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Graphical abstract

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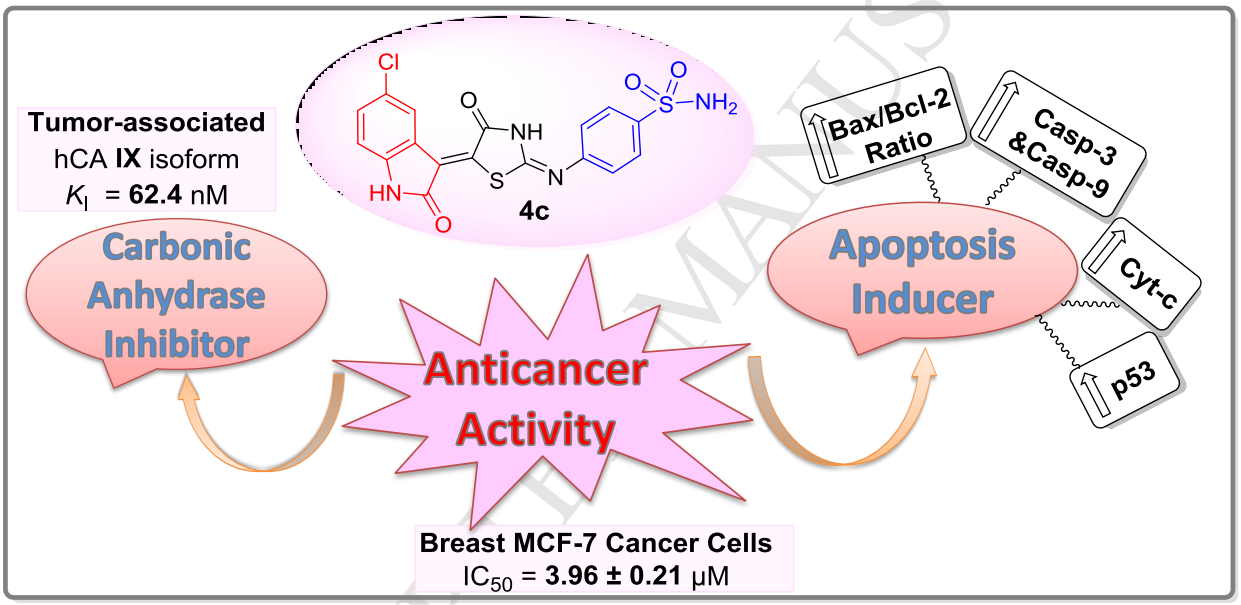
Two different series of novel isatin-based benzenesulfonamide were synthesized and evaluated for their inhibitory activity against a panel of carbonic anhydrase isoforms, hCA I, II, IV and IX. Also, they were evaluated for their anti-proliferative activity against breast cancer MCF-7 and colorectal cancer Caco-2 cell lines.



Novel 4/3-((4-oxo-5-(2-oxoindolin-3-ylidene)thiazolidin-2-ylidene)amino) benzenesulfonamides: Synthesis, carbonic anhydrase inhibitory activity, anticancer activity and molecular modelling studies

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Abstract

Herein we report the synthesis of two series of novel 4/3-((4-oxo-5-(2-oxoindolin-3-ylidene)thiazolidin-2-ylidene)amino)benzenesulfonamides (**4a-m** and **7a-g**). All the newly prepared sulfonamides were *in vitro* investigated as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) isoforms hCA I, II, IV and IX, using a stopped-flow CO_2 hydrase assay. In particular, hCA isoforms II and IX (tumor-associated) were more susceptible to inhibition by the synthesized derivatives, with K_Is in the range of 2.6–598.2 nM for hCA II, and of 16.1–321 nM for hCA IX. All compounds (**4a-m** and **7a-g**) were evaluated for their anti-proliferative activity against breast cancer MCF-7 and colorectal

cancer Caco-2 cell lines. Compound **4c** was found to be the most potent derivative against MCF-7 (IC_{50} = 3.96 ± 0.21 µM)**,** while **4j** was the most active member against Caco-2 cells (IC_{50} = 5.87 ± 0.37 µM). Compound **4c** induced the intrinsic apoptotic mitochondrial pathway in MCF-7 cells; evidenced by the enhanced expression of the pro-apoptotic protein Bax and the reduced expression of the anti-apoptotic protein Bcl-2, and the up-regulated active caspase-9 and caspase-3 levels.

Keywords: Carbonic anhydrase inhibitors; Isatin; Benzenesulfonamide; Anticancer; Apoptosis.

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1-Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1), the prevalent metalloenzymes in all life kingdoms, catalyze the efficient interconversion between CO_2 and bicarbonate [1-3]. In humans, the CA-catalyzed reaction encompasses three simple chemical entities, CO_2, HCO_3^-, and H^+ essential in a host of physiological and pathological processes, such as electrolyte secretion, bone resorption, calcification, respiration, pH and CO_2 homeostasis, biosynthetic reactions (as gluconeogenesis and lipogenesis), tumorigenicity and others [1-3]. Seven distinct genetic enzymatic families have been identified so far: the α-, β-, γ-, δ-, ζ-. η- and θ-Cas [1-3]. The sixteen human (h) isoforms of CA, which all belong to the α-class, displayed different patterns of location and tissue distribution, thus cytosolic (I, II, III, VII, and XIII), membrane-bound (IV, IX, XII, and XIV), secreted (VI) and mitochondrial (VA and VB) forms have been described in mammals [1-3].

Such isozymes represent valuable biological targets for the design of CA inhibitors (CAIs) with many biomedical applications [2-4]. Noteworthy, many sulfonamides-CAIs are clinically used for decades to treat several diseases (e.g., glaucoma, edema, epilepsy). The ubiquitous hCAI and CAII, which are involved in many physiological processes, were found to be off-target in the treatment of several pathologies [1-3], since they are the main responsible of most side effects of non-selective inhibitors. The membrane-associated isoform hCA IV is a drug target for retinitis pigmentosa and stroke, in addition to glaucoma, together with hCA II [5]. The transmembrane hCA IX is overexpressed in many solid tumors associated with the hypoxic phenotype, where it is pivotal in the growth of solid tumors, tuning the pH to support an extracellular hypoxic microenvironment suited for hypoxic tumor cell survival and proliferation, thus emerged as an innovative target for the development of anticancer agents [6,7].

Encouraged by these findings, the goal of developing isoform-selective hCAIs has been nurtured over the latter years with several studies [1,4]. The most exploited method to overcome the typical lack in selectivity of sulfonamide-like CAIs against the different human isozymes turned out to be the “tail approach” which consists in modulating the functionality (tail) appended to the aromatic/heterocyclic ring present in the scaffold of the CAIs, in order to differently interact with the middle and the rim parts of the active

site cavity, which is the most variable region among the sixteen isoforms mentioned above [8-10].

Over the last few years, isatin (1*H*-indole-2,3-dione) stood out as a promising tail scaffold to design compounds with interesting inhibitory activity profiles towards different carbonic anhydrase isoforms. Numerous studies have developed diverse isatin-based derivatives as potent CA inhibitors, compounds **I-IV** (**Fig. 1**) [11-15]. Moreover, isatin, as a privileged scaffold, is endowed with excellent anticancer profile [16, 17] and represents an important pharmacophore in two clinically approved anticancer drugs; Sunitinib **V** (Sutent®) and Nintedanib **VI** (Ofev®) (**Fig. 2**) [18, 19]. Consequently, design and synthesis of various effective isatin-based anticancer agents have attracted considerable attention in the current medical era [20-25]. Several research groups explored the anti-proliferative activity of many isatin-thiazolidine/thiazolidinone analogs, such as compounds **VII-IX** (**Fig. 2**) [26-30].

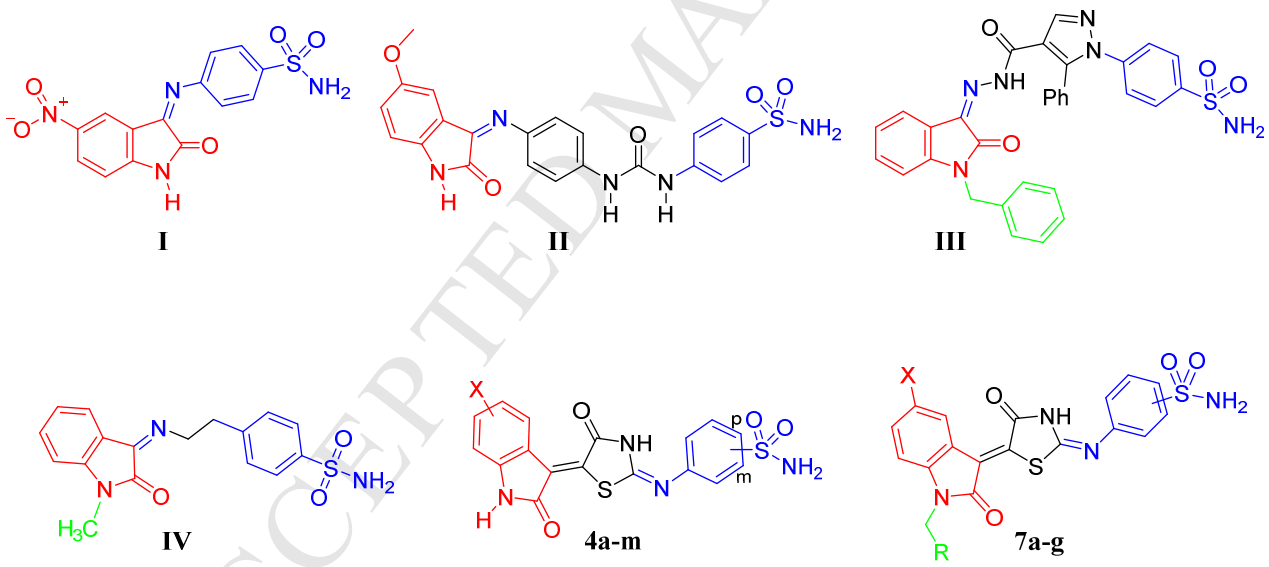


Figure 1. Structures of isatin-based carbonic anhydrase inhibitors **I-IV** and the target sulfonamides **4a-m** and **7a-g**.

Taking the above into account, herein we report two novel series of 4/3-((4-oxo-5-(2-oxoindolin-3-ylidene)thiazolidin-2-ylidene)amino)benzenesulfonamides (**4a-m** and **7a-g**)

(**Fig. 1**), with the prime aim of combining a strong hCAIX inhibition together with the intrinsic anti-tumor activity of the isatin scaffold. Indeed, all the newly synthesized sulfonamides were evaluated *in vitro* for their inhibitory activity against a panel of hCA I, II, IV and IX isoforms, using stopped-flow CO_2 hydrase assay. Furthermore, compounds **4a-m** and **7a-g** were evaluated for their anti-proliferative activity against breast cancer MCF-7 and colorectal cancer Caco-2 cell lines. Compound **4c** was further investigated for its apoptosis induction potential in MCF-7 cells, to gain mechanistic insights into the anti-proliferative activity of the newly prepared sulfonamides.

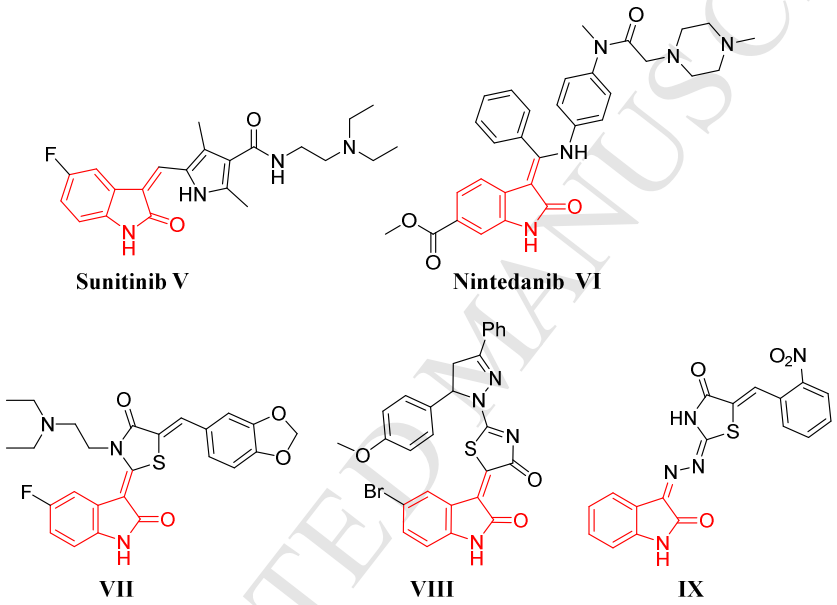


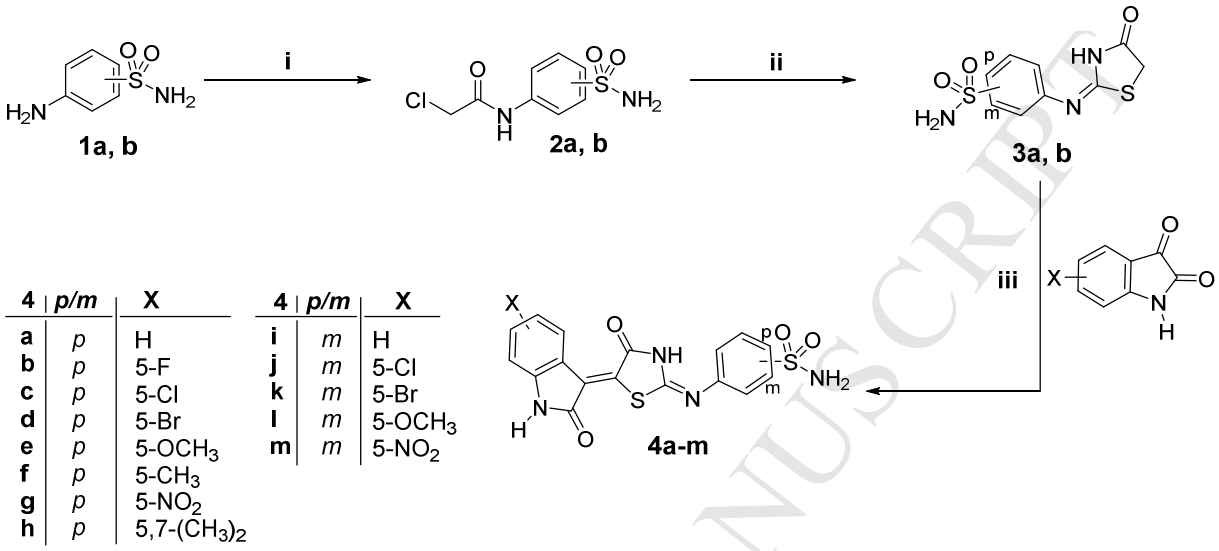
Figure 2. Structures of isatin-based approved anticancer drugs (**V**& **VI**) and some reported isatin-based derivatives with potent anti-proliferative activity (**VII - IX**).

2. Results and Discussion

2.1. Chemistry

The synthesis of the new sulfonamides in this study is outlined in Schemes **1** and **2**. In scheme **1**, synthesis of the target derivatives was started by acetylation of sulfanilamides **1a,b** with chloroacetyl chloride in dry DMF to produce derivatives **2a, b**, which subsequently cyclized in absolute ethanol with ammonium thiocyanate to furnish the key

intermediates **3a, b**. The later condensed with different isatins in acetic acid in the presence of sodium acetate to afford the target sulfonamides **4a-m** (Scheme **1**).

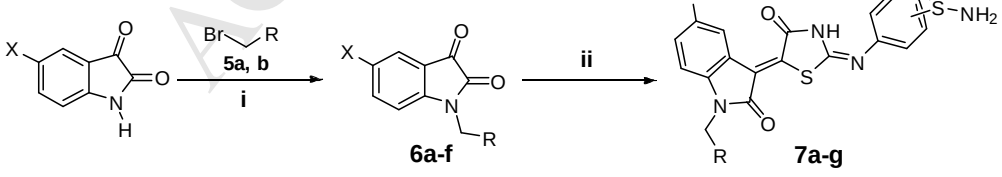


4	*p/m*	X
a	*p*	H
b	*p*	5-F
c	*p*	5-Cl
d	*p*	5-Br
e	*p*	5-OCH_3
f	*p*	5-CH_3
g	*p*	5-NO_2
h	*p*	5,7-$(CH_3)_2$

4	*p/m*	X
i	*m*	H
j	*m*	5-Cl
k	*m*	5-Br
l	*m*	5-OCH_3
m	*m*	5-NO_2

Scheme 1. Synthesis of target sulfonamides **4a-m**; *Reagents and conditions*: (**i**) Dry DMF / $ClCOCH_2Cl$ / r.t. 2 h, (**ii**) NH_4SCN / Absolute ethyl alcohol / reflux 4 h, (**iii**) Glacial acetic acid / Sodium acetate / reflux 3 h.

Scheme **2** depicts the synthesis protocol followed to get sulfonamides **7a-g**. It started by *N* alkylation of different isatins *via* methyl bromide **5a** or benzyl bromide **5b** to afford derivatives **6a-f**, which subsequently treated at reflux temperature with the key intermediates **3a, b** in glacial acetic acid in the presence of sodium acetate to furnish the target compounds **7a-g** (Scheme **2**).



7	*p/m*	R	X
a	*p*	H	H
b	*p*	H	Cl
c	*p*	H	Br
d	*p*	C_6H_5	H
e	*p*	C_6H_5	Cl
f	*p*	C_6H_5	Br
g	*m*	C_6H_5	H

Scheme 2. Synthesis of sulfonamides **7a-g**; *Reagents and conditions*: (**i**) DMF, K_2CO_3, reflux 3 h, (**ii**) Glacial acetic acid / Sodium acetate / reflux 3 h.

Postulated structures of the newly synthesized sulfonamides **4a-m** and **7a-g** were in full agreement with their spectral and elemental analyses data.

2.2. Biological Evaluation

2.2.1. Carbonic anhydrase inhibition

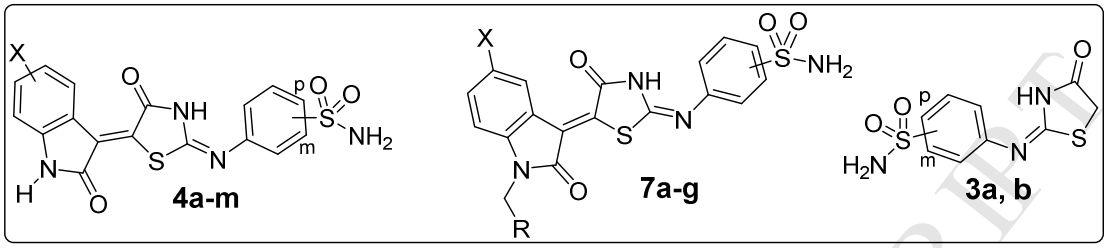
The newly prepared sulfonamides **3a-b**, **4a-m** and **7a-g** were evaluated for their ability to inhibit the physiologically relevant hCA isoforms, hCA I, II (cytosolic) and hCA IV (transmembrane) as well as the tumor-associated hCA IX (transmembrane), by a stopped-flow CO_2 hydrase assays [31], in comparison to the clinically used acetazolamide (**AAZ**) as standard CAI. The following structure–activity relationship (SAR) can be compiled from the inhibition data reported in **Table 1**:

(i) The cytosolic isoform hCA I was moderately inhibited by most of the sulfonamides with inhibition constants (K_Is) ranging in the high nanomolar - low micromolar range, in detail, between 513.7 and 9269.7 nM, except for the *p*-substituted precursor **3a** which arose as the best hCA I inhibitor with a K_I of 91.9 nM.

(ii) The physiologically dominant isoform hCA II was very potently inhibited by most of the *p*-substituted derivatives **3a**, **4a-h**, **7a-7f** (K_I values ranging between 1.8 and 69.6 nM, Table 1), apart from the *N*-unsubstituted 5-Br and 5,7-$(CH_3)_2$ isatin-bearing derivatives **4d** and **4h**, which exhibited a slightly reduced inhibitory efficacy (K_I values of 210.5 and 466.0 nM). The incorporation of a methyl group on the isatin core (**7a-c**) did not substantially interfere with the derivatives hCA II inhibitory potency, whereas an analogue benzylic substitution (**7d-f**) led to a three-fold to twenty-fold efficacy enhancement likened to the *N*-unsubstituted compounds (**4a-4g**).

Conversely, it is noteworthy that all the *m*-substituted derivatives (**4i-m**) were found to possess a generally ten-fold diminished hCA II inhibition efficacy (K_Is ranging between 176.4 and 598.2 nM) in comparison to their *p*-substituted analogues. Likewise, the *meta*-substituted precursor **3b** (K_I of 73.2 nM) was forty times less potent than **3a** (K_I of 1.8 nM). On the other hand, the *N*-benzyl moiety furnished the 3-substituted **7g** with a comparable inhibitory potency likened to the 4-substituted *N*-benzyl analogues (**7d-f**).

Table 1. Inhibition data of human CA isoforms hCA I, II, IV and IX with sulfonamides **3a-b**, **4a-m** and **7a-g** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO2 hydrase assay[31]



Comp.	*p/m*	X	R	K_I (nM)*			
				hCA I	**hCA II**	**hCA IV**	**hCA IX**
4a	*p*	H	-	630.8	23.0	>10000	47.0
4b	*p*	5-F	-	513.7	7.1	4290.5	26.6
4c	*p*	5-Cl	-	2290.4	69.6	3256.1	62.4
4d	*p*	5-Br	-	3611.4	210.5	2986.4	66.6
4e	*p*	5-OCH_3	-	2881.6	24.3	>10000	23.1
4f	*p*	5-CH_3	-	4243.7	57.4	2864.4	72.1
4g	*p*	5-NO_2	-	2883.7	35.7	3682.3	34.1
4h	*p*	5,7-$(CH_3)_2$	-	7602.0	466.0	4751.0	100.1
4i	*m*	H	-	2662.6	273.8	1972.7	16.1
4j	*m*	5-Cl	-	728.5	598.2	395.5	62.0
4k	*m*	5-Br	-	718.7	416.0	235.0	15.9
4l	*m*	5-OCH_3	-	3424.4	176.4	2526.6	24.7
4m	*m*	5-NO_2	-	884.2	506.6	441.9	48.0
7a	*p*	H	H	4001.7	43.5	4712.2	146.3
7b	*p*	5-Cl	H	923.9	38.4	3040.7	123.3
7c	*p*	5-Br	H	872.7	17.8	2634.0	38.9
7d	*p*	H	C_6H_5	5630.7	8.3	3299.6	117.5
7e	*p*	5-Cl	C_6H_5	881.1	2.6	4305.8	90.6
7f	*p*	5-Br	C_6H_5	9269.7	59.8	2897.3	321.1
7g	*m*	H	C_6H_5	856.4	18.2	2244.6	169.8
3a	*p*		-	91.9	1.8	348.3	4.5
3b	*m*		-	741.4	73.2	101.9	17.7
AAZ	-	-	-	250	12	74	25

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

(iii) The data reported in Table 1 ascribed to the most reported sulfonamides poor efficacy in inhibiting the trans-membrane isoform hCA IV. Indeed, the *p*-substituted derivatives **4a-h**, **7a-f** were shown to inhibit such isozyme in the micromolar range (K_Is ranging between 2634.0 and 4751.0 nM), except **4a** and **4e** which did not inhibit hCA IV up to 10000 nM. It is worth highlighting that the 3-substituted **4i-m** showed a generally improved inhibitory profile against hCA IV, in particular derivatives **4j**, **4k** and **4m (**K_Is of 395.5, 235.0 and 441.9 nM), which were endowed with electro-withdrawing substituents at the 5- position of the isatin scaffold (respectively 5-Cl, 5-Br, 5-NO_2). The precursors **3a** and **3b**, devoid of the isatin core appended at the oxothiazolidin-2-ylidene moiety, arose among the best inhibitors against such trans-membrane isozyme, again with the *m*-substitution preferred to the *p*-substituted one (K_Is of 348.3 vs. 101.9 nM).

(iv) The target tumor-associated isoform hCA IX was effectively inhibited by all the isatin-bearing sulfonamides herein reported. Furthermore, the inhibition profiles were found to be rather flat, since the measures K_Is ranged between 15.9 and 169.8 nM, aside from derivative **7f** whose efficacy raised at slightly higher concentration (K_I of 321.1 nM). It is worth stressing the comparable inhibitory effectiveness of the *N*-unsubstituted 3- and 4-pendant bearing derivatives (although slightly heightened for derivatives **4i-4m**; K_Is ranging between 16.1 and 62.0 nM), unlike arose for the cytosolic hCA II.

The incorporation of a methyl or benzyl group on the isatin N atom elicit a worsening of effectiveness against hCA IX both for the *p*-substituted derivatives **7a-7f** (except **7c**, 5-Br; K_I of 38.9 nM) and the *m*-pendant bearing **7g** (K_I 169.8 nM). Finally, the isatin-devoid precursor **3a** and **3b** inhibited hCA IX in the low nanomolar range with K_Is of 4.5 and 17.7 nM.

(v) The inhibitory profiles reported in Table 1 undeniably ascribed to the m-substituted amminobenzenesulfonamide **4i-4m** a rather selective hCA IX/II inhibitory efficacy. Indeed, it is satisfying to note that such compounds displayed a selectivity ratio hCA IX/hCA II which spanned from 7.1 and 26.2. Considering that isoform hCA IX is a validated target for the diagnosis and treatment of cancers, discovery of selective inhibitors represents a promising step to unveil a more effective cancer therapy devoid of the classical side effects owing to hCA I/II inhibition.

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

Anti-proliferative activity of the newly synthesized sulfonamides (**4a-m** and **7a-g**) was evaluated against breast cancer MCF-7 and colorectal cancer Caco-2 cell lines. Doxorubicin was included in the experiment as a reference drug. The results were expressed as IC_{50} values, and listed in **Table 2**.

Table 2. *In vitro* anti-proliferative activity of sulfonamides **4a-m** and **7a-g** against breast MCF-7 and colorectal Caco-2 cancer cell lines.

Comp.	**IC_{50} (µM)**[a]	
	MCF-7	**Caco-2**
4a	NA[b]	NA[b]
4b	20.50 ± 2.25	59.90 ± 4.01
4c	3.96 ± 0.21	20.30 ± 1.35
4d	11.3 ± 0.77	42.30 ± 2.57
4e	125.0 ± 10.53	98.30 ± 6.28
4f	NA[b]	174.0 ± 11.09
4g	NA[b]	13.80 ± 1.16
4h	NA[b]	146 ± 9.44
4i	NA[b]	12.10 ± 1.05
4j	NA[b]	5.87 ± 0.37
4k	NA[b]	10.80 ± 0.81
4l	NA[b]	8.39 ± 0.55
4m	NA[b]	20.62 ± 1.64
7a	51.9 ± 4.74	163 ± 14.74
7b	NA[b]	42.30 ± 4.02
7c	196 ± 9.75	13.20 ± 1.16
7d	NA[b]	NA[b]
7e	124 ± 6.21	13.70 ± 1.24
7f	NA[b]	57.80 ± 3.10
7g	NA[b]	103 ± 8.25
Dox.	2.37 ± 0.09	3.83 ± 0.19

[a] IC_{50} values are the mean ± S.D. of three separate experiments.
[b] NA: Compounds having IC_{50} value >200 µM.

As shown in table 2, the Caco-2 cell line is more susceptible to the synthesized sulfonamides than MCF-7 cancer cell line. Investigations of the anti-proliferative activity against MCF-7 indicated that compounds **4c** and **4d**, only, displayed good activity with IC_{50} values of 3.96±0.21 and 11.3±0.77 µM, respectively. While compounds **4b** and **7a**

were moderately active with IC_{50} = 20.50±2.25 and 51.9±4.74 µM, respectively. Also, compounds **4e**, **7c** and **7e** elicited weak activity with IC_{50} values ranging from 124±6.21 to 196 ± 9.75 µM.

Concerning activity against Caco-2 cells, the synthesized sulfonamides showed excellent to modest growth inhibitory activity. Compound **4j** (IC_{50} = 5.87±0.37 µM) was found to be the most potent derivative overall the tested compounds against Caco-2 as it was 1.5 times less active than doxorubicin (IC_{50} = 3.83±0.19). Besides, compounds **4g**, **4i**, **4k**, **4l**, **7c** and **7e** displayed good activity with IC_{50} values of 13.80±1.16, 12.10±1.05, 10.8±0.81, 8.39±0.55, 13.20±1.16 and 13.70±1.24 µM, respectively. Moreover, compounds **4b-d**, **4m**, **7b** and **7f** elicited moderate anti-proliferative activity (IC_{50}: 20.30±1.35 - 59.90±4.01 µM). Furthermore, compounds **4e**, **4f**, **4h**, **7a** and **7g** showed fair anti-proliferative against Caco-2 with IC_{50} values ranging from 98.30±6.28 to 174 ± 11.09 µM.

2.2.3. Induction of apoptosis in breast cancer MCF-7 cells

2.2.3.1. Effects on mitochondrial apoptosis pathway (Bcl-2 family) proteins

The Bcl-2 family, the best characterized protein family involved in the regulation of apoptosis, is consisting of anti-apoptotic and pro-apoptotic members [32]. The anti-apoptotic members, such as Bcl-2, attenuate apoptosis either by preventing the release of mitochondrial apoptogenic factors like cytochrome c into the cytoplasm or by sequestering pro-forms of the caspases. On the other hand, pro-apoptotic members of Bcl-2 family, such as Bax, trigger the release of caspases [32]. Thence, the Bcl-2 family of proteins acts as a crucial life–death decision point within the common pathway of apoptosis.

Herein, the study was further substantiated to investigate the ability of compound **4c** to provoke apoptosis in MCF-7 cells. Treatment of MCF-7 cells with compound **4c** significantly increased the expression levels of the pro-apoptotic molecule Bax by 8.5 folds compared to control (**Table 3**). In contrast, exposure of MCF-7 cells to compound **4c** resulted in significant decrease in the protein expression levels of the anti-apoptotic protein Bcl-2 by about 45 % compared to control (**Table 3**). Analyzing the results revealed that sulfonamide **4c** boosted the Bax/Bcl-2 ratio fifteen folds in comparison to the control,

where the bax/bcl-2 ratio serving to as a decisive value to determine cell susceptibility to apoptosis [33].

Table 3. Effect of compound **4c** on the expression levels of Bax, Bcl-2, active caspases-3 and -9, cytochrome C and p53 levels in MCF-7 breast cancer cells treated with the compound at its IC_{50} concentration.

Comp.	**Bax** ng/ml	**Bcl-2** ng/ml	**Bax/Bcl-2 ratio**	**Caspase-3** ng/ml	**Caspase-9** ng/ml	**Cyt-c** ng/ml	**p53** ng/ml
Control	1.64	0.46	3.56	0.049	1.64	0.234	172
4c	14.03***	0.26*	53.96	0.195*	14.03***	0.395*	860.8**

Data are mean ± SD of three separate experiments. * Significantly different from control (1% DMSO) at P <0.05. ** Significantly different from control at P <0.01. *** Significantly different from control at P <0.001.

2.2.3.2. Effects on the levels of active caspase-3 and caspase-9

Caspases, cysteine-containing aspartic acid-specific proteases, provide pivotal links in cell regulatory networks controlling cell death [34]. Caspase-3 is the key executioner protease which activated by upstream initiator caspases as caspase-9 [34]. Therefore, the elevated Bax/Bcl-2 ratios aroused the investigation of the protein expression levels of active caspases-3 and caspases-9. Treatment of MCF-7 cells with compound **4c** produced a significant elevation in the level of active caspase-3 and caspases-9 by about 4 and 8.5 folds, respectively, compared to control (**Table 3**).

2.2.3.3. Effect on the level of Cytochrome C

As a result of Bax/Bcl2 increments, mitochondrial cytochrome C is released into the cytosol where it potentiates a cascade of caspases that finally triggers the executioner caspase; caspase 3 [35]. Thus, we measured the level of cytochrome C to assure the adoption of the intrinsic pathway. Results revealed that cytochrome C level increased by 1.7 folds, compared to control, upon treatment of MCF-7 cells with the IC_{50} of sulfonamide **4c** (**Table 3**).

2.2.3.4. Effect on the level of p53

The p53 tumor suppressor gene is critically involved in cell cycle regulation, DNA repair, and programmed cell death [36]. Finally, in this study we analyzed p53 expression in breast

MCF-7 cancer cells after treatment with **4c**. Results confirmed that **4c** has strong ability to enhance tumor suppressor p53 expression by 5 folds compared to control (**Table 3**).

In conclusion, the ability of compound **4c** to down regulate Bcl-2 level while boosting Bax, caspase-3, caspase-9, cytochrome C and p53 levels confirms its effectiveness as apoptosis inducer through, at least in part, inducing the mitochondrial pathway of apoptosis.

2.3. Molecular modeling studies

The inhibitory profiles of the derivatives in Table 1 were further investigated carrying out docking and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) calculation to predict their binding mode and free energy as inhibitors of the hCA II (PDB 5LJT) [9] and IX (PDB 5FL4) [37a] isoforms.

As it could be foreseen, all docking solutions oriented the benzenesulfonamide moieties deeply into the active site region of both the isozymes. Indeed, it is worth highlighting that all the reported derivatives were found to inhibit both hCA II and IX in the nanomolar range. In detail, the sulfonamide established two hydrogen bonds with the Thr199 residue, its negatively charged nitrogen atom coordinated the zinc ion and the phenyl ring was involved in several hydrophobic contacts (V121, H94 and L198).

Despite the different size of the hydrophobic pocket in hCA II and hCA IX isoforms, mainly due to the Phe131/Val131 mutation, the tails of the *p*-substituted derivatives **3a**, **4a-4h** and **7a-7f** located comparably within the two isozyme binding sites (**Fig**.**3a** and **4a**). In detail, the heterocyclic tails were found to lie in the hydrophobic areas defined by Phe131, Ile91, Val121, Gln92 and Val131, Leu91, Val121 in hCA II and hCA IX, respectively. The C=O moiety of the oxothiazolidin-2-ylidene was involved in a three-centre H-bond involving the HE/Gln92 and HD/Asn67 (hCA II) or HE/Gln71 (hCA IX) hydrogen atoms as donor groups. The isatin scaffolds was stabilized by edge-to-face and π-alkyl hydrophobic interactions occurring with Phe131 (V131 in hCA IX) and Ile91. X-substitution on the isatin benzene ring did not substantially affect ligands positioning within the hydrophobic pockets, although they differently interacted with the residues nearby (**Fig**.**3b** and **4b**). It is worth noting that the contacts involving the *N*-methyl and *N*-benzyl groups of derivatives **7a-7f** within the aforementioned hydrophobic regions contributed in increasing the inhibitory

efficacy against the cytosolic isozyme, whereas they had no, or at least little, influence on the inhibitory efficacy of the hCA IX.

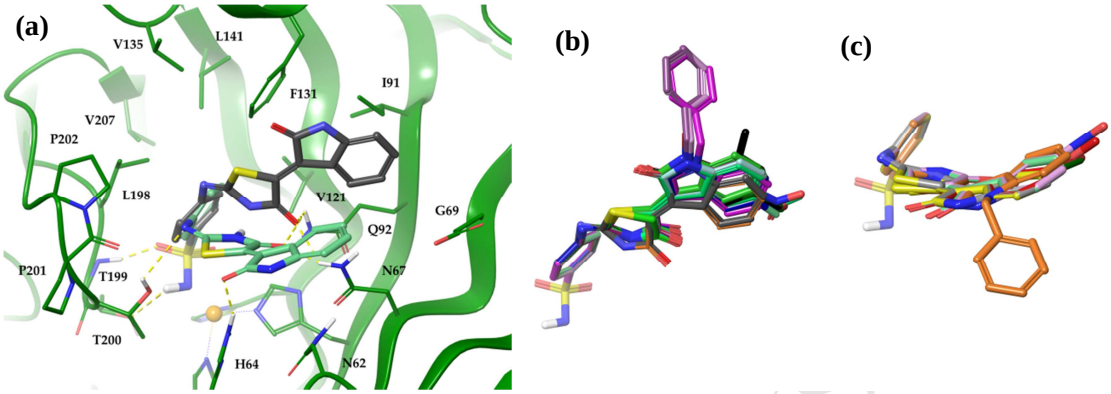


Figure 3. (**a**) Simulated binding modes of compounds **4a** (4-substituted, grey) and **4i** (3-substituted, aquamarine) within hCA II active site; (**b**) 4-substituted and (**c**) 3-substituted benzenesulfonamides docked within hCA II active site.

Conversely, docking solutions found for the 3- substituted derivatives **3a**, **4i-4m** and **7g** identified two cavities, which differ depending on the enzyme isoform considered, within which the N-pendant on the amminobenzensulfonamide moieties locate (**Fig.3a** and **4a**). In hCA IX, the pocket defined by Val131, Leu91, Gln67, Ser69 and Gln92 is roomy enough to accommodate the N-pendant, though rotated by 90° (**Fig**. **4a**). Again, the endocyclic carbonyl of the oxothiazolidin-2-ylidene acts as H-bond acceptor to HE/Gln67 which, in turn, is involved in weak C-H···π H-bonds with Val121 and Leu91 (**Fig**. **4a**). The isatin benzene ring additional formed π-alkyl hydrophobic interactions with Ser69.

In hCA II, the *N*-pendant of the amminobenzensulfonamide was oriented towards Asn62 and His64, and was engaged in several H-bonds (**Fig.3a**). The imine nitrogen act as H-bond acceptor from HG/Thr200, the carbonyl of the oxothiazolidin-2-ylidene was part of bifurcated H-bonds with donor being HE/Gln92 and HD/Asn67, whereas the C=O of the isatin accepts the HE/His64. The isatin benzene ring formed additional π- alkyl hydrophobic contacts with Leu60. The introduction of a benzyl group on the nitrogen atom of the isatin provided the 3-substituted compound **7g** with an inhibitory efficacy against hCA II comparable to the one observed for the 4-substituted N-benzyl derivatives **7d**-**7f**. Again, the

type and nature of the X-substitution at the isatin benzene ring did not substantially affect ligands positioning within both the isozymes binding pockets.

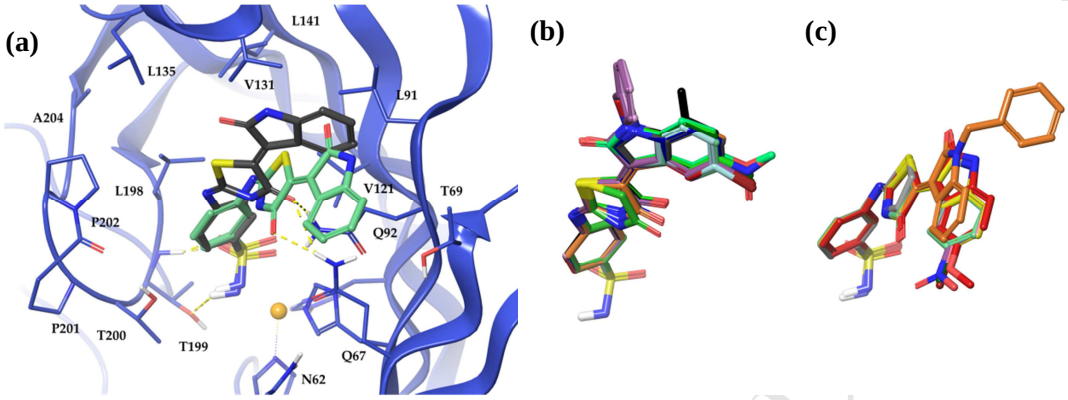


Figure 4. (**a**) Simulated binding modes of compounds **4a** (4-substituted, grey) and **4i** (3-substituted, aquamarine) within hCA IX active site; (**b**) 4-substituted and (**c**) 3-substituted benzenesulfonamides docked within hCA IX active site.

Analysing the results obtained from docking simulations, it is possible to assume that switching the substituents from the *para* to the *meta* position caused the increase in the steric hindrance that, together with a torsional strain, prevent the heterocyclic tails of the 3-substituted derivatives to lie within the hCA II hydrophobic cavity, thus worsening the inhibitory efficacy of the compounds against such isoform.

Basing on the enzyme-inhibitor complexes obtained from molecular docking, ΔG binding energy were calculated using the Prime MM-GBSA approach [37b], either or not defining a 4Å shell from the ligands within which hCA II and hCA IX residues were relaxed during the computations (**Table 4**).

For all derivatives, but **7d-f** in the case of hCA II, the ranking of the ligands based on the calculated MM-GBSA $\Delta G_{binding}$ values substantially agreed with ranking based on experimental inhibition data. As far as it concerns the hCA IX isoform, the ΔG binding values found reflected the experimental inhibition data in Table1 (**Fig. 5**). In the case of hCA II, a better agreement was achieved not considering the **7d-f** (featured by the *N*-benzyl substitution at the isatin scaffold).

Within the here considered congeneric series of sulfonamides, the MM-GBSA approach has shown good success in rank-ordering the inhibitor potencies of the studied compounds making it possible to prioritize derivatives for synthesis as inhibitors against hCA II and hCA IX enzyme isoforms.

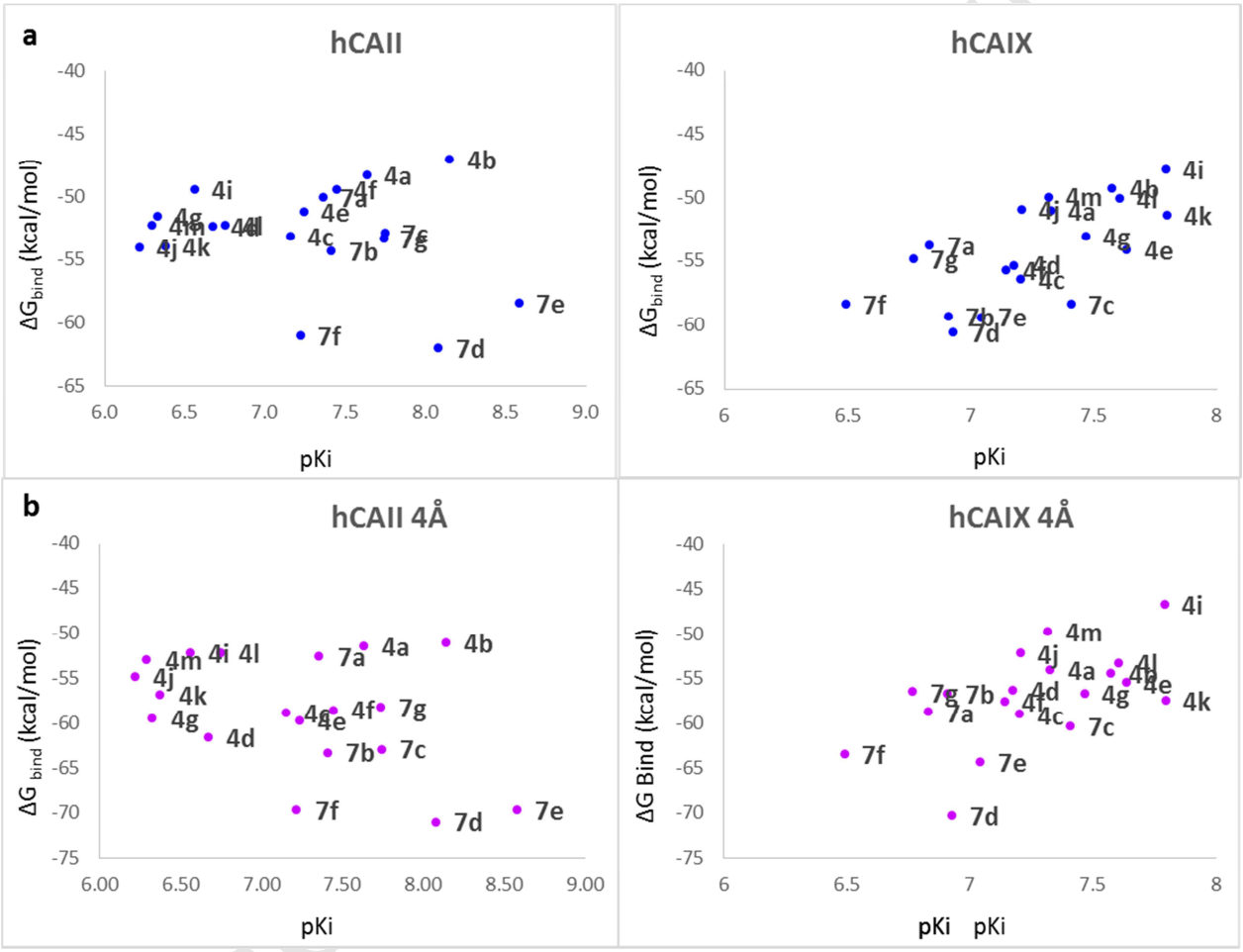


Figure 5. MMGBSA ΔG binding energy vs. pKi values. Because of the absence of the isatin moiety, derivatives **3a** and **3b**, were not considered. Energy values were computed (**a**) not allowing residues to relax and (**b**) defining an enzyme flexible region (4Å from the ligand).

Table 4. Results of the MM-GBSA calculation on hCA II (5LJT) and hCA IX (5FL4)

Compound	ΔG_{exp} (kcal/mol)			
	5LJT	5LJT 4Å [(a)]	5FL4	5FL4 4Å[(a)]
3a	-33.965	-42.301	-34.196	-34.543
3b	-37.229	-39.449	-37.523	-36.224
4a	-48.294	-51.442	-51.102	-54.033
4b	-47.054	-51.073	-49.334	-54.442
4c	-53.238	-58.898	-56.486	-59.021
4d	-52.393	-61.659	-55.364	-56.322
4e	-52.470	-58.807	-54.195	-55.507
4f	-51.247	-59.706	-55.772	-57.587
4g	-49.431	-58.535	-53.066	-56.657
4h	-51.580	-59.519	-56.823	-59.265
4i	-49.434	-52.147	-47.805	-46.780
4j	-54.111	-54.874	-51.015	-52.226
4k	-54.057	-56.809	-51.408	-57.458
4l	-52.317	-52.146	-50.098	-53.287
4m	-52.289	-52.928	-49.969	-49.812
7a	-50.096	-52.559	-53.794	-58.783
7b	-54.387	-63.293	-59.336	-56.696
7c	-52.935	-62.923	-58.048	-60.361
7d	-58.443	-71.069	-60.630	-70.339
7e	-62.064	-69.749	-59.418	-64.349
7f	-61.063	-69.790	-58.381	-63.488
7g	-53.362	-58.269	-54.903	-56.508

[(a)] Residues within 4Å from the ligand were treated as flexible

3. Conclusion

In summary, this study reports the synthesis of two series of novel sulfonamides (**4a-m** and **7a-g**) incorporating substituted indolin-2-one moieties linked to benzenesulfonamide (as zinc anchoring moieties). The newly synthesized sulfonamides were evaluated *in vitro* for their inhibitory activity against hCA I, II, IV and IX. All these isoforms were inhibited by the sulfonamides reported here in variable degrees. hCA isoforms II and IX were more susceptible to the synthesized sulfonamides, with K_Is in the range of 2.6–598.2 nM for hCA II, and of 16.1–321 nM for hCA IX. Noteworthy, *m*-substituted derivatives **4i-m** were found to exhibit a selective hCA IX/II inhibitory efficacy. Moreover, all sulfonamides were examined for their anti-proliferative activity against breast cancer MCF-7 and colorectal cancer Caco-2 cell lines. In particular, compound **4c** was the most potent derivative against MCF-7 (IC_{50} = 3.96 ± 0.21)**,** while **4j** was the most active member against Caco-2 cells (IC_{50} = 5.87 ± 0.37). Interestingly, compound **4c** provoked the intrinsic apoptotic mitochondrial pathway in MCF-7 cells; evidenced by the enhanced expression of the pro-apoptotic protein Bax and the reduced expression of the anti-apoptotic protein Bcl-2, and the up-regulated active caspase-3 and caspase-9, cytochrome C and p53 levels. Finally, the inhibitory profiles of the synthesized derivatives were further investigated carrying out docking and Molecular Mechanics Generalized Born Surface Area (MM-GBSA) calculation to predict their binding mode and free energy as inhibitors of the hCA II (PDB: 5LJT) and IX (PDB: 5FL4) isoforms.

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were measured with a Stuart melting point apparatus and were uncorrected. Infrared spectra were recorded on Schimadzu FT-IR 8400S spectrophotometer. The NMR spectra were recorded by Varian Mercury or Bruker spectrophotometers at 400 MHz. ^{13}C NMR spectra were run at 100 MHz in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts (δ_H) are reported relative to TMS as

internal standard. All coupling constant (*J*) values are given in hertz. Chemical shifts (δ_C) are reported relative to DMSO-d_6 as internal standards. Elemental analyses were carried out at the Regional Center for Microbiology and Biotechnology, Al-Azhar University, Cairo, Egypt. Unless otherwise noted, all solvents and reagents were commercially available and used without further purification.

4.1.2. General procedure for preparation of 2-chloro-N-(4/3-sulfamoylphenyl)acetamides **2a**, **b**.

To a solution of sulfanilamides **1a**, **b** (1.72 gm, 10 mmol) in dry DMF, chloroacetyl chloride (0.8 mL, 10 mmol) was added dropwise at 0°C then stirred at room temperature for 2 h. The reaction mixture was then poured into ice-water. The obtained solid was filtered off, washed with water several times and crystallized from dioxane to give compounds **2a**, **b** [38-40].

4.1.3. General procedure for preparation of 4/3-((4-oxothiazolidin-2-ylidene)amino) benzenesulfonamides **3a,b**.

To a hot solution of derivatives **2a**,**b** (1.24 gm, 5 mmol) in absolute ethyl alcohol, ammonium thiocyanate (0.76 gm, 10 mmol) was added. The reaction mixture was refluxed for 4 h then allowed to cool to room temperature. The formed precipitate was filtered off, washed with water and recrystallized from ethanol to furnish the key intermediates **3a**, **b**.

4.1.3.1. 4-((4-Oxothiazolidin-2-ylidene)amino)benzenesulfonamide (**3a**). [41]

4.1.3.2. 3-((4-Oxothiazolidin-2-ylidene)amino)benzenesulfonamide (**3b**). White powder (yield 80%), m.p. > 300 °C ; IR (KBr, ν cm^{-1}): 3183 (NH), 1675 (C=O) and 1331, 1159 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 4.00 (s, 2H, C$\underline{H_2}$), 7.14 (d, 1H, Ar-H, *J* = 8.4 Hz), 7.37 (s, 2H, NH_2, D_2O exchangeable), 7.54-7.56 (m, 2H, Ar-H), 7.85 (d, 1H, Ar-H, *J* = 8.48 Hz), 11.39 (s, 1H, NH, D_2O exchangeable); Anal. Calcd. (Found) For $C_9H_9N_3O_3S_2$: C, 39.84 (40.11); H, 3.34 (3.40); N, 15.49 (15.82).

4.1.4. General procedure for synthesis of the target sulfonamides **4a-m.**

The appropriate isatin derivatives was added to a hot stirred solution of the key intermediates **3a, b** (0.27 gm, 1 mmol) and sodium acetate (0.16 gm, 2 mmol) in glacial acetic acid (15 mL). The reaction mixture was heated under reflux for 3 h. The formed solid was filtered off while hot, washed with hot ethanol, dried and recrystallized from DMF/ethanol to afford the target sulfonamides **4a-m**.

4.1.4.1. 4-((4-Oxo-5-(-2-oxoindolin-3-ylidene)thiazolidin-2-ylidene)amino) benzenesulfonamide (**4a**). Red powder (yield 72%); m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3290, 3255 (NH, NH_2), 1663 (C=O) and 1330, 1153 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.89-7.09 (m, 2H, Ar-H), 7.20 (d, 0.5H, Ar-H, J = 8.0 Hz), 7.32-7.39 (m, 1.5H, Ar-H), 7.52 (s, 2H, NH_2, D_2O exchangeable), 7.65 (d, 1H, Ar-H, J = 8.4 Hz), 7.86 (t, 1H, Ar-H, J = 8.36 Hz), 7.96 (d, 1H, Ar-H, J = 8.32 Hz), 8.71 (d, 0.5H, Ar-H, J = 7.88 Hz), 8.80 (d, 0.25H, Ar-H, J = 7.84 Hz), 8.94 (d, 0.25H, Ar-H, J = 7.52 Hz), 10.02, 12.03 (2s, 1H, NH isatin, D_2O exchangeable), 11.15, 12.58 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 110.71, 120.60, 120.85, 121.19, 122.09, 122.23, 122.37, 124.11, 125.32, 126.77, 127.47, 127.71, 128.12, 128.43, 129.90, 131.98, 132.25, 132.37, 133.05, 133.82, 137.74, 140.63, 143.57, 143.63, 144.50, 156.61, 165.75, 168.91, 169.04, 169.33; MS, *m/z* [%]: 400 [M^+, 2.97]; Anal. Calcd. (Found) for $C_{17}H_{12}N_4O_4S_2$: C, 50.99 (51.23); H, 3.02 (3.08); N, 13.99 (14.21).

4.1.4.2. 4-((5-(5-Fluoro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4b**). Red powder (yield 70%); m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3294, 3271 (NH, NH_2), 1693 (C=O) and 1334, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.86-6.94 (m, 1H, Ar-H), 7.17-7.21 (m, 1H, Ar-H), 7.35-7.39 (m, 1H, Ar-H), 7.52 (s, 2H, NH_2, D_2O exchangeable), 7.65 (d, 1H, Ar-H, J = 8.4 Hz), 7.86-7.99 (m, 2H, Ar-H), 8.48 (d, 0.5H, Ar-H, J = 10.28 Hz), 8.58 (d, 0.25H, Ar-H, J = 10.04 Hz), 8.72 (d, 0.25H, Ar-H, J = 9.4 Hz), 10.11, 11.99 (2s, 1H, NH isatin, D_2O exchangeable), 11.16, 12.74 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 111.38, 111.45, 114.53, 114.80, 115.14, 118.13, 118.37, 121.29, 121.40, 122.08, 123.46, 126.80, 127.48, 127.72, 129.87, 134.80, 135.68, 137.59, 139.89, 139.98, 140.05, 140.88, 144.58, 156.31, 156.74, 159.07, 165.78, 168.86, 169.01, 169.28; MS, *m/z* [%]: 418 [M^+,

4.8]; Anal. Calcd. (Found) for $C_{17}H_{11}FN_4O_4S_2$: C, 48.80 (48.97); H, 2.65 (2.63); N, 13.39 (13.60).

4.1.4.3. 4-((5-(5-Chloro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4c**). Red powder (yield 62%); m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3295, 3249 (NH, NH_2), 1690 (C=O) and 1330, 1153 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.88-6.95 (m, 1H, Ar-H), 7.20 (d, 1H, Ar-H, *J* = 8.2 Hz), 7.35-7.39 (m, 1H , Ar-H), 7.52 (s, 2H, NH_2, D_2O exchangeable), 7.65 (d,1H, Ar-H , *J* = 8.4 Hz), 7.86-7.99 (m, 1H, Ar-H), 7.95-7.99 (m, 1H, Ar-H), 8.72 (s, 0.25H, Ar-H), 8.81 (s, 0.5H, Ar-H), 8.96 (s, 0.25 H, Ar-H), 10.12, 11.97 (2s, 1H, NH isatin, D_2O exchangeable), 11.26, 12.71 (2s, 1H, NH thiazolidinone, D_2O exchangeable); Anal. Calcd. (Found) for $C_{17}H_{11}ClN_4O_4S_2$: C, 46.95 (47.32); H, 2.55 (2.59); N, 12.88 (13.04).

4.1.4.4. ((5-(5-Bromo-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4d**). Red powder (yield 75%); m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3332, 3251 (NH, NH_2), 1693 (C=O) and 1334, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.83-6.90 (m, 1H, Ar-H), 7.20 (d, 1H, Ar-H, *J* = 7.36 Hz), 7.35-7.39 (m, 1H, Ar-H), 7.47-7.52 (m, 3H, 1H Ar-H and 2H of NH_2, D_2O exchangeable), 7.65 (d, 0.5H, Ar-H, *J* = 8.0 Hz), 7.86-7.99 (m, 1.5H, Ar-H), 8.85 (s, 0.25H, Ar-H), 8.94 (s, 0.5H, Ar-H), 9.09 (s, 0.25H, Ar-H), 10.12, 12.30 (2s, 1H, NH1isatin, D_2O exchangeable), 11.25, 12.62 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 112.52, 113.89, 113.96, 121.30, 122.09, 122.35, 122.54, 123.91, 126.82, 127.46, 127.72, 129.85, 130.12, 130.46, 133.96, 134.23, 134.33, 135.12, 135.91, 137.57, 140.73, 140.84, 142.60, 142.68, 144.58, 156.24, 165.74, 168.50, 168.63, 168.92; Anal. Calcd. (Found) for $C_{17}H_{11}BrN_4O_4S_2$: C, 42.60 (42.76); H, 2.31 (2.24); N, 11.69 (11.87).

4.1.4.5. 4-((5-(5-Methoxy-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4e**). Red powder (yield 55%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3321, 3248 (NH, NH_2), 1708 (C=O) and 1330, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 3.71 (s, 3H, $OC\underline{H_3}$), 6.79-7.82 (m, 7H, 4 Ar-H and 2H, NH_2, D_2O exchangeable), 8.39-8.63 (m, 2H, Ar-H), 10.89 (s, 1H, NH isatin, D_2O exchangeable),

12.29 (2s, H, NH thiazolidinone, D_2O exchangeable); MS, *m/z* [%]: 430 [M^+, 8.18]; Anal. Calcd. (Found) for $C_{18}H_{14}N_4O_5S_2$: C, 50.23 (50.60); H, 3.28 (3.37); N, 13.02 (13.34).

4.1.4.6. 4-((5-(5-Methyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4f**). Red powder (yield 73%), m.p. > 300 °C ; IR (KBr, *v* cm^{-1}): 3321, 3294 (NH, NH_2), 1693 (C=O) and 1330, 1157 (SO_2); ^1H (DMSO-d_6, 400 MHz) δ *ppm:* 2.48 (s, 3H, $C\underline{H_3}$), 6.75-6.82 (m, 1H, Ar-H), 7.12-7.19 (m, 1H, Ar-H), 7.31-7.35 (m, 1H, Ar-H), 7.49 (s, 2H, NH_2, D_2O exchangeable), 7.62-7.64 (m,1H, Ar-H), 7.82-7.96 (m, 2H, Ar-H), 8.54 (s, 0.5H, Ar-H), 8.62 (s, 0.25H, Ar-H), 8.75 (s, 0.25H, Ar-H), 9.97, 11.86 (2s, 1H, NH isatin , D_2O exchangeable), 11.00, 12.59 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 21.34, 21.44, 110.38, 120.63, 120.71, 121.13, 122.07, 124.36, 126.77, 127.44, 127.67, 128.53, 128.83, 129.92, 130.91, 130.99, 132.38, 132.67, 133.36, 137.75, 140.60, 141.34, 141.41, 141.47, 144.52, 156.66, 165.76, 168.92, 169.06, 169.45, 169.92; MS, *m/z* [%]: 414 [M^+, 6.69]; Anal. Calcd. (Found) for $C_{18}H_{14}N_4O_4S_2$: C, 52.16 (52.32); H, 3.40 (3.46); N, 13.52 (13.75).

4.1.4.7. 4-((5-(5-Nitro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4g**). Red powder (yield 62%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3352, 3263 (NH, NH_2), 1693 (C=O), 1527, 1362 (NO_2) and 1342, 1157 (SO_2); ^1H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 7.00-7.07 (m, 1H, Ar-H), 7.18 (d, 1H, Ar-H, *J* = 8.4 Hz), 7.33-7.36 (m, 1H, Ar-H), 7.48 (s, 2H, NH_2, D_2O exchangeable), 7.64-7.66 (m, 1H, Ar-H), 7.84-7.98 (m, 1.5H, Ar-H), 8.18-8.23 (m, 0.5H, Ar-H), 9.53 (s, 0.5H, Ar-H), 9.63 (s, 0.25H, Ar-H), 9.75 (s, 0.25H, Ar-H), 10.22 (s, 1H, NH isatin, D_2O exchangeable), 11.73 (t, 1H, NH thiazolidinone, D_2O exchangeable, *J* = 8.8 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 110.80, 110.86, 120.47, 120.57, 120.72, 121.42, 121.74, 122.06, 122.96, 123.23, 123.46, 123.59, 126.86, 127.42, 127.59, 127.71, 127.84, 129.82, 137.43, 137.82, 140.83, 140.90, 142.46, 142.55, 144.63, 148.61, 148.65, 155.71, 165.60, 169.17, 169.62; Anal. Calcd. (Found) for $C_{17}H_{11}N_5O_6S_2$: C, 45.84 (46.13); H, 2.49 (2.53); N, 15.72 (15.98).

4.1.4.8. 4-((5-(5,7-Dimethyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4h**). Red powder (yield 70%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3332, 3224 (NH, NH_2), 1685 (C=O) and 1323, 1153 (SO_2); ^1H NMR (DMSO-d_6, 400

MHz) δ *ppm*: 2.14 (s, 3H, C<u>H</u>$_3$), 2.26 (s, 3H, C<u>H</u>$_3$), 6.97 (s, 1H, H-6 isatin), 7.48 (s, 2H, NH_2, D_2O exchangeable), 7.61 (d, 2H, H-3 and H-5 of -C_6H_4-SO_2NH_2, *J* = 8.4 Hz), 7.93 (d, 2H, H-2 and H-6 of -C_6H_4-SO_2NH_2, *J* = 8.4 Hz), 8.4 (s, 1H, H-4 isatin) , 9.94 (s, 1H, NH isatin , D_2O exchangeable), 11.03 (s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 16.76, 21.24, 119.50, 120.47, 124.80, 126.03, 126.76, 129.93, 130.75, 133.15, 133.89, 137.78, 139.94, 144.50, 156.73, 165.76, 169.52; MS, *m/z* [%]: 428 [M^+, 3.99]; Anal. Calcd. (Found) for $C_{19}H_{16}N_4O_4S_2$: C, 53.26 (53.52); H, 3.76 (3.80); N, 13.08 (13.29).

4.1.4.9. 3-((4-Oxo-5-(2-oxoindolin-3-ylidene)thiazolidin-2-ylidene)amino) benzenesulfonamide (**4i**). Red powder (yield 75%); m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3307, 3217 (NH, NH_2), 1688 (C=O) and 1334, 1156 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.91 (m, 1H, Ar-H), 7.04 (q, 1H, Ar-H, *J* = 7.76 Hz), 7.26 (d, 0.5H, Ar-H, *J* = 7.32 Hz), 7.33 (q, 1.5H, Ar-H, *J* = 8.08 Hz), 7.45,7.50 (s, 2H, NH_2, D_2O exchangeable), 7.59-7.68 (m, 2H, Ar-H), 7.98 (s br, 1H, Ar-H), 8.33 (s, 0.5H, Ar-H), 8.81 (d, 0.25H, Ar-H, *J* = 7.84 Hz), 8.95 (d, 0.25H, Ar-H, *J* = 7.88 Hz), 11.15, 12.55 (2s, 1H, NH isatin, D_2O exchangeable), 11.89, 12.68 (2s, 1H, NH thiazolidinone, D_2O exchangeable); MS, *m/z* [%]: 400 [M^+, 3.83]; Anal. Calcd. (Found) for $C_{17}H_{12}N_4O_4S_2$: C, 50.99 (51.27); H, 3.02 (3.09); N, 13.99 (14.23).

4.1.4.10. 3-((5-(5-Chloro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4j**). Red powder (yield 72%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3317, 3275 (NH, NH_2), 1685 (C=O) and 1330, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.88-6.95 (m, 1H, Ar-H,), 7.23 (d, 0.5H, Ar-H, *J* = 7.2 Hz), 7.35-7.43 (m, 1.5H, Ar-H), 7.48 (s, 2H, NH_2, D_2O exchangeable), 7.57-7.66 (m, 2H, Ar-H), 7.95 (d, 0.5H, Ar-H, *J* = 6.8 Hz), 8.29 (s, 0.5H, Ar-H), 8.82 (s, 0.5H, Ar-H), 8.96 (s, 0.5H, Ar-H), 10.82, 11.94 (2s, 1H, NH isatin , D_2O exchangeable), 11.24, 12.76 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 112.06, 118.2, 118.92, 121.85, 122.08, 122.08, 122.34, 122.99, 123.33, 124.03, 124.35, 124.90, 125.16, 126.15, 127.55, 127.70, 130.58, 130.66, 131.46, 131.55, 135.40, 138.04, 138.90, 142.29, 142.33, 143.37, 145.55, 145.78, 146.91, 168.62, 169.08; Anal. Calcd. (Found) for $C_{17}H_{11}ClN_4O_4S_2$: C, 46.95 (47.18); H, 2.55 (2.57); N, 12.88 (13.07).

4.1.4.11. 3-((5-(5-Bromo-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4k**). Red powder (yield 72%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3282, 3209 (NH, NH_2), 1678 (C=O) and 1330, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 6.83-6.89 (m, 1H, Ar-H), 7.23 (s br, 0.5H, Ar-H), 7.43-7.51 (m, 3.5H, 1.5Ar-H and 2H of NH_2, D_2O exchangeable), 7.57-7.65 (m, 2H, Ar-H), 7.95 (s, 0.5H, Ar-H), 8.29 (s, 0.5H, Ar-H), 8.95 (s, 0.5H, Ar-H), 9.09 (s, 0.5H, Ar-H), 11.10, 11.94 (2s, 1H, NH isatin , D_2O exchangeable), 11.24, 12.76 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 112.53 (2 C), 113.90, 113.93, 118.26, 118.93, 121.85, 122.32, 122.55, 122.98, 123.90, 124.34, 125.02, 126.20, 127.55, 130.33, 130.46, 130.58, 130.66, 134.23, 134.23, 134.32, 135.24, 138.01, 138.90, 142.63, 142.67, 143.37, 145.54, 145.78, 168.49, 168.94, 174.94, 180.76; Anal. Calcd. (Found) for $C_{17}H_{11}BrN_4O_4S_2$: C, 42.60 (42.56); H, 2.31 (2.27); N, 11.69 (11.88).

4.1.4.12. 3-((5-(5-Methoxy-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4l**). Red powder (yield 70%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3309, 3275 (NH, NH_2), 1678 (C=O) and 1330, 1157 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 3.73, 3.75 (s, 3H, OC$\underline{H_3}$), 6.78-6.85 (m, 1H, Ar-H, *J* = 8.4 Hz), 6.92-6.97 (m, 1H, Ar-H), 7.24 (d, 0.5H, Ar-H, *J* = 6.8 Hz), 7.42,7.47 (2s, 2H, NH_2, D_2O exchangeable), 7.57-7.65 (m, 2.5H, Ar-H), 7.94 (d, 0.5H, Ar-H, *J* = 6 Hz), 8.32 (s, 0.5H, Ar-H), 8.50 (s, 0.5H, Ar-H), 8.65 (s, 0.5H, Ar-H), 10.92 (s, 1H, NH isatin , D_2O exchangeable), 11.87, 12.70 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 55.93, 110.98, 114.41, 117.77, 118.20, 118.90, 121.22, 121.47, 122.25, 122.84, 124.23, 124.97, 125.78, 126.91, 130.52, 130.64, 136.23, 137.46, 139.00, 145.55, 145.77, 154.98, 155.01, 168.84, 168.29, 175.19, 180.83; MS, *m/z* [%]: 430 [M^+, 1.6]; Anal. Calcd. (Found) for $C_{18}H_{14}N_4O_5S_2$: C, 50.23 (50.49); H, 3.28 (3.34); N, 13.02 (13.16).

4.1.4.13. 3-((5-(5-Nitro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**4m**). Red powder (yield 55%), m.p. > 300 °C; IR (KBr, *v* cm^{-1}): 3286, 3244 (NH, NH_2), 1693 (C=O) and 1330, 1153 (SO_2); ^{1}H NMR (DSMO-d6) δ *ppm*: 7.04 (q, 1H, Ar-H, *J* = 8.8 Hz), 7.25 (d, 1H, Ar-H, *J* = 8 Hz), 7.43, 7.48 (2s, 2H, NH_2, D_2O exchangeable), 7.58-7.66 (m, 2H, Ar-H), 7.95 (s, 1H, Ar-H), 8.22-8.29 (m,1H,Ar-H),), 9.68 (s, 0.5H, Ar-H), 9.84 (s, 0.5H, Ar-H), 11.77, 12.03 (2s, 1H, NH isatin, D_2O

exchangeable), 11.79, 12.95 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 110.84, 118.35, 120.56, 120.78, 123.07, 123.65, 124.42, 127.89, 127.98, 130.59, 130.71, 142.59, 142.62, 145.56, 145.81, 148.74, 148.80, 169.23, 169.71, 180.66; Anal. Calcd. (Found) for $C_{17}H_{11}N_5O_6S_2$: C, 45.84 (46.12); H, 2.49 (3.47); N, 15.72 (15.94).

4.1.5. N-Methyyl/Benzyl isatin **6a-f**.

Compounds **6a-f** were prepared according to the literature procedure [11].

4.1.6. General procedure for synthesis of targeted compounds **7a-g.**

To a hot stirred solution of the key intermediates **3a, b** (0.27 gm, 1 mmol) and sodium acetate (0.16 gm, 2 mmol) in acetic acid (15 mL), *N*-substituted isatins **6a-f** were added and this mixture was heated under reflux for 3 h. The formed solid was filtered while hot, dried and recrystallized from DMF/ethanol to obtain the target compounds **7a-g**.

4.1.6.1. 4-((5-(1-Methyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7a**). Red powder (yield 68%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3266, 3227 (NH, NH_2), 1675 (C=O) and 1335, 1151 (SO_2); ^1H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 3.29 (s, 3H, *N*-C<u>H</u>$_3$), 7.02 (t, 1H, H-5 isatin, *J* = 7.76 Hz), 7.13 (d, 1H, H-7 isatin, *J* = 7.8 Hz), 7.43 (t, 1H, H-6 isatin, *J* = 7.68 Hz), 7.51 (s, 2H, NH_2, D_2O exchangeable), 7.65 (d, 2H, H-3 and H-5 of -C_6H_4-SO_2NH_2, *J* = 8.44 Hz), 7.96 (d, 2H, H-2 and H-6 of -C_6H_4-SO_2NH_2, *J* = 8.44 Hz), 8.77 (d, 1H, H-4 isatin, *J* = 7.84 Hz), 10.08 (s, 1H, NH thiazolidinone, D_2O exchangeable); MS, *m/z* [%]: 414 [M^+, 3.06]; Anal. Calcd. (Found) for $C_{18}H_{14}N_4O_4S_2$: C, 52.16 (52.43); H, 3.40 (3.45); N, 13.52 (13.81).

4.1.6.2. 4-((5-(5-Chloro-1-methyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7b**). Red powder (yield 75%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3275, 3224 (NH, NH_2), 1678 (C=O) and 1327, 1153 (SO_2); ^1H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 3.25 (s, 3H, *N*-C<u>H</u>$_3$), 7.02-7.08 (m, 1H, Ar-H), 7.17 (d, 1H, Ar-H, *J* = 7.6 Hz), 7.32 (d, 1H, Ar-H, *J* = 8 Hz), 7.48 (s, 2H, NH_2, D_2O exchangeable) 7.56-7.65 (m, 1H, Ar-H), 7.83-7.96 (m, 2H, Ar-H), 8.87 (s, 0.5H, Ar-H), 8.95 (s, 0.25H, Ar-H), 9.09 (s, 0.25H, Ar-H), 10.15, 12.05 (2s, 1H, NH thiazolidinone, D_2O

exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 26.93, 111.42, 114.51, 121.31, 121.56, 126.80, 127.44, 127.69, 129.82, 129.86, 133.83, 136.66, 137.49, 143.52, 144.59, 155.88, 165.60, 167.08; Anal. Calcd. (Found) for $C_{18}H_{13}ClN_4O_4S_2$: C, 48.16 (48.40); H, 2.92 (2.89); N, 12.48 (12.69).

4.1.6.3. 4-((5-(5-Bromo-1-methyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7c**). Red powder (yield 59%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3275, 3221 (NH, NH_2), 1678 (C=O) and 1330, 1153 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 3.25 (s, 3H, *N*-C<u>H</u>$_3$), 7.12 (d, 1H, H-7 isatin, J = 8.44 Hz), 7.46 (dd, 1H, H-6 isatin, J = 2.08- 8.4 Hz), 7.52 (s, 2H, NH_2, D_2O exchangeable), 7.66 (d, 2H, H-3 and H-5 of -C_6H_4-SO_2NH_2, J = 8.48 Hz), 7.97 (d, 2H, H-2 and H-6 of -C_6H_4-SO_2NH_2, J = 8.48 Hz), 8.75 (s, 1H, H-4 isatin), 10.19 (s, 1H, NH thiazolidinone, D_2O exchangeable); Anal. Calcd. (Found) for $C_{18}H_{13}BrN_4O_4S_2$: C, 43.82 (43.97); H, 2.66 (2.71); N, 11.36 (11.58).

4.1.6.4. 4-((5-(1-Benzyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7d**). Red powder (yield 75%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3352, 3244 (NH, NH_2), 1674 (C=O) and 1396, 1153 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 5.05 (s, 2H, benzylic C<u>H</u>$_2$), 7.03-7.07 (m, 1H, Ar-H), 7.24-7.35 (m, 7H, Ar-H), 7.48 (s, 2H, NH_2, D_2O exchangeable), 7.63 (d, 2H, Ar-H, J = 8.8 Hz), 7.94 (d, 2H, Ar-H, J = 8.4 Hz), 8.77 (s, 0.5H, Ar-H), 8.80 (s, 0.5H, Ar-H), 10.09 (s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 43.47, 110.08, 120.15, 122.83, 122.95, 123.45, 126.76, 127.59, 128.01, 129.19, 129.86, 131.86, 135.80, 136.51, 137.68, 143.46, 144.54, 156.09, 165.59, 167.71; MS, *m/z* [%]: 490 [M^+, 1.68]; Anal. Calcd. (Found) for $C_{24}H_{18}N_4O_4S_2$: C, 58.76 (58.98); H, 3.70 (3.79); N, 11.42 (11.71).

4.1.6.5. 4-((5-(1-Benzyl-5-chloro-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7e**).Red powder (yield 65%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3269, 3230 (NH, NH_2), 1683 (C=O) and 1344, 1156 (SO_2); ^{1}H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 4.96, 5.05 (2s, 2H, benzylic C<u>H</u>$_2$), 7.05 (t, 1H, Ar-H, J = 8.8 Hz), 7.20-7.32 (m, 7H, Ar-H), 7.37 (s, 2H, NH_2, D_2O exchangeable), 7.42-7.45 (m, 1H, Ar-H), 7.83-7.89 (m, 1H, Ar-H), 7.94 (d, 1H, Ar-H, J = 8.8 Hz),), 8.91 (s, 0.5H, Ar-H), 9.06

(s, 0.5H, Ar-H), 12.07, 12.96 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 43.54, 111.50, 114.75, 121.33, 121.37, 127.02, 127.49, 127.58, 127.70, 127.75, 128.05, 129.17, 129.20, 131.28, 136.12, 141.45, 142.18, 155.73, 165.63, 167.30; Anal. Calcd. (Found) for $C_{24}H_{17}ClN_4O_4S_2$: C, 54.91 (55.23); H, 3.26 (3.30); N, 10.67 (10.92).

4.1.6.6. 4-((5-(1-Benzyl-5-bromo-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7f**). Red powder (yield 66%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3271, 3232 (NH, NH_2), 1693 (C=O) and 1384, 1157 (SO_2); ^1H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 5.05 (s, 2H,benzylic C$\underline{H_2}$), 7.02 (d, 1H, H-7 isatin, J = 8.4 Hz), 7.24-7.34 (m, 5H, phenyl ring), 7.48 (s, 2H, NH_2, D_2O exchangeable), 7.52 (d, 1H, H-6 isatin, J = 8.4 Hz), 7.63 (d, 2H, H-3 and H-5 of -C_6H_4-SO_2NH_2, J = 8.4 Hz), 7.94 (d, 2H, H-2 and H-6 of -C_6H_4-SO_2NH_2, J = 8.4 Hz), 8.96 (s, 1H, H-7 isatin), 10.20 (s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 43.54, 112.00, 114.75, 121.33, 121.87, 126.83, 127.55, 128.08, 129.22, 129.84, 130.13, 133.80, 136.20, 137.53, 137.69, 142.45, 144.63, 155.73, 165.63, 167.36; Anal. Calcd. (Found) for $C_{24}H_{17}BrN_4O_4S_2$: C, 50.62 (50.86); H, 3.01 (3.07); N, 9.84 (10.01).

4.1.6.7. 3-((5-(1-Benzyl-2-oxoindolin-3-ylidene)-4-oxothiazolidin-2-ylidene)amino) benzenesulfonamide (**7g**). Red powder (yield 70%), m.p. > 300 °C; IR (KBr, ν cm^{-1}): 3282, 3201 (NH, NH_2), 1678 (C=O) and 1311, 1149 (SO_2); ^1H NMR (DMSO-d_6, 400 MHz) δ *ppm*: 4.96, 5.05 (2s, 2H,benzylic C$\underline{H_2}$), 7.03 (t, 1H, Ar-H, J = 7.04 Hz), 7.09 (q,1H, Ar-H, J = 7.8 Hz), 7.25-7.38 (m, 5H, Ar-H), 7.46-7.50 (m, 3H, 1H Ar-H and 2H, NH_2, D_2O exchangeable), 7.59-7.68 (m, 2H, Ar-H), 7.95 (d, 1H, Ar-H, J = 7.36 Hz), 8.31 (s, 1H, Ar-H), 8.89 (d, 0.5H, Ar-H, J = 7.8 Hz), 9.03 (d, 0.5H, Ar-H, J = 7.8 Hz), 12.36, 12.41 (2s, 1H, NH thiazolidinone, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz) δ *ppm*: 43.52, 43.68, 110.00, 118.47, 119.00, 120.15, 120.44, 122.27, 122.67, 123.04, 123.84, 124.56, 125.02, 127.62, 127.75, 128.00, 128.22, 128.37, 129.20, 130.46, 130.64, 131.97, 136.44, 136.53, 143.48, 145.49, 145.78, 167.55, 168.18; Anal. Calcd. (Found) for $C_{24}H_{18}N_4O_4S_2$: C, 58.76 (58.94); H, 3.70 (3.67); N, 11.42 (11.59).

4.2. Biological Evaluation

4.2.1. CA inhibitory assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity, as reported earlier [31]. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation as reported earlier [42], and represent the mean from at least three different determinations. The four tested CA isofoms were recombinant ones obtained in-house as reported earlier [43].

4.2.2. Anti-proliferative activity against MCF-7

MCF-7 cells (human breast cancer cell line), were obtained from VACSERA Tissue Culture Unit. The cells were propagated in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two times a week. Cytotoxicity was determined following a reported procedure [44]. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound**.** The 50% inhibitory concentration (IC_{50}) was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA). The data presented are the mean of at least three separate experiments.

4.2.3. ELISA immunoassay

The levels of the apoptotic markers Bax, caspase-3, caspase-9, cytochrome C and p53 as well as the anti-apoptotic marker Bcl-2 were assessed using ELISA colorimetric kits per the manufacturer's instructions, as reported earlier [20, 45].

4.2.4. Statistical analysis

Data are presented as the mean ±S.D. Comparisons were carried out using one-way analysis of variance followed by Tukey–Kramer's test for post hoc analysis. Statistical significance was considered to be $P<0.05$. All statistical analyses were performed using

GraphPad InStat software, version 3.05 (GraphPad Software, La Jolla, CA). Graphs were plotted using GraphPad Prism software, version 5.00 (GraphPad Software, La Jolla, CA).

4.3. Molecular modelling studies

The crystal structures of hCA II (5LJT) [37a] and hCA IX (5FL4 monomer A; residue numbering as in 3IAI) [37b,46] were prepared for docking calculations using the Protein Preparation Wizard protocol [47a] in Maestro 10.5 [47b], deleting water molecules, using default input parameters and Prime for adding missing side chains or loops. The prediction of the side chains hetero groups ionization and tautomeric states was performed using Epik [47c]. The ligands 3D structures were generated with Maestro 10.5 [47b], evaluated for their ionization states at pH 7.4±1.0 with Epik [47c] and geometrically minimized with MacroModel [47d] (FF OPLS_2005) [48] for a maximum number of 2500 conjugate gradient iterations and setting a convergence criterion of 0.05 kcal mol-1Å-1.

Docking studies were carried out with the program Glide [47e]. Grids for docking were centred in the centroid of the complexed ligand. The standard precision (SP) mode of the GlideScore function was applied to evaluate the predicted binding poses. The best three poses for each compound were re-docked by means of Prime MM-GBSA calculations [37c] with a VSGB solvatation model [49]. The pictures were generated with Maestro 10.5 [47b].

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Highlights

- Two different series of novel isatin-based sulfonamides were developed.

- Inhibitory activity of such sulfonamides was evaluated toward hCA I, II, IV and IX isoforms.

- Anti-proliferative activity against MCF-7 and Caco-2 cancer cell lines was evaluated.

- Compound **4c** induced the intrinsic apoptotic mitochondrial pathway in MCF-7 cells.

- Molecular docking and MM-GBSA calculations were carried out.