenyl), 7.08 (d, 1H, J = 8.2 Hz, CH-8 chromanone), 7.13 (t, 1H, J = 8.5 Hz, CH-6 chromanone), 7.51 (d, 2H, J = 8.7 Hz, CH-2, CH-6 of phenyl), 7.58 (d, 1H, J = 8.8 Hz, CH-5 chromanone), 7.86 (t, 1H, J = 8.7 Hz, CH-7 chromanone), 8.12 (d, 1H, J = 12.0, CH enamine), 11.35 (s, 1H, SO_2NH, exchanged by D_2O), 11.73 (d, 1H, J = 12.0, NH, exchanged with D_2O). ^{13}C NMR δ: 12.4 (CH_3 of isoxazole ring), 15.3 (CH_3, $-OCH_2\underline{CH_3}$), 63.6 (CH_2, $-O\underline{CH_2}CH_3$), 95.6 (C-4 of isoxazole), 100.2 (C-2 of chromanone), 105.8 (C-3+C-5 of phenyl), 113.0 (C-8 of chromanone), 117.0 (C-3 of chromanone), 120.4, 122.5, 124.4, 125.7, 126.2, 127.2, 129.2, 133.7, 135.5, 136.7 (aromatic Cs), 153.5 (C-3 of isoxazole), 157.8 (C-8a of chromanone), 170.5 (C-5 of isoxazole), 181.3 (C-4 of chromanone). EI-MS m/z (%): 455.38 (M^+, 22.2%). Anal. Calcd. for $C_{22}H_{21}N_3O_6S$ (455.48): C, 58.01; H, 4.65; N, 9.23. Found: C, 58.03; H, 4.67; N, 9.29.

*4.1.1.3. 4-(((2-Ethoxy-4-oxochroman-3-ylidene)methyl)amino)-N-(pyrimidin-2-yl)benzenesulfonamide (**3c**).* (Yield: 73%, m.p. 244–245 °C as reported) [39].

*4.1.1.4. N-(4,6-Dimethylpyrimidin-2-yl)-4-(((2-ethoxy-4-oxochroman-3-ylidene)methyl)amino)benzenesulfonamide (**3d**).* Yield: 75%; m.p. 288–290. IR (cm^{-1}): 3230 (NH), 3082 (aromatic CH), 2974 (aliphatic CH), 1651(C=O), 1280(SO_2^{asym}), 1155 (SO_2^{sym}). ^1H NMR (400 MHz) δ:1.08 (t, 3H, J = 7.2 Hz, $CH_2\underline{CH_3}$), 2.26 (s, 6H, $2CH_3$), 3.67 (q, 2H, J = 7.2 Hz, $\underline{CH_2}CH_3$), 5.95 (s, 1H, CH-2, chromanone), 6.77 (s, 1H, CH, pyrimidine), 7.05–7.16 (m, 2H, ArH), 7.44–7.57 (m, 3H, ArH), 7.82–7.98 (m, 4H, ArH), 8.13 (d, 1H, J = 12.0 Hz, CH enamine), 11.76 (d, 1H, J = 12.0 Hz, NH, exchanged with D_2O). ^{13}C NMR δ: 15.4 (CH_3), 63.5 (CH_2), 100.3 (C-2 of chromanone), 105.5 (C-5 of diazine), 116.0 (C-3 + C-5 of phenyl), 118.5 (C-8 of chromanone), 122.4, 125.7, 130.4, 130.6, 135.1, 135.2, 138.9 (aromatic Cs), 143.6 (methine), 144.3 (C-4 of phenyl), 156.2 (C-8a of chromanone), 156.6 (C-4 + C-6 of diazine), 167.8 (C-2 of diazine), 181.1 (C-4 of chromanone). EI-MS m/z (%): 480.5 (M^+, 0.1%). Anal. Calcd. for $C_{24}H_{24}N_4O_5S$ (480.54): C, 59.99; H, 5.03; N, 11.66. Found: C, 60.08; H, 5.07; N, 11.79.

*4.1.1.5. 4-(((2-Ethoxy-4-oxochroman-3-ylidene)methyl)amino)-N-(thiazol-2-yl)benzenesulfonamide (**3e**).* Yield: 80%; m.p. 284–288 °C. IR (cm^{-1}): 3118 (NH), 3012 (aromatic CH), 2972 (aliphatic CH), 1658(C=O), 1278(SO_2^{asym}), 1147 (SO_2^{sym}). ^1H NMR (400 MHz) δ:1.07 (t, 3H, J = 7.1 Hz, $CH_2\underline{CH_3}$), 3.68 (q, 2H, J = 7.1 Hz, $\underline{CH_2}CH_3$), 5.95 (s, 1H, CH-2, chromanone), 6.45–6.54 (m, 1H, ArH), 6.82–7.05 (m, 3H, ArH), 7.11–7.50 (m, 3H, ArH), 7.61–7.82 (m, 3H, ArH), 8.06 (d, 1H, J = 12.0 Hz, CH enamine), 11.77 (d, 1H, J = 12.0 Hz, NH, exchanged by D_2O), 12.72 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 15.4 (CH_3), 63.5 (CH_2), 100.3 (C-2 of chromanone), 105.3 (C-5 of thiazole), 108.6 (C-3+C-5 of phenyl), 116.9 (C-8 of chromanone), 118.5 (C-3 of chromanone), 122.3, 123.0, 124.9, 126.1, 126.5, 128.2, 135.2 (aromatic Cs). 137.4 (C-4 of thiazole), 143.2 (methine), 144.5 (C-4 of phenyl), 156.1 (C-8a of chromanone), 178.9 (C-2 of thiazole), 181.0 (C-4 of chromanone). EI-MS m/z (%): 459.30 (M^+ + 2, 8.7%), 458.35(M^+ + 1, 20.16%), 457.35 (M^+, 20.14%). Anal. Calcd. for $C_{21}H_{19}N_3O_5S_2$ (457.52): C, 55.13; H, 4.19; N, 9.18. Found: C, 55.31; H, 4.24; N, 9.34.

*4.1.1.6. 4-(((2-Ethoxy-4-oxochroman-3-ylidene)methyl)amino)-N-(pyridin-2-yl)benzenesulfonamide (**3f**).* Yield: 82%; m.p. 185–187 °C. IR (cm^{-1}): 3304 (NH), 3030 (aromatic CH), 2927 (aliphatic CH), 1653 (C=O), 1280 (SO_2^{asym}), 1159 (SO_2^{sym}). ^1H NMR (400 MHz) δ:1.06 (t, 3H, J = 7.2 Hz, $CH_2\underline{CH_3}$), 3.65 (q, 2H, J = 7.2 Hz, $\underline{CH_2}CH_3$), 5.94 (s, 1H, CH-2, chromanone), 7.09–8.05 (m, 13H, ArH), 8.11 (d, 1H, J = 12.0 Hz, CH enamine), 11.74 (d, 1H, J = 12.0 Hz, NH, exchanged with D_2O). ^{13}C NMR δ: 14.8(CH_3), 63.0 (CH_2), 99.8 (C-2 of chromanone), 113.6 (C-3 of pyridine), 115.7 (C-3+ C-5 of phenyl), 116.7(C-8 of chromanone), 119.9, 121.2, 122.3, 125.2, 128.5, 129.1, 134.7, 136.3 (aromatic Cs), 139.0 (C-4 of pyridine), 142.9 (methine), 145.6 (C-4 of phenyl), 152.3 (C-6 of pyridine), 155.5 (C-2 of pyridine), 156.3 (C-8a of chromanone), 180.9 (C-4 of chromanone). Anal. Calcd. for $C_{23}H_{21}N_3O_5S$ (451.50): C, 61.19; H, 4.69; N, 9.31. Found: C, 60.09; H, 4.76; N, 9.54.

*4.1.2. General method for the synthesis of compounds (**4a**–**f**)*

A mixture of the appropriate enamine derivatives **3a**–**f** (10 mmol) and sodium borohydride (3.7 g, 10 mmol) in absolute ethanol (20 mL) was stirred for 24 h at room temperature. The reaction mixture was diluted with water, neutralized with diluted HCl and the separated solid was filtered off, washed with water and crystallized from ethanol.

*4.1.2.1. 4-(((4-Oxo-4H-chromen-3-yl)methyl)amino)benzenesulfonamide (**4a**).* Yield: 60%; m.p. 242–242 °C. IR (cm^{-1}): 3304, 3224 (NH, NH_2), 3118 (aromatic CH), 2920 (aliphatic CH), 1635 (C=O), 1573 (C=C), 1315(SO_2^{asym}), 1147 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 4.14 (d, 2H, J = 5.7 Hz, CH_2), 6.60 (t, 1H, J = 6.0 Hz, NH, exchanged with D_2O), 6.89 (s, 2H, SO_2NH_2, exchanged with D_2O), 6.68 (d, 1H, J = 8.7, ArH), 7.48 (m, 4H, ArH), 7.62 (d, 1H, J = 8.7, ArH), 7.78 (m, 1H, ArH), 8.08 (d, 1H, ArH), 8.31 (s. 1H, CH-2, chromone). ^{13}C NMR δ: 38.4(CH_2), 111.6 (C-3+C-5 of phenyl), 118.9 (C-8 of chromone), 121.0, 123.6, 125.4, 125.9, 127.8, 131.0, 134.7 (aromatic Cs), 151.3 (C-4 of phenyl), 154.9 (C-2 of chromone), 156.4 (C-8a of chromone), 176.85 (C-4 of chromone). EI-MS m/z (%): 331 (M^++1, 55.1%), 330 (M^+,16.33%). Anal. Calcd. for $C_{16}H_{14}N_2O_4S$ (330.36): C, 58.17; H, 4.27; N, 8.48. Found: C, 58.24; H, 4.31; N, 8.56.

*4.1.2.2. N-(5-Methylisoxazol-3-yl)-4-(((4-oxo-4H-chromen-3-yl)methyl) amino)benzenesulfonamide (**4b**).* Yield: 75%; m.p. 204–206 °C. IR (cm^{-1}): 3348 (NH), 3070 (aromatic CH), 2974 (aliphatic CH), 1643 (C=O), 1604 (C=C), 1327(SO_2^{asym}), 1161(SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.27 (s, 3H, CH_3), 4.12 (d, 2H, J = 5.7 Hz, CH_2), 6.08 (s, 1H, CH, isoxazole), 6.85 (t, 1H, J = 6.0 Hz, NH, exchanged with D_2O), 6.69–8.07 (m, 8H, ArH), 8.31 (s. 1H, CH-2, chromone), 10.92 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 11.4 (CH_3), 37.2 (CH_2), 94.6 (C-4 of ioxazole ring), 110.6 (C-3+C-5 of phenyl), 119.7 (C-8 of chromone), 120.4, 122.6, 124.1, 124.3, 124.8, 124.9, 128.5, 133.6 (aromatic Cs), 151.4(C-3 of isoxazole), 154.0 (C-2 of chromone ring), 155.3(C-4 of phenyl), 157.2 (C-8a of chromone), 169.3 (C-5 of isoxazole), 175.7 (C-4 of chromone). EI-MS m/z (%): 411 (M^+, 53.0%). Anal. Calcd. for $C_{20}H_{17}N_3O_5S$ (411.43): C, 58.39; H, 4.16; N, 10.21. Found: C, 58.47; H, 4.20; N, 10.36.

*4.1.2.3. 4-(((4-Oxo-4H-chromen-3-yl)methyl)amino)-N-(pyrimidin-2-yl)benzenesulfonamide (**4c**).* Yield: 40%; m.p. 220–222 °C. IR (cm^{-1}): 3369 (NH), 3086 (aromatic CH), 2929 (aliphatic CH), 1635 (C=O), 1558 (C=C), 1317 (SO_2^{asym}), 1149 (SO_2^{sym}).^1H NMR (400 MHz) δ: 4.12 (d, 2H, J = 5.6 Hz, CH_2), 6.78 (t, 1H, J = 6.0 Hz, NH, exchanged

with D_2O), 6.59–8.09 (m, 11H, ArH), 8.33 (s. 1H, CH-2, chromone), 11.04 (s,1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 38.3 (CH_2), 111.0 (C-3+ C-5 of phenyl), 114.3 (C-5 of diazine), 117.1 (C-8 of chromone), 118.9, 120.9, 121.5, 123.6, 125.9, 129.6, 134.7 (aromatic Cs), 150.7 (C-2 of chromone), 152.1 (C-4 of phenyl), 155.0 (C-8a of chromone), 157.0 (C-4 + C-6 of diazine), 167.8 (C-2 of diazine), 176.8 (C-4 of chromone). Anal. Calcd. for $C_{20}H_{16}N_4O_4S$ (408.43): C, 58.82; H, 3.95; N, 13.72. Found; C, 58.97; H, 3.94; N, 13.88.

*4.1.2.4. N-(4,6-Dimethylpyrimidin-2-yl)-4-(((4-oxo-4H-chromen-3-yl)methyl)amino)benzenesulfonamide (**4d**).* Yield: 40%; m.p. 165–166 °C. IR (cm^{-1}): 3392 (NH), 3080 (aromatic CH), 2926, (aliphatic CH), 1635 (C=O), 1558 (C=C), 1319 (SO_2^{asym}), 1149 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 2.23 (s, 6H, $2CH_3$), 4.12 (d, 2H, J = 5.6 Hz,CH_2), 6.71 (t, 1H, J = 6.0 Hz, NH, exchanged with D_2O), 6.67–8.17 (m, 9H, ArH), 8.33 (s. 1H, CH-2, chromone), 11.77 (s,1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 14.8 (CH_3), 38.6 (CH_2), 110.2 (C-5 of pyrimidine), 116.6, 117.9, 120.2, 122.7, 125.4, 126.1, 128.2, 130.3 (aromatic Cs), 150.2 (C-2 of chromone + C-4 of phenyl), 152.2 (C-8a of chromone), 156.6 (C-4 of chromone). Anal. Calcd. for $C_{22}H_{20}N_4O_4S$ (436.49): C, 60.54; H, 4.62; N, 12.84. Found: C, 60.62; H; 4.65; N, 12.97.

*4.1.2.5. 4-(((4-Oxo-4H-chromen-3-yl)methyl)amino)-N-(thiazol-2-yl)benzenesulfonamide (**4e**).* Yield: 65%; m.p. 165–166 °C. IR (cm^{-1}): 3425 (NH), 3099 (aromatic CH), 2928 (aliphatic CH), 1643 (C=O), 1577 (C=C), 1292 (SO_2^{asym}), 1139 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 4.11 (d, 2H, J = 5.7 Hz, CH_2), 6.72 (t, 1H, NH, J = 6.0 Hz, exchanged with D_2O), 6.65–8.10 (m, 10H, ArH), 8.30 (s. 1H, CH-2, chromone), 12.40 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 37.9 (CH_2), 107.4(C-3+C-5 of phenyl), 110.8 (C-5 of thiazole), 111.1 (C-8 of chromone), 116.6, 118.4, 120.4, 120.9, 123.1, 124.2, 124.8, 125.4, 134.1 (aromatic Cs), 151.1(C-2 of chromone), 155.9 (C-8a of chromone), 167.9 (C-2 of thiazole), 176.3 (C-4 of chromone). EI-MS m/z (%): 413 (M^+, 8,7). Anal. Calcd. for $C_{19}H_{15}N_3O_4S_2$ (413.47): C, 55.19; H, 3.66; N, 10.16. Found: C, 55.26; H, 3.72; N, 10.30.

*4.1.2.6. 4-(((4-Oxo-4H-chromen-3-yl)methyl)amino)-N-(pyridin-2-yl)benzenesulfonamide (**4f**).* Yield: 40%; Mp 240–242 °C. IR (cm^{-1}): 3352 (NH), 3070 (aromatic CH), 2925 (aliphatic CH), 1643 (C=O), 1465 (C=C), 1276 (SO_2^{asym}), 1141 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 4.10 (d, 2H, J = 5.6 Hz, CH_2), 6.76 (t, 1H, NH, J = 5.6 Hz, exchanged with D_2O), 6.66–8.09 (m, 12H, ArH), 8.32 (s. 1H, CH-2, chromone), 11.02 (s, 1H, SO_2NH, exchanged with D_2O). EI-MS m/z (%): 407 (M^+, 10.0%). Anal. Calcd. for $C_{21}H_{17}N_3O_4S$ (407.44): C, 61.91; H, 4.21; N, 10.31. Found: C, 62.03; H, 4.27; N, 10.43.

*4.1.3. General method for the synthesis of compounds (**6a**–**f**)*

To a solution of the appropriate *N*-chloroacetyl sulfonamide derivatives **5a**–**f** (10 mmol) in ethanol (20 mL), ammonium thiocyanate (0.75 g, 10 mmol) was added and the reaction mixture was refluxed for 3–12 h. The reaction mixture was filtered while hot and the obtained solid was crystallized from dioxane to afford the title compounds **6a**–**f**.

*4.1.3.1. 4-((4-Oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (**6a**).* (Yield: 90%; mp. 244–246 °C; reflux time: 3 h. as reported) [50].

*4.1.3.2. N-(5-Methylisoxazol-3-yl)-4-((4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (**6b**).* Yield: 85%; m.p. 259–261 °C; reflux time: 8 h. IR (cm^{-1}): 3334 (NH), 3089 (aromatic CH), 2933 (aliphatic CH), 1689 (C=O), 1244 (SO_2^{asym}), 1170 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.29 (s, 3H, CH_3), 4.02 (s, 2H, CH_2), 6.11 (s, 1H, CH, isoxazole), 7.74–7.85 (m, 4H, ArH), 11.35 (s, 1H, NH, exchanged with D_2O), 11.89 (s, 1H, SO_2NH, exchanged with D_2O). EI-MS m/z (%): 352 (M^+ + 1.1%). Anal. Calcd. for $C_{13}H_{12}N_4O_4S_2$ (352.38): C, 44.31; H, 3.43; N, 15.90. Found: C, 44.39; H, 3.47; N; 16.04.

*4.1.3.3. 4-((4-Oxo-4,5-dihydrothiazol-2-yl)amino)-N-(pyrimidin-2-yl)benzenesulfonamide (**6c**).* Yield: 73%; m.p. 297(dec.); reflux time: 10 h. IR (cm^{-1}): 3277 (NH), 3039 (aromatic CH), 2941 (aliphatic CH), 1687 (C=O), 1330 (SO_2^{asym}), 1161 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 4.03 (s, 2H, CH_2), 7.03–7.1 (m, 2H, ArH), 7.73–7.97 (m, 3H, ArH), 8.49–8.54 (m, 2H, ArH), 11.79 (br s, 2H, NH and SO_2NH, exchanged with D_2O). EI-MS m/z (%): 349 (M^+, 0.5%). Anal. Calcd. for $C_{13}H_{11}N_5O_3S_2$ (349.38): C, 44.69; H, 3.17; N, 20.05. Found: C, 44.81; H, 3.20; N, 20.21.

*4.1.3.4. N-(4,6-Dimethylpyrimidin-2-yl)-4-((4-oxo-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (**6d**).* Yield: 68%; m.p. 275–277 °C; reflux time: 12 h. IR (cm^{-1}): 3263 (NH), 3041 (aromatic CH), 2978 (aliphatic CH), 1670 (C=O), 1244(SO_2^{asym}), 1143, (SO_2^{sym}). ^1H NMR (400 MHz) δ: 2.25 (s, 6H, 2 CH_3), 4.02 (s, 2H, CH_2), 7.72–7.96 (m, 5H, ArH), 11.76 (br, s, 2H, NH and SO_2NH, exchanged with D_2O). EI-MS m/z (%): 378 (M^+ + 1, 0.6%), 377 (M^+, 0.6%). Anal. Calcd. for $C_{15}H_{15}N_5O_3S_2$ (377.44): C, 47.73; H, 4.01; N, 18.56. Found: C, 47.88; H, 4.07; N, 18.69.

*4.1.3.5. 4-((4-Oxo-4,5-dihydrothiazol-2-yl)amino)-N-(thiazol-2-ylbenzenesulfonamide (**6e**).* Yield: 88%; m.p. > 300 °C; reflux time: 10 h. IR (cm^{-1}): 3269 (NH), 3045 (aromatic CH), 2989 (aliphatic CH), 1687 (C=O), 1240 (SO_2^{asym}), 1136 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 4.02 (s, 2H, CH_2), 6.81 (d, 1H, J = 4.0 Hz, CH-5, thiazole), 7.25 (d, 1H, J = 4.0 Hz, CH-4, thiazole), 7.71–7.90 (m, 4H, ArH), 11.48 (s, 1H, NH exchanged with D_2O), 12.64 (s, 1H, SO_2NH exchanged with D_2O). EI-MS m/z (%): 354 (M^+, 37.7%). Anal. Calcd. for $C_{12}H_{10}N_4O_3S_3$ (354.42): C, 40.67; H, 2.84; N, 15.81. Found: C, 40.75; H, 2.82; N, 15.97.

*4.1.3.6. 4-((4-Oxo-4,5-dihydrothiazol-2-yl)amino)-N-(pyridin-2-yl) benzenesulfonamide (**6f**).* (Yield: 85%; m.p. 240–242 °C; reflux time: 5 h as reported) [51].

*4.1.4. General method for the synthesis of compounds (**7a**–**f**)*

A mixture of chromone-3-carboxaldehyde **2** (1.74 g, 10 mmol), the appropriate thiazolinone derivative **6a**–**f** (10 mmol) and anhydrous sodium acetate (0.82 g, 10 mmol) in glacial acetic acid (50 mL) was heated in a boiling water bath for 3–12 h. The separated solid was filtered off while hot, washed with hot water and crystallized from DMF/H_2O.

*4.1.4.1. 4-((4-Oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (**7a**).* Yield: 80%; m.p. < 300 °C; reaction time: 3 h. IR (cm^{-1}): 3325 (NH), 3290, 3242 (NH_2), 3049 (aromatic CH), 2950 (aliphatic CH), 1676 (C=O amide), 1643 (C=O, chromone), 1290(SO_2^{asym}), 1159 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 7.34 (s, 2H, SO_2NH_2, exchanged with D_2O), 7.44–8.16 (m, 9H, ArH + methine H), 8.81 (s, 1H, CH-2, chromone), 12.12 (s, 1H, NH exchanged with D_2O). ^{13}C NMR δ: 118.9 (C-3 of chromone), 119.0 (C-8 of chromone), 121.3, 122.0, 123.4, 125.2, 125.9, 126.7, 127.0, 127.6, 129.7, 135.5, 138.0 (aromatic Cs), 144.3 (C-9, methine), 154.4 (C-2 of chromone), 155.8 (C-8a of chromone), 160.4 (C-2 of thiazolinone), 166.04 (C-4, C=O of chromone), 175.27 (C-4, C=O of thiazolinone). EI-MS m/z (%): 428 (M^++1, 2.3%), 427 (M^+, 0.5%). Anal. Calcd. for $C_{19}H_{13}N_3O_5S_2$ (427.45): C, 53.39; H; 3.07; N, 9.83. Found: C, 53.48; H, 3.12; N, 9.98.

*4.1.4.2. N-(5-Methylisoxazol-3-yl)-4-((4-oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)benzenesulfonamide (**7b**).* Yield: 85%; m.p. < 300 °C; reaction time: 8 h. IR

(cm^{-1}): 3263 (NH), 3051(aromatic CH), 2964 (aliphatic CH), 1680 (C=O, amide), 1651 (C=O, chromone), 1238 (SO_2^{asym}), 1161 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.31 (s, 3H, CH_3), 6.16 (s, 1H,CH, isoxazole), 7.20 (m, 1H, ArH), 7.50–7.70 (m, 3H, ArH), 7.82–8.10 (m, 6H, ArH + methine H + NH, exchanged with D_2O), 8.79 (s, 1H, CH-2, chromone), 11.44 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 11.4 (CH_3), 94.8 (C-4 of isoxazole), 117.4 (C-8 of chromone), 119.9, 121.4, 122.3, 124.9, 125.7, 127.7, 134.3 (aromatic Cs), 154.7 (C-2 of chromone), 156.4 (C-8a of chromone), 160.0 (C-2 of thiazolinone), 161.7 (C-4 of chromone), 169.7 (C-4 of thiazolinone). EI-MS *m/z* (%): 509 (M^++1, 48%), 508 (M^+, 48%). Anal. Calcd. for $C_{23}H_{16}N_4O_6S_2$ (508.52): C, 54.32; H, 3.17; N, 11.02. Found: C, 54.39; H, 3.21; N, 11.13.

*4.1.4.3. 4-((4-Oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)-N-(pyrimidin-2-yl)benzenesulfonamide (**7c**).* Yield: 78%; m.p. < 300 °C; reaction time: 12 h. IR (cm^{-1}): 3250 (NH), 3041 (aromatic CH), 2955 (aliphatic CH), 1680 (C=O, amide), 1649 (C=O, chromone), 1247 (SO_2^{asym}), 115 7(SO_2^{sym}). ^1H NMR (300 MHz) δ: 7.04–7.18 (m, 2H, ArH), 7.53–7.71 (m, 3H, ArH), 7.83–8.11 (m, 5H, ArH), 8.51–8.53 (m, 2H, ArH, +methine H), 8.79 (s, 1H, CH-2, chromone), 11.85 (s, 2H, NH and SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 116.2 (C-8 of chromone), 118.5, 119.0, 120.5, 121.9, 123.0, 123.3, 126.0, 126.7, 129.6, 135.4 (aromatic Cs), 155.8 (C-8a of chromone), 157.3 (C-2 of thiazolinone + C-4 and C-6 of diazine), 158.8 (C-4 of thiazolinone + C-2 of diazine), 160.9 (C-4 of chromone). EI-MS *m/z* (%): 505 (M^+, 5%). Anal. Calcd. for $C_{23}H_{15}N_5O_5S_2$ (505.52): C, 54.65; H, 2.99; N, 13.85. Found: C, 54.72; H, 3.02; N, 14.03.

*4.1.4.4. N-(4,6-Dimethylpyrimidin-2-yl)-4-((4-oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)benzene sulfonamide (**7d**).* Yield: 69%; m.p. < 300 °C; reaction time: 12 h. IR (cm^{-1}): 3313 (NH), 3043 (aromatic CH), 2957 (aliphatic CH), 1680 (C=O, amide), 1647 (C=O, chromone), 1246 (SO_2^{asym}), 1163 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 2.27 (s, 6H, $2CH_3$), 6.76 (s, 1H, CH-5, pyrimidine), 7.45–7.56 (m, 3H, ArH + methine H), 7.69–7.85 (m, 3H, ArH), 7.94–8.12 (m, 3H, ArH), 8.69 (s, 1H, CH-2, chromone), 11.89 (s, 2H, NH and SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 22.7 (CH_3), 113.2 (C-5 of pyrimidine), 118.4 (C-3+C-5 of phenyl), 121.0 (C-8 of chromone), 122.3, 122.9, 125.1, 126.0, 126.4, 127.1, 128.2, 128.9, 130.4, 131.2, 134.8 (aromatic Cs), 155.2 (C-2 of chromone), 156.0 (C-8a of chromone), 160.3 (C-2 of thiazolinone), 169.6 (C-4+C-6 of pyrimidine), 185.7 (C-4 of thiazolinone), 193.2 (C-4 of chromone). EI-MS *m/z* (%): 533 (M^+, 12.0%). Anal. Calcd. for $C_{25}H_{19}N_5O_5S_2$ (533.58): C, 56.28; H, 3.59; N, 13.13. Found: C, 56.39; H, 3.64; N, 13.28.

*4.1.4.5. 4-((4-Oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)-N-(thiazol-2-yl)benzenesulfonamide (**7e**).* Yield: 65%; m.p. < 300 °C; reaction time: 8 h. IR (cm^{-1}): 3246 (NH), 3043 (aromatic CH), 2950 (aliphatic CH), 1674 (C=O, amide), 1651 (C=O chromone), 1321 (SO_2^{asym}),1184 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.84 (d, 1H, J = 4.0 Hz, CH-5, thiazole), 7.14–7.15 (m, 1H, ArH), 7.27 (d, 1H, J = 4.0 Hz, CH-4, thiazole), 7.50–7.92 (m, 7H, ArH), 8.08 (s, 1H, methine H), 8.79 (s, 1H, CH-2, chromone), 12.40, 12.75 (s, 2H, NH and SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 108.7 (C-5 of thiazole + C-3 and C-5 of phenyl), 118.5 (C-8 of chromone), 119.0, 120.7, 122.0, 122.8, 123.4, 124.9, 126.0, 126.7, 127.7, 135.4, 138.7 (aromatic Cs), 155.8 (C-8a of chromone), 160.9 (C-4 of thiazolinone), 169.2 (C-4 of chromone). EI-MS *m/z* (%): 510 (M^+, 2.8%). Anal. Calcd. for $C_{22}H_{14}N_4O_5S_3$ (510.56): C, 51.76; H, 2.76; N, 10.97. Found: C, 51.79; H, 2.78; N, 11.13.

*4.1.4.6. 4-((4-Oxo-5-((4-oxo-4H-chromen-3-yl)methylene)-4,5-dihydrothiazol-2-yl)amino)-N-(pyridin-2-yl)benzenesulfonamide (**7f**).* Yield: 70%; m.p. < 300 °C; reaction time: 4 h. IR (cm^{-1}): 3244 (NH), 3022 (aromatic CH), 2945 (aliphatic CH), 1681 (C=O, amide), 1649 (C=O chromone), 1280 (SO_2^{asym}), 1178 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.80–6.90 (m, 1H, ArH), 7.17–7.20 (m, 2H, ArH), 7.47–7.52 (m, 2H, ArH), 7.66–7.87 (m, 6H, ArH), 8.02–8.04 (m, 2H, ArH + methine H), 8.71 (s, 1H, CH-2, chromone), 12.00 (s, 2H, NH and SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 102.1(C-3 of pyridine), 113.7 (C-3+C-5 of phenyl), 115.5 (C-8 of chromone), 118.4, 118.7, 120.5, 121.3, 122.8, 125.4, 126.1, 126.8, 127.5, 128.3, 129.4, 130.5, 134.7 (aromatic Cs), 140.2 (C-6 of pyridine), 147.4 (C-2 of chromone), 153.0 (C-8a of chromone), 155.3 (C-4 of thiazolinone), 170.0 (C-4 of chromone). Anal. Calcd. for $C_{24}H_{16}N_4O_5S_2$ (504.54): C, 57.13; H, 3.20; N, 11.10. Found: C, 57.27; H, 3.18; N, 11.27.

*4.1.5. General method for the synthesis of compounds (**10a–f**) and (**11a–f**)*

An equimolar mixture of the oxazolone **9a** or **9b** and the appropriate sulfonamide derivative (10 mmol) and anhydrous sodium acetate (0.03 g, 0.36 mmol) in glacial acetic acid (20 mL) was heated in a boiling water bath while stirring for 8–24 h. The separated solid was filtered off while hot, washed with water, and crystallized from DMF/H_2O.

*4.1.5.1. 4-(5-Oxo-4-((4-oxo-4H-chromen-3-yl)methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl)benzenesulfonamide (**10a**).* Yield: 82%; m.p. 292–294 °C; reaction time: 8 h. IR (cm^{-1}): 3311, 3265 (NH_2), 3050 (aromatic CH), 2950 (aliphatic CH), 1683 (C=O, imidazolinone), 1653 (C=O, chromone), 1593 (C=C), 1315 (SO_2^{asym}), 1159 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.79–7.10 (m, 2H, ArH), 7.23 (s, 2H, SO_2NH_2, exchanged with D_2O), 7.52–7.99 (m, 12H, ArH), 8.72 (s, 1H, CH-2 of chromone). ^{13}C NMR δ: 119.9 (C-8 of chromone), 123.6, 126.0, 126.5, 126.9, 128.2, 128.9, 132.4, 133.6, 139.0 (aromatic Cs), 142.6 (C-2 of chromone), 155.8 (C-8a of chromone), 158.5 (C-2 of imidazolinone), 164.6 (C-4 of imidazolinone), 175.9 (C-4 of chromone). EI-MS *m/z* (%): 472 (M^+ + 1, 4.6%). Anal. Calcd. for $C_{25}H_{17}N_3O_5S$ (471.49): C, 63.69; H, 3.63; N, 8.91. Found: C, 63.81; H, 3.65; N, 8.99.

*4.1.5.2. N-(5-Methylisoxazol-3-yl)-4-(5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl)benzenesulfonamide (**10b**).* Yield: 75%; m.p. 295–296 °C; reaction time: 8 h. IR (cm^{-1}): 3251 (NH), 3010 (aromatic CH), 2926 (aliphatic CH), 1687 (C=O, imidazolinone), 1656 (C=O, chromone), 1591 (C=C), 1317 (SO_2^{asym}), 1166 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.30 (s, 3H, CH_3), 6.12 (s, 1H, CH, isoxazole), 6.76 (s, 1H, methine H), 7.51–8.19 (m, 13H, ArH), 8.73 (s, 1H, CH-2 of chromone), 10.60 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 12.0 (CH_3), 95.3 (C-4 of isoxazole), 115.4 (C-8 of chromone), 119.6, 123.1, 125.4, 126.0, 127.7, 127.8, 128.4, 131.9, 133.1, 133.1, 133.2, 134.6 (aromatic Cs), 149.5 (C-1 of phenyl sulfonamide), 155.3 (C-2 of chromone), 157.5 (C-3 of isoxazole), 157.5 (C-8a of chromone), 158.1 (C-2 of imidazolinone), 164.3 (C-5 of isoxazole), 170.2 (C-4 of imidazolinone), 175.4 (C-4 of chromone). EI-MS *m/z* (%): 552 (M^+, 40.7%). Anal. Calcd. for $C_{29}H_{20}N_4O_6S$ (552.56): C, 63.04; H, 3.65; N, 10.14. Found: C, 63.08; H, 3.64; N, 10.22.

*4.1.5.3. 4-(5-Oxo-4-((4-oxo-4H-chromen-3-yl)methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl)-N-(pyrimidin-2-yl)benzenesulfonamide (**10c**).* Yield: 60%; m.p. 280–282 °C; reaction time: 10 h. IR (cm^{-1}): 3421 (NH), 3034 (aromatic CH), 2932 (aliphatic CH), 1680 (C=O, imidazolinone), 1653 (C=O, chromone), 1593 (C=C), 1319(SO_2^{asym}), 1165 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.75 (s, 1H, methine H), 7.03 (m, 1H, ArH), 7.48–7.53 (m, 12H, ArH), 8.16–8.17 (m, 1H, ArH), 8.49–8.51 (m, 2H, ArH), 8.72 (s, 1H, CH-2 of chromone), 10.55 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 115.4 (C-5 of pyrimidine), 118.4 (C-8 of chromone), 118.8, 119.1, 120.2,

122.2, 122.9, 123.8, 125.7, 127.1, 128.6, 129.5, 130.4, 132.7, 133.1, 134.6 (aromatic Cs), 143.1 (C-2 of chromone), 155.2 (C-8a of chromone), 158.1 (C-4 + C-6 of pyrimidine + C-2 of imidazolinone), 164.2 (C-2 of pyrimidine), 164.9 (C-4 of imidazolinone), 175.4 (C-4 of chromone). Anal. Calcd. for $C_{29}H_{19}N_5O_5S$ (559.56): C, 63.38; H, 3.48; N, 12.74. Found: C, 63.52; H, 3.46; N, 12.91.

*4.1.5.4. N-(4,6-Dimethylpyrimidin-2-yl)-4-(5-oxo-4-((4-oxo-4H-chromen-3-yl)methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl) benzenesulfonamide (**10d**).* Yield: 45%; m.p. 212–213 °C; reaction time: 12 h. IR (cm^{-1}): 3242 (NH), 3062 (aromatic CH), 2926 (aliphatic CH), 1718 (C=O, imidazolinone), 1651 (C=O, chromone), 1595 (C=C), 1313(SO_2^{asym}), 1159(SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.24 (s, 6H, $2CH_3$), 6.75 (s, 1H, methine H), 7.41–8.01 (m, 14H, ArH), 8.70 (s, 1H, CH-2 of chromone), 10.60 (s, 1H, SO_2NH, exchanged with D_2O). EI-MS *m/z* (%): 577 (M^+, 8.0%). Anal. Calcd. for $C_{31}H_{23}N_5O_5S$ (577.62): C, 64.46; H, 4.01; N, 12.12; S, 5.55. Found: C, 64.59; H, 4.09; N, 12.21; S, 5.62.

*4.1.5.5. 4-(5-Oxo-4-((4-oxo-4H-chromen-3-yl)methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl)-N-(thiazol-2-yl)benzenesulfonamide (**10e**).* Yield: 80%; m.p. 241–246 °C; reaction time: 8 h. IR (cm^{-1}): 3379 (NH), 3032(aromatic CH), 2925 (aliphatic CH), 1672 (C=O, imidazolinone), 1649 (C=O, chromone), 1587 (C=C), 1317(SO_2^{asym}), 1143 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 6.77 (s, 1H, methine H), 6.81 (d,1H, CH-5, J = 4.0 Hz, thiazole), 7.24 (d,1H, CH-4, J = 4.0 Hz, thiazole), 7.52–7.61 (m, 4H, ArH), 7.71–7.78 (m, 3H, ArH), 7.85–7.89 (m, 3H, ArH), 7.97–7.99 (m, 2H, ArH), 8.17–8.19 (m, 1H, ArH), 8.73 (s, 1H, CH-2 of chromone), 10.53 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 108.3 (C-5 of thiazole), 115.8 (C-8 of chromone), 118.7, 119.1, 119.7, 123.4, 125.8, 126.3, 127.0, 128.0, 128.7, 132.2, 133.5, 135.0, 136.8 (aromatic Cs), 142.7 (C-4 of thiazole + C-4 of phenyl), 148.4 (C-2 of chromone), 155.6 (C-8a of chromone), 158.3 (C-2 of imidazolinone), 164.4 (C-4 of imidazolinone), 171.3 (C-2 of thiazole), 175.8 (C-4 of chromone). EI-MS *m/z* (%): 554 (M^+, 1.1%). Anal. Calcd. for $C_{28}H_{18}N_4O_5S_2$ (554.60): C, 60.64; H, 3.27; N, 10.10. Found: C, 60.68; H, 3.29; N, 11.13.

*4.1.5.6. 4-(5-Oxo-4-((4-oxo-4H-chromen-3-yl)methylene)-2-phenyl-4,5-dihydro-1H-imidazol-1-yl)-N-(pyridin-2-yl)benzenesulfonamide (**10f**).* Yield: 70%; m.p. 246–248 °C; reaction time: 8 h. IR (cm^{-1}): 3261 (NH), 3051 (aromatic CH), 2931 (aliphatic CH), 1685 (C=O, imidazolinone), 1655 (C=O, chromone), 1591 (C=C), 1319(SO_2^{asym}), 1163 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.75 (s, 1H, methine H), 7.15–7.98 (m, 17H, ArH), 8.71 (s, 1H, CH-2 of chromone), 10.50 (s, 1H, SO_2NH, exchanged with D_2O).^{13}C NMR δ: 115.9 (C-3 of pyridine), 119.9 (C-8 of chromone), 123.6, 126.0, 126.5, 128.1, 128.3, 128.9, 132.4, 133.6, 135.2 (aromatic Cs), 143.0 (C-6 of pyridine), 155.8 (C-2 of chromone), 158.5 (C-8a of chromone), 164.6 (C-2 of imidazolinone), 165.5 (C-4 of imidazolinone), 175.9 (C-4 of chromone). EI-MS *m/z* (%): 548 (M^+, 65.3%). Anal. Calcd. for $C_{30}H_{20}N_4O_5S$ (548.57): C, 65.68; H, 3.67; N, 10.21. Found: C, 65.76; H, 3.64; N, 10.40.

*4.1.5.7. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)benzenesulfonamide (**11a**).* Yield: 62%; m.p. > 300 °C; reaction time: 18 h. IR (cm^{-1}): 3338, 3288 (NH_2), 3059 (aromatic CH), 2930 (aliphatic CH), 1681 (C=O, imidazolinone), 1656 (C=O, chromone), 1595 (C=C), 1300 (SO_2^{asym}), 1161 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.83 (s, 1H, methine H), 7.24 (s, 2H, SO_2NH_2, exchanged with D_2O), 7.55–7.97 (m, 11H, ArH), 8.15–8.19 (m, 1H, ArH), 8.72 (s, 1H, CH-2, chromone). ^{13}C NMR δ: 115.8 (C-8 of chromone), 119.6, 123.1, 125.4, 126.0, 127.8, 128.5, 129.6, 132.0, 132.8, 133.3, 134.6, 136.6 (aromatic Cs), 143.4 (C-2 of chromone), 157.5 (C-8a of chromone), 158.1 (C-2 of imidazolinone), 164.1 (C-4 of imidazolinone), 175.8 (C-4 of chromone). EI-MS *m/z* (%): 507.02 (M^++2, 0.7), 505.03 (M^+, 2.0%). Anal. Calcd. for $C_{25}H_{16}ClN_3O_5S$ (505.93): C, 59.35; H, 3.19; N, 8.31. Found: C, 59.42; H, 3.21; N, 8.44.

*4.1.5.8. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (**11b**).* Yield: 75%; m.p. 295–297 °C; reaction time: 12 h. IR (cm^{-1}): 3250 (NH), 3161 (aromatic CH), 2924 (aliphatic CH), 1687 (C=O, imidazolinone), 1654 (C=O, chromone), 1591 (C=C), 1317(SO_2^{asym}), 1165 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 2.29 (s, 3H, CH_3), 6.10 (s, 1H, CH, isoxazole), 6.79 (s, 1H, methine H), 7.55–7.99 (m, 11H, ArH), 8.17–8.18 (m, 1H, ArH), 8.73 (s, 1H, CH-2 chromone), 10.57 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 12.0 (CH_3), 95.3 (C-4 of isoxazole), 115.9 (C-8 of chromone), 119.6, 123.1, 125.4, 126.0, 127.8, 128.5, 129.6, 132.0, 132.8, 133.3, 134.6, 136.8 (aromatic Cs), 155.3 (C-2 of chromone), 157.5 (C-3 of isoxazole), 158.1 (C-8a of chromone + C-2 of imidazolinone), 164.1 (C-5 of isoxazole), 170.2 (C-4 of imidazolinone), 175.3 (C-4 of chromone). EI-MS *m/z* (%): 588.01 (M^++2, 2.2%) 586 (M^+, 6.9%). Anal. Calcd. for $C_{29}H_{19}ClN_4O_6S$ (586.07): C, 59.34; H, 3.26; N, 9.54. Found: C, 59.42; H, 3.29; N, 9.68.

*4.1.5.9. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)-N-(pyrimidin-2-yl)benzenesulfonamide (**11c**).* Yield: 40%; m.p. 284–288 °C; reaction time: 14 h. IR (cm^{-1}): 3284 (NH), 3041 (aromatic CH), 2950 (aliphatic CH), 1683 (C=O, imidazolinone), 1656 (C=O, chromone), 1591 (C=C), 1315(SO_2^{asym}), 1163 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.79 (s, 1H, methine H), 7.01–7.04 (m, 2H, ArH), 7.52–7.99 (m, 8H, ArH), 8.15–8.18 (m, 2H, ArH), 8.49–8.51 (m, 3H, ArH), 8.73 (s, 1H, CH-2, chromone), 10.54 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 115.9 (C-5 of pyrimidine), 118.4 (C-8 of chromone), 118.7 (C-3 of chromone), 119.2, 123.1, 125.4, 126.0, 128.5, 128.6, 129.6, 132.0, 132.8, 134.3, 134.6, 136.7 (aromatic Cs), 143.0 (C-1 of phenyl), 155.3 (C-2 of chromone), 156.9 (C-8a of chromone), 158.0 (C-4 + C-6 of pyrimidine + C-2 of imidazolone), 158.2 (C-2 of pyrimidine), 164.0 (C-4 of imidazolinone), 175.3 (C-4 of chromone). Anal. Calcd. for $C_{29}H_{18}ClN_5O_5S$ (583.07): C, 59.64; H, 3.11; N, 11.99. Found: C, 59.73; H, 3.14; N, 12.13.

*4.1.5.10. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)-N-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide (**11d**).* Yield: 43%; m.p. 261–268 °C; reaction time: 24 h. IR (cm^{-1}): 3363 (NH), 3089 (aromatic CH), 2927 (aliphatic CH), 1697 (C=O, imidazolinone), 1653 (C=O, chromone), 1591 (C=C), 1315(SO_2^{asym}), 1149 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 2.26 (s, 6H, $2CH_3$), 6.76 (s, 1H, ArH, pyrimidine), 6.80 (s, 1H, methine H), 7.54–7.58 (m, 3H, ArH), 7.61–7.63 (m, 1H, ArH),7.71–7.73 (m, 3H, ArH), 7.95–7.99 (m, 4H, ArH),8.15–8.17 (m, 1H, ArH), 8.73 (s, 1H, CH-2 chromone), 10.54 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 23.3 (CH_3), 114.0 (C-5 of pyrimidine), 118.9 (C-8 of chromone), 119.2, 119.3, 123.6, 125.9, 126.5, 129.0, 129.5, 130.2, 132.5, 133.3, 135.1, 137.3 (aromatic Cs), 143.3 (C-2 of chromone), 155.8 (C-8a of chromone), 156.7 (C-2 of imidazolinone), 158.5 (C-4 + C-6 of pyrimidine), 164.5 (C-2 of pyrimidine), 164.6 (C-4 of imidazolinone), 175.8 (C-4 of chromone). Anal. Calcd. for $C_{31}H_{22}ClN_5O_5S$ (611.06): C, 60.83; H, 3.62; N 11.44; S, 5.24. Found: C, 60.90; H, 3.69; N, 11.58; S, 5.33.

*4.1.5.11. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)-N-(thiazol-2-yl)benzenesulfonamide (**11e**).* Yield: 72%; m.p. 268–271 °C; reaction time: 24 h. IR (cm^{-1}): 3300 (NH), 3084 (aromatic CH), 2935 (aliphatic CH), 1672 (C=O, imidazolinone), 1651 (C=O, chromone), 1587 (C=C), 1313(SO_2^{asym}), 1143 (SO_2^{sym}). ^1H NMR (400 MHz) δ: 6.80 (s,

1H, methine H), 6.81 (d, 1H, CH-5, $J = 4.0$ Hz, thiazole), 7.43 (d, 1H, CH-4, $J = 4.0$ Hz, thiazole), 7.46–7.99 (m, 11H, ArH), 8.15–8.17 (m, 1H, ArH), 8.73 (s, 1H, CH-2, chromone),10.52 (s, 1H, SO_2NH, exchanged with D_2O). ^{13}C NMR δ: 108.5 (C-5 of thiazole), 116.5 (C-8 of chromone), 119.9, 123.6, 124.8, 125.9, 126.5, 127.2, 127.4, 128.6, 129.0, 130.1, 131.4, 132.5, 133.3, 135.1, 137.0, 137.2 (aromatic Cs), 138.4 (C-4 of thiazole), 142.8 (C-2 of chromone), 155.8 (C-8a of chromone), 158.5 (C-2 of imidazolinone), 164.5 (C-4 of imidazolinone), 168.7 (C-2 of thiazole), 175.8 (C-4 of chromone). EI-MS *m/z* (%): 590 (M^++2, 7.5%), 588.01 (M^+, 22.8%). Anal. Calcd. for $C_{28}H_{17}ClN_4O_5S_2$ (589.04): C, 57.09; H, 2.91; N, 9.51. Found: C, 57.20; H, 2.97; N, 9.62.

*4.1.5.12. 4-(2-(4-Chlorophenyl)-5-oxo-4-((4-oxo-4H-chromen-3-yl) methylene)-4,5-dihydro-1H-imidazol-1-yl)-N-(pyridin-2-yl)benzene sulfonamide (**11f**).* Yield: 45%; m.p. 285–287 °C; reaction time: 15 h. IR (cm^{-1}): 3269 (NH), 3059 (aromatic CH), 2926 (aliphatic CH), 1683 (C=O, imidazolinone), 1653 (C=O, chromone), 1593 (C=C), 1313(SO_2^{asym}), 1166 (SO_2^{sym}). ^1H NMR (300 MHz) δ: 6.79 (s, 1H, methine H), 7.03–7.04 (m, 1H, ArH), 7.55–7.99 (m, 14H, ArH), 8.15–8.17 (m, 1H, ArH), 8.73 (s, 1H, CH-2, chromone), 10.54 (s, 1H, SO_2NH, exchanged with D_2O). EI-MS *m/z* (%): 584 (M^+ + 2, 3.5%), 582 (M^+, 10.7%). Anal. Calcd. for $C_{30}H_{19}ClN_4O_5S$ (583.01): C, 61.80; H, 3.28, N; 9.61; S, 5.50. Found: C, 61.92; H, 3.24; N,9.73; S, 5.61.

4.2. In vitro cytotoxic activity

4.2.1. Cell culture

MCF-7 human breast cancer cells, grown in RPMI-1640 medium; and A-549 human lung cancer cells, grown in DMEM, were supplemented with 10% heat inactivated FBS, 50 units/mL of penicillin and 50 mg/mL of streptomycin and were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were maintained as "monolayer culture" by serial subculturing.

4.2.2. Sulforhodamine B assay (SRB)

Exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at 1000–2000 cells/well in RPMI supplemented medium or in DMEM supplemented medium. After 24 h, the cells were incubated for 72 h with various concentrations of the tested compounds. Following 72 h treatment, the cells were fixed with 10% trichloroacetic acid for 1 h at 4 °C. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h and the dye was solubilized with Tris–HCl for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). The IC_{50} values were calculated according to the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models (Graph Pad, Prism Version 5).

4.3. Carbonic anhydrase inhibition

An applied photophysics stopped-flow instrument was used for assaying the CA catalysed CO_2 hydration activity [49]. Phenol red (at a concentration of 0.2 mM) was 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 10–100 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 was 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 constants were obtained by non-linear least-squares methods using the Cheng-Prusoff equation, as reported earlier [52–54], and represent the mean from at least three different determinations. All four CA isoforms were recombinant ones obtained in-house as reported earlier [52–54].

4.4. Apoptosis study

MCF-7 or A-549 cells (1×10^5 cells/mL) were plated in a 24-well culture plate (Corning) for 24 h at 37 °C with 5% CO_2 and 95% humidity. The tested compound **4a** was added. Cells were incubated for another 24 h. Cells were trypsinized with 0.25% trypsin in Ca^{2+}/Mg^{2+}-free 2% BSA-PBS (Sigma, St. Louis, MO,USA) and washed twice with Ca^{2+}/Mg^{2+}-free PBS. Cells were stained and adjusted to 1×10^6 cells/ml with annexin V-FITC/PI. The reaction was allowed to proceed in the dark for 30 min at room temperature. Finally, 400 mL of 1 × binding buffer was added and flow cytometry carried out within 1 h.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.033.

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