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Benzofuran-based carboxylic acids as carbonic anhydrase inhibitors and antiproliferative agents against breast cancer

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KEYWORDS: anticancer; benzofurans; carbonic anhydrases; carboxylic acids; synthesis.

ABSTRACT: Pursuing on our effort for developing effective inhibitors of the cancer-related hCA IX isoform, here we describe the synthesis of novel benzofuran-based carboxylic acid derivatives, featuring the benzoic (**9a-f**) or hippuric (**11a,b**) acid moieties linked to 2-methylbenzofuran or 5-bromobenzofuran tails via an ureido linker. The target carboxylic acids were evaluated for the potential inhibitory action against hCAs I, II, IX and XII. Superiorly, benzofuran-containing carboxylic acid derivatives **9b**, **9e** and **9f** acted as submicromolar hCA IX inhibitors with KIs = 0.91, 0.79 and 0.56 μM, respectively, with selective inhibitory profile against the target hCA IX over the off-target isoforms hCA I and II (*SI*s: 2 – > 63 and 4 – 47, respectively). Compounds **9b**, **9e** and **9f** were examined for their anti-proliferative action against human breast cancer (MCF-7 and MDA-MB-231) cell lines. In particular, **9e** displayed promising anti-proliferative (IC_{50} = 2.52±0.39 μM), cell cycle disturbance and pro-apoptotic actions in MDA-MB-231 cells.

Carbonic anhydrases (CAs, EC 4.2.1.1) are considered as the most widespread metalloenzymes present in the living organisms that play a vital role in catalyzing the efficacious inter-conversion between carbon dioxide and bicarbonate.[1] Such a simple CA-catalyzed reaction is crucial for diverse physiological and pathological events associated with pH and CO_2 homeostasis, electrolyte secretion, tumorigenicity and others.[2] Up to now, fifteen diverse human (*h*) CA isoforms (α-CAs) have been described and identified. Among these, twelve isoforms only are catalytically active with distinct kinetic properties, tissue distributions and subcellular localizations; cytosolic (I, II, III, VII, and XIII), mitochondrial (VA and VB), secreted (VI), and membrane-bound (IV, IX, XII, and XIV).[3]

During last decades it was well-established that modulators of these metalloenzymes represent an important class of therapeutics such as diuretics,[4] anti-epileptics,[5] anti-glaucoma agents,[6] and anticancer agents.[7,8] Moreover *h*CA IX isoform, not considerably expressed in most human normal tissues, is up-regulated in the hypoxic tumors upon induction *via* HIF-1α, and thus considered as a crucial element in tumor cells proliferation, invasiveness, survival, and metastasis.[8] Accordingly, selective inhibition of *h*CA IX emerged out as a valuable therapeutic approach for targeting and treatment of different human malignancies.[8] SLC-0111 (**Figure 1**) is a front-runner carbonic anhydrase inhibitor that is currently in Phase II clinical trials for management of advanced hypoxic tumors, with a preferential *h*CA IX inhibitory action.[9]

Carboxylic acids are among the most versatile CA inhibitor (CAI) chemotypes.[10] They are capable of interacting with the CAs through a variety of inhibition mechanisms, such as coordination to the metal ion likely as carboxylate anions,[11] anchoring to the zinc-bound water/hydroxide ion,[12] occluding the entrance of the carbonic anhydrase active site cavity,[13] and inhibiting CAs binding out of the active site.[14]

Recently, Abdelrahman *et al*. developed a novel series of benzofuran-based CAIs exhibiting the zinc anchoring sulfonamide group connected to a benzofuran tail through hydrazido and hydrazino spacers, compounds **I** and **II** (**Figure 1**).[15] The arylsulfonehydrazones derivatives exerted effective and selective inhibitory activities toward target *h*CA IX and XII isoforms over off-target *h*CA I and II isoforms.

Pursuing on our endeavour toward developing efficient inhibitors for the cancer-related isoform *h*CA IX,[16-20] here we present the design and synthesis of novel benzofuran-based carboxylic acid derivatives, featuring the benzoic acid (**9a-f**) or hippuric acid (**11a**, **b**) moieties linked to 2-methylbenzofuran or 5-bromobenzofuran tails *via* an ureido linker (**Figure 1**).

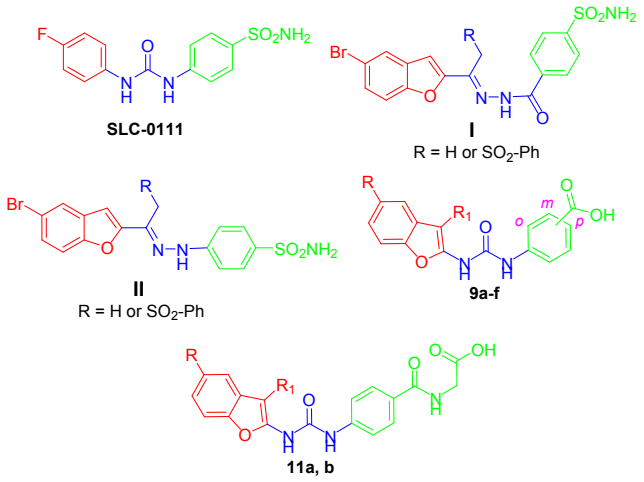


Figure 1. Structures for SLC-0111 and CAIs **I** and **II**, and target benzofuran-based carboxylic acid derivatives **9a-f** and **11a**, **b**.

Herein reported benzofuran-based carboxylic acid derivatives (**9a-f** and **11a**, **b**) were synthesized following the procedures outlined in **Schemes 1** and **2**. The ethyl benzofuran-2-carboxylate derivatives **4a**, **b** were generated through a cyclocondensation of intermediates **3a**, **b** yielded by an *o*-alkylation reaction for sodium phenolate **1a** and 2-hydroxybenzaldehyde **1b** with ethyl 2-chloroacetoacetate **2a** and ethyl bromoacetate **2b**, respectively.[21,22] Thereafter, hydrazinolysis of ethyl benzofuran-2-carboxylates **4a**, **b** was performed *via* their refluxing with an excess of 99% hydrazine hydrate in ethyl alcohol to yield benzofuran-2-carbohydrazides **5a, b**, respectively.

The benzofuran-2-carbonyl azides **6a**,**b** were provided by stirring of benzofuran-2-carbohydrazides **5a**,**b** with sodium nitrite ($NaNO_2$) in an ice-cold acetic acid (**Scheme 1**), which was subsequently subjected to Curtius rearrangement *via* stirring under reflux temperature in dry xylene to furnish the corresponding key intermediates isocyanatobenzofuran derivatives **7a**,**b** (**Scheme 2**).[23] Finally, the target benzofuran-based carboxylic acids **9a-f** and **11a**,**b** were obtained through addition of aminobenzoic acids **8a-c** or *para*-aminohippuric acid **10** to the hot stirred solution of isocyanatobenzofurans **7a**,**b** in dry xylene, with moderate yields (68–85%) (**Scheme 2**).

Elucidation of the structures for the newly synthesized benzofuran-based carboxylic acids (**9a-f** and **11a**,**b**) were supported by the elemental and spectral data which was in consistence with the postulated structures

All the prepared benzofuran-based carboxylic acid derivatives **9a-f** and **11a**, **b** were assessed for their potential inhibitory actions against the cytosolic hCA I and II, in addition to the trans membrane cancer-related hCA IX and XII isoforms, by the use of an instrument of applied photophysics stopped-flow.[24] Thereafter, the anti-proliferative activities for the best efficient and selective *h*CA IX inhibitors in this study were screened toward two breast cancer (MCF-7 and MDA-MB-231) cell lines. Furthermore, the impact of benzofuran-based benzoic acid derivative **9e** on distribution of the cell cycle phases in breast cancer MDA-MB-231 cell line was assessed, in addition to assessment of its ability to induce the early and late apoptosis *via* AnnexinV-FITC/PI binding assay.

Inhibition data for the tested hCA isoforms (I, II, IX and XII) are displayed in **Table 1**.

The slow cytosolic isoform *h*CA I (mainly considered as an off-target isoform when CAIs are developed as a potential anticancer agents) was moderately or weakly inhibited by five of the investigated benzofuran-based carboxylic acids; **9b**, **9c**, **9e**, **9f** and **11a** which showed K_Is spanning in the range of 4.5 and 64.7 μM, whereas benzofuran derivatives **9a**, **9d** and **11b** could not inhibit the cytosolic isoform *h*CA I up to 100 μM.

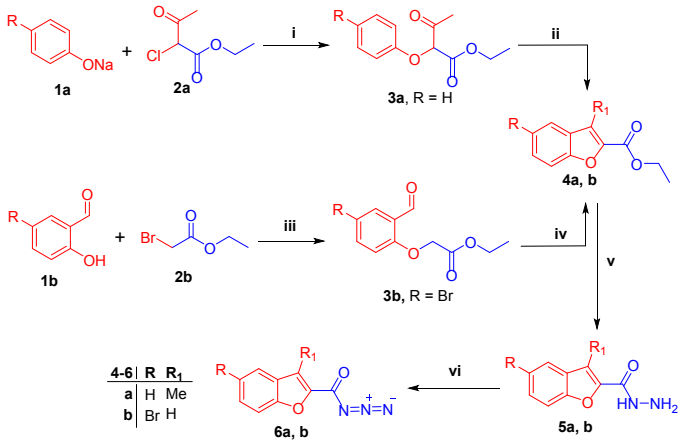
It is worth stressing that grafting carboxylic acid functionality at the *ortho*-position in both 2-methylbenzofuran (**9a**) and 5-bromobenzofuran (**9d**) scaffolds resulted in a diminished *h*CA I inhibitory activity. Moreover, replacement of the benzoic acid moiety with the hippuric acid one led to a significant worsening (for the 2-methylbenzofuran scaffold; **11a**: K_I = 64.7 μM) or led to an abolished (for the 5-bromobenzofuran scaffold; **11b**: K_I >100 μM) inhibitory activity against *h*CA I.

Inhibition of the most physiologically relevant cytosolic isoform *h*CA II was ranged from moderate to weak, with K_I values in the range of 3.1 – 67.1 μM. In particular benzofuran derivatives **9a**, **9c**, **9d** and **9f** were the best herein reported *h*CA II inhibitors with single-digit micromolar inhibitory activity (K_Is = 7.9, 3.1, 4.1 and 7.2 μM, respectively), **Table 1**. It is worth stressing that *meta*-substituted 2-methylbenzofuran derivative (**9b**) exhibited a slightly reduced inhibitory efficacy (K_I = 10.1 μM) than it's *ortho*-substituted (**9a**: K_I = 7.9 μM) and *para*-substituted (**9c**: K_I = 3.1 μM) analogues, likewise, the *meta*-substituted 5-bromobenzofuran derivative (**9e**) showed a weaker *h*CA II inhibitory activity (K_I = 37.0 μM) than both *ortho*-substituted (**9d**: K_I = 4.1 μM) and *para*-substituted (**9f**: K_I = 7.2 μM) counterparts. Furthermore, incorporation of the hippuric acid moiety resulted in a decreased activity for both 2-methylbenzofuran and 5-bromobenzofuran scaffolds (**11a** and **11b**: K_Is = 25.8 and 67.1 μM, respectively) in comparison to their benzoic acid-containing analogues (**9c** and **9f**: K_Is = 3.1 and 7.2 μM, respectively), **Table 1**. These structure-activity relationships (SARs) highlighted that appending *ortho*- and *para*-benzoic acids is more advantageous for *h*CA II inhibitory action than incorporation of *meta*-benzoic acid or hippuric acid.

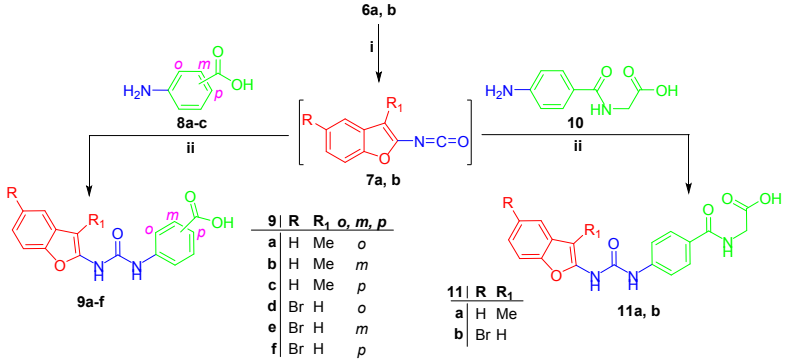
Table 1. Inhibition data (K_Is) of *h*CAs I, II, IX and XII with carboxylic acids **9a-f** and **11a**,**b** and **AAZ** as reference inhibitor by a stopped-flow CO2 hydrase assay.[24]

Cmp	R	R_1	*o*/*m*/*p*	K_I (μM)			
				CA I	CA II	CA IX	CA XII
9a	H	CH_3	*o*	>100	7.9	1.6	3.4
9b	H	CH_3	*m*	32.8	10.1	0.91	2.2
9c	H	CH_3	*p*	4.5	3.1	5.1	0.88
9d	Br	H	*o*	>100	4.1	5.1	8.0
9e	Br	H	*m*	33.2	37.0	0.79	2.3
9f	Br	H	*p*	20.5	7.2	0.56	1.6
11a	H	CH_3	-	64.7	25.8	35.7	2.7
11b	Br	H	-	>100	67.1	19.0	10.1
AAZ	-	-	-	0.25	0.01	0.02	0.006

a. Mean data from 3 different assays. SD: standard deviations ranged from ±5% to ±10% of the indicated KI values.



Scheme 1. *Reagent and conditions:* (i) dry toluene, reflux 4 hrs, 91%; (ii) H_2SO_4, stirring 2 hrs (0-5 °C), 84%; (iii) NaH, DMF, stirring 2.5 hrs at 0 °C, 80%; (iv) a) EtONa, EtOH, reflux 3 hrs, b) EtOH, H_2SO_4, reflux 4 hrs, 72%; (v) 99% $NH_2NH_2{\cdot}H_2O$, EtOH, reflux 4 hrs, 79-84%; (vi) $NaNO_2$, AcOH, stirring 1 hr (0 °C) then 1.5 hrs (r.t.), 76-81%.



Scheme 2. *Conditions and reagent:* (i) Dry xylene, reflux 1 hr, 95%; (ii) Dry xylene, reflux 5 hrs, 68-85%.

The obtained K_I values pointed out that the main antitumor target isoform *h*CA IX was effectively inhibited by the here reported benzofuran-based derivatives decorated with benzoic acid moiety **9a-f** with K_Is ranging between 0.56 and 5.1 µM, whereas *h*CA IX was moderately affected by the benzofuran-based carboxylic acids decorated with hippuric acid moiety (**11a** and **11b**) with K_Is values equal 35.7 and 19.0 µM, respectively. Superiorly, benzofuran-based carboxylic acids **9b**, **9e** and **9f** emerged as submicromolar *h*CA IX inhibitors with K_Is = 0.91, 0.79 and 0.56 µM, respectively.

Table 2. Selectivity index for inhibition of isoforms hCA IX and XII over hCA I and II, for carboxylic acids **9a-f** and **11a,b**.

Cmp	I / IX	II / IX	I / XII	II / XII
9a	> 63	5	> 29	2
9b	36	**11**	15	5
9c	-	-	5	4
9d	20	-	13	-
9e	42	**47**	14	16
9f	37	**13**	13	5
11a	2	-	24	10
11b	5	4	10	7

It is noteworthy to mention that the order of activities within the 2-methylbenzofuran-based regioisomers **9a-c** toward *h*CA IX was decreased in the order of *meta* isomer (**9b**) > *ortho* isomer (**9a**) > *para* isomer (**9c**), whereas, the order of *h*CA IX inhibitory activities for the 5-bromobenzofuran-based regioisomers **9d-f** toward was decreased in the order *para* isomer (**9f**) > *meta* isomer (**9e**) > *ortho* isomer (**9d**).

The second cancer-related target CA isoform examined here is *h*CA XII. As can be seen from the results presented in **Table 1**, *h*CA XII was efficiently inhibited by the herein reported benzofuran-based acids with K_Is in the range of 0.88–3.4 µM, aside from benzofuran derivatives **9d** and **11b** whose potency raised at slightly higher concentration (K_Is = 8.0 and 10.1 µM, respectively). The 2-methylbenzofuran-based derivative **9c** with K_I equals 0.88 µM, is the only submicromolar CA inhibitor identified toward *h*CA XII isoform in this study (**Table 1**). Remarkably, the deduced SAR suggested that utilizing of the 2-methylbenzofuran scaffold elicited an enhancement of effectiveness toward *h*CA XII for both benzoic acid-containing derivatives **9a-c** (K_Is = 3.4, 2.2 and 0.88 µM, respectively) and hippuric acid-containing derivative **11a** (K_I = 2.7 µM) in comparison to their corresponding 5-bromobenzofuran counterparts **9d-f** (K_Is = 8.0, 2.3 and 1.6 µM, respectively) and **11b** (K_I = 10.1 µM). With regard to the impact of regioisomerism, the order of potencies within the 2-methylbenzofuran-based regioisomers **9a-c** and 5-bromobenzofuran-based regioisomers **9d-f** against *h*CA XII was lowered as the following order; *para* isomers > *meta* isomers > *ortho* isomers.

Concerning the target/off-target CAs selectivity indexes (*SI*s) of action for target benzofuran-based carboxylic acid

derivatives (**9a-f** and **11a**,**b**), all compounds, except **9c**, exhibited adequate selectivity profiles for *h*CA IX over *h*CA I (*SI*: 2 – >63). In addition, compounds **9a**, **9b**, **9e**, **9f** and **11b** displayed remarkable II/IX inhibitory specificity with *SI*s span the range of 4 – 47, **Table 2**.

Table 3. Anti-proliferative activities of benzofuran-based carboxylic acids **9b**, **9e** and **9f** against two breast cancer cell lines; MCF-7 and MDA-MB-231

Cmp	IC_{50} (μM)[a]	
	MCF-7	MDA-MB-231
9b	NA[b]	37.60 ± 1.86
9e	14.91 ± 1.04	2.52 ± 0.39
9f	19.70 ± 2.06	11.50 ± 1.05
Dox	1.43 ± 0.12	2.36 ± 0.18

a. IC50 values are the mean ± S.D. of three separate experiments.
b. NA: Derivatives possessing having IC50 value > 100 μM.

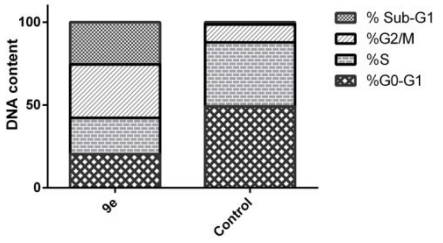


Figure 2. Effect of benzofuran-based carboxylic acid **9e** on the phases of cell cycle in breast cancer MDA-MB-231cells.

Benzofuran-based carboxylic acids **9b**, **9e** and **9f** displayed effective and selective inhibitory actions toward the cancer-associated hCA IX / XII isoforms over the off-target isoforms CA I and II (**Tables 1** and **2**). Therefore, the three benzofuran derivatives have been chosen to be tested for their possible *in vitro* anti-proliferative actions toward the breast cancer (MCF-7 and MDA-MB-231) cell lines, using the SRB colorimetric reduction method as reported by Skehan *et al*.[25] The results from this assay have been expressed as IC_{50} values and presented in **Table 3**.

The obtained IC_{50} values from the SRB analysis (**Table 3**) indicated that the tested benzofuran derivatives **9b**, **9e** and **9f** were more effective toward MDA-MB-231 (IC_{50} values = 37.60 ± 1.86, 2.52 ± 0.39 and 11.50 ± 1.05 μM, respectively) than MCF-7 cells (IC_{50} values = > 100 μM, 14.91 ± 1.04 and 19.70 ± 2.06 μM, respectively). Superiorly, the 5-bromobenzofuran-based derivative **9e** was the most effective anti-proliferative agent toward MDA-MB-231 cells with IC_{50} value = 2.52 ± 0.39 μM, with a comparable potency to Doxorubicin (Dox), the reference drug, (IC_{50} = 2.36 ± 0.18 μM).

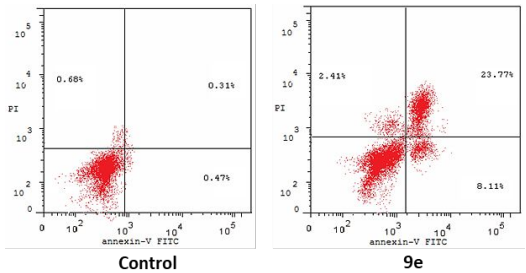


Figure 3. Influence of benzofuran-based carboxylic acid derivative **9e** over the AV-FITC-positive staining percentages in breast cancer MDA-MB-231 cells. (Lower right: early apoptotic; upper right: late apoptotic; lower left: viable; upper left: necrotic).

The promising anticancer effect of benzofuran-based carboxylic acid derivative **9e** toward MDA-MB-231 breast cancer cell line (**Table 3**) motivated a further examination for its growth inhibitory activity. The effect of carboxylic acid derivative **9e** on cell cycle phases distribution, upon incubation with breast cancer MDA-MB-231 cells for 24 hrs at its IC_{50} concentration (2.52 μM), was assessed by the use of a DNA flow cytometry assay (**Figure 2**).

The assay outcomes revealed that MDA-MB-231 cancer cells treated with benzofuran-based acid derivative **9e** were significantly arrested at the G2-M phase showing a cell population increase from 10.80 % (for the control cells) to 32.30 % (in **9e**-treated MDA-MB-231 cells). In addition, the number of cells in the sub-G1 phase was strikingly increased from 1.43 % (for the control cells) to 25.53 % (in **9e**-treated MDA-MB-231 cells), **Figure 2**.

Table 4. Distribution of apoptotic MDA-MB-231 cells after incubation with benzofuran derivative **9e** in the AV-FITC/PI staining assay

Cmp	Total %	Early Apoptosis	Late Apoptosis %	Necrosis
9e	34.29	8.11	23.77	2.41
Ctrl	1.46	0.47	0.31	0.68

The AV/PI staining assay has been adopted in order to investigate the effect of benzofuran-based carboxylic acid derivative **9e** on the early and late-apoptosis percentages in human breast MDA-MB-231 cancer cells (**Figure 3**, **Table 4**). The results of this assay pointed out that incubation of MDA-MB-231 cells with acid derivative **9e** provoked apoptosis in such cells, evidenced by the significant increase in the percentages of apoptotic MDA-MB-231 cells for both the early apoptosis phase (from 0.47 %, for the control, to 8.11 %), and the late apoptosis phase (from 0.31 %, for the control, to 23.77 %) (**Table 4**).

In summary, we explored in this letter the design and synthesis for novel benzofuran-based carboxylic acid derivatives, featuring the benzoic (**9a-f**) or hippuric (**11a**, **b**) acid moieties linked to 2-methylbenzofuran or 5-bromobenzofuran tails *via* an ureido linker. All the target carboxylic acid derivatives were tested for their potential inhibitory action against four *h*CA isoforms (I, II, IX and XII). The cancer-related *h*CA IX isoform was effectively affected by the all prepared benzofuran derivatives decorated with benzoic acid moiety **9a-f** with K_Is ranging between 0.56 and 5.1 μM, Superiorly, compounds **9b**, **9e** and **9f** emerged as submicromolar *h*CA IX inhibitors with K_Is = 0.91, 0.79 and 0.56 μM, respectively. Moreover, all compounds, except **9d** and **11b**, inhibited *h*CA XII isoform with K_Is in the range: 0.88–3.4 μM. Regarding the target/off-target CAs *SI*s of action for the target benzofuran-based carboxylic acid derivatives, compounds **9b**, **9e** and **9f** showed good selective inhibitory action toward *h*CA IX over the off-target *h*CA I and II (*SI*s: 2–>63 and 4–47, respectively). The

concluded SAR revealed that replacement of the benzoic acid moiety with the hippuric acid one led to a worsening or abolishment of inhibitory activity against all the tested *h*CA isoforms, whereas, utilizing of the 2-methylbenzofuran scaffold elicited an enhancement of effectiveness toward *h*CA XII for both benzoic and hippuric acid-containing derivatives in comparison to the corresponding 5-bromobenzofuran-based counterparts **9d-f**. Moreover, compounds **9b**, **9e** and **9f** were tested for their potential growth inhibitory actions toward two human breast cancer (MCF-7 and MDA-MB-231) cell lines. In particular, **9e** displayed the best anti-proliferative action toward MDA-MB-231 cancer cells (IC_{50} = 2.52 ± 0.39 μM) that is comparable to Doxorubicin (IC_{50} = 2.36 ± 0.18 μM). Furthermore, **9e** disrupted the cell cycle (through arrest of G_2-M stage and alteration of Sub-G_1 phase), and significantly boosted the percentage of AV-FITC positive MDA-MB-231 apoptotic cells (from 0.78 to 31.88%).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: synthetic procedures, compounds characterization, *in vitro* kinetic method (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CA, carbonic anhydrase; AAZ, acetazolamide; SRB, sulfo-rhodamine-B; AV, annexinV; PI, propidium iodide.

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