Discovery of 2,4-thiazolidinedione-tethered coumarins as novel selective inhibitors for carbonic anhydrase IX and XII isoforms

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ABSTRACT

Different 2,4-thiazolidinedione-tethered coumarins 5a-b, 10a-n and 11a-d were synthesised and evaluated for their inhibitory action against the cancer-associated hCAs IX and XII, as well as the physiologically dominant hCAs I and II to explore their selectivity. Un-substituted phenyl-bearing coumarins 10a, 10h, and 2-thienyl/furyl-bearing coumarins 11a-c exhibited the best hCA IX (Ks between 0.48 and 0.93 μM) and hCA XII (Ks between 0.44 and 1.1 μM) inhibitory actions. Interestingly, none of the coumarins had any inhibitory effect on the off-target hCA I and II isoforms. The sub-micromolar compounds from the biochemical assay, coumarins 10a, 10h and 11a-c, were assessed in an in vitro antiproliferative assay, and then the most potent antiproliferative agent 11a was tested to explore its impact on the cell cycle phases and apoptosis in MCF-7 breast cancer cells to provide more insights into the anticancer activity of these compounds.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes found in all living organisms and are responsible for the catalysis of the biologically crucial reversible hydration of carbon dioxide to bicarbonate and proton. This is a simple but pivotal physiological reaction which is essential for normal and pathological processes such as CO2 and pH homeostasis, respiration, gluconeogenesis, calcification, bone resorption, fluid secretion and tumorigenesis [1,2]. CAs are grouped into different families, amongst them α-CAs which are present in all vertebrates and are further sub-classified into fifteen isoforms (herein referred to as human CAs or hCAs) that differ by molecular features, expression levels, kinetic properties and cellular distribution in the different tissues [3]. Of note, only twelve hCAs are catalytically active (I-IV, VA, VB, VI, VII, IX, XII-XIV) with an active site containing three histidine residues in a triple coordination with a zinc ion [4]. Regarding the subcellular distribution of the catalytically active hCAs, they can be categorised into different subsets: cytosolic (I, II, III, VII and XIII), trans-membrane (IV, IX, XII, and XIV), mitochondrial (VA and VB), while VI is secreted in saliva and milk [5]. The overexpressed levels and/or dysfunctions of hCAs can lead to many disorders, hence CA inhibitors (CAIs) are utilised for the treatment of glaucoma (targeting hCA II, IV and XII), edoema (targeting hCA II, IV and XIV), mental disorders (targeting hCA II, VII and XIV) and obesity (targeting hCA VA and VB) [4,6,7].

It should be stressed that the trans-membranous hCA IX and XII are hypoxia-induced tumours-associated isozymes and overexpressed in most cancer cells compared to the normal ones [8]. While the overexpressed hCA IX isozyme is mainly linked to cancer poor prognosis and limited to hypoxic tumours, hCA XII can be found in some normal tissues like kidney and colon alongside the hypoxic tumours [9,10]. Interestingly, the tumour growth, angiogenesis, proliferation and metastasis are attributed to the overexpressed levels of hCA IX and XII suggesting a strategy for targeting of such enzymes as a new approach in cancer chemotherapy [8,11]. In this context, selective inhibition of the tumour-associated hCA IX and XII isozymes over the other isoforms, particularly the most prevalent cytosolic hCA I and II is highly desirable and will result in cancer treatment with fewer side effects [12].

In view of this, intensive efforts are being conducted for the development of hCA IX/XII selective inhibitors as a validated approach for cancer treatment [13–15]. hCA IX and XII can be inhibited by different strategies such as coordination to the zinc
ion situated in the catalytic active site. Molecules in this class are exemplified by sulfonamide-derived hCAIs and their bioisosters. In addition, the occlusion of the catalytic active cleft is explored and this approach has been explored using coumarins as a newly discovered hCAIs class [16,17].

Coumarin I is a naturally-derived, privileged heterocyclic scaffold and molecules containing it show numerous biological properties such as inhibition of CK2, EGFR and PI3K-AKT-mTOR signalling. Moreover, coumarins are known to have anticoagulation, monoamine oxidase inhibition, anti-infective, antioxidant, anti-inflammatory and anticancer activities [14,18–20]. Coumarins are recently discovered as a novel class of hCAIs with inhibitory mechanism different from the sulfonamide-based inhibitors. Coumarin acts as prodrug undergoing hydrolysis by the esterase activity of CA to yield 2-hydroxycinnamic acid derivative II which can bind to the active site cleft occluding its entrance. Figure 1 [15,21]. Since coumarins binding sites are the most heterologous region of the active site between all CAs isoforms, it is not surprising that these chemotypes display very high selectivity for specific CAs isoforms. Furthermore, the chemical simplicity of coumarins permits the facile incorporation of diverse substituents, leading to generation of a large number of derivatives with interesting biological profiles [22]. Consequently, many ongoing efforts have focussed on developing novel coumarin derivatives as selective hCAs IX/XII inhibitors that could be used for cancer therapy. For instance, diverse coumarins III–X have been reported as selective hCAs IX/XII inhibitors with nanomolar $K_i$ [8,10,13,17,21–24].

Inspired by these findings, we prepared a series of 2,4-thiazolidinedione-tethered coumarins, compounds 5a–b, 10a–n and 11a–d, and evaluated their inhibitory action against the cancer-associated hCAs IX and XII, and selectivity over inhibition of the physiologically dominant hCAs I and II to explore their selectivity in order to the cancer-related isoforms. Moreover, the efficient hCAs IX/XII inhibitors 10a, 10h and 11a–c were subjected to in vitro antiproliferative assay under hypoxic conditions and most potent antiproliferative agent 11a was tested to explore its impact on the cell cycle phases and apoptosis in MCF-7 breast cancer cells furnishing more insights on the anticancer activity of such compounds.

2. Experimental
2.1. Chemistry
2.1.1. General
The NMR spectra were recorded by Bruker 400 MHz spectrometer. $^1$H and $^{13}$C spectra were run at 400 and 100 MHz, respectively, in deuterated dimethylsulphoxide (DMSO-$d_6$) or deuterated trifluoroacetic acid. All coupling constant ($J$) values are given in hertz. IR spectra were recorded with a Bruker FT-IR spectrophotometer. Reaction courses and product mixtures were routinely monitored by thin layer chromatography (TLC) on silica gel precoated F$254$ Merck plates. Unless otherwise mentioned, all reagents and solvents are commercially available and have been used without further purification. Compounds 2 and 3 are previously reported [25,26].

2.1.2. General procedures for synthesis of 3–(2-oxo-2–(2-oxo-2H-chromen-3-yl)ethyl)thiazolidine-2,4-dione derivatives (5a–b)
To a stirred solution of 3–(2-bromoacetyl)-2H-chromen-2-one 3a (0.27 g, 1.0 mmol) or 6-bromo-3–(2-bromoacetyl)-2H-chromen-2-one 3b (0.34 g, 1.0 mmol) in DMF (7 ml), thiazolidine-2,4-dione 4 (0.12 g, 1.0 mmol), anhydrous K$_2$CO$_3$ (0.28 g, 2.0 mmol) and KI (cat.) were added. The reaction mixture was heated on a water bath for 8h, then was poured over crushed ice. The precipitate was filtered, dried, and crystallized from hot ethanol to give the corresponding key intermediates 5a–b, respectively.
2.1.3. General procedures for preparation of the intermediates 8a–g and 9a–b

To a solution of thiazolidine-2,4-dione 4 (0.12 g, 1 mmol) in glacial acetic acid (5 mL), anhydrous sodium acetate (0.08 g, 1 mmol) and the appropriate aldehyde derivative (6a–g and 7a–b) were added. The resulting reaction mixture was allowed to stir under reflux for 3 h. The precipitated solid was collected by filtration while hot, washed with cold ethanol and water, and dried to afford intermediates 8a–g and 9a–b.

2.1.4. General procedures for preparation of coumarins 10a–n and 11a–d

The appropriate benzylidene derivative 8a–g (2 mmol) was added to a hot stirred mixture of 3-(bromoacetyl)coumarin derivatives 3a–b (2 mmol), K$_2$CO$_3$ (0.55 g, 4 mmol), Kl (2 mmol) in DMF (8 mL), then the resulting mixture was stirred under reflux for 8 h. The formed precipitates were collected by filtration, washed with water, dried and recrystallized from DMF/water to yield the final target coumarin-based CAls 10a–n and 11a–d.

2.1.4.1. 5-Benzylidene-3–(2-oxo-2-(2-oxo-2H-chromen-3-yl)ethyl)thiazolidine-2,4-dione 10a

Grey crystals (yield, 70%); m. p. = 249–251°C; $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.17 (s, 1H, ArH), 8.27 (s, 1H, –CH=), 8.01–7.97 (m, 2H, ArH), 7.70–7.61 (m, 7H, ArH), 5.64 (s, 2H, CH$_2$, –N–CH$_2$–CO–); $^{13}$C NMR (101 MHz, DMSO) δ 173.27, 172.54, 168.52, 168.28, 153.27, 139.52, 139.52, 137.19, 132.00, 131.79, 131.19, 131.04, 129.01, 126.36, 118.57, 116.70, 115.75, 54.32 (–N(–CH$_2$–)); Anal. Calcld. for C$_{25}$H$_{19}$NO$_7$S (451.45): C, 61.19; H, 3.80; N, 3.10; Found: C, 61.19; H, 3.82; N, 3.10.

2.1.4.2. 5–(4-Methylbenzylidene)-3–(2-oxo-2-(2-oxo-2H-chromen-3-yl)ethyl)thiazolidine-2,4-dione 10b

Yellow crystals (yield, 85%); m. p. = 227–229°C; $^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.61 (s, 1H, ArH), 8.04 (d, J = 8.0 Hz, 1H, ArH), 7.79 (t, J = 7.6 Hz, 1H, H–7 ArH), 7.72 (s, 1H, –CH=), 7.51 (d, J = 8.0 Hz, 1H, ArH), 7.47 (d, J = 8.0 Hz, 2H, ArH), 7.48 (t, J = 7.6 Hz, 1H, ArH), 7.33 (d, J = 8.0 Hz, 2H, ArH), 5.13 (s, 2H, CH$_2$, –N–CH$_2$–CO–), 2.41 (s, 3H, CH$_3$); Anal. Calcld. for C$_{25}$H$_{19}$NO$_7$S (451.45): C, 61.19; H, 3.80; N, 3.10; Found: C, 61.20; H, 3.80; N, 3.10.
1.1.5. General procedures for preparation of target coumarins 11a-d

5-(Thiophen-2-ylmethylene)thiazolidine-2,4-dione 7a (15 mmol) and/or 5-(5-(methylfuran-2-ylmethylene)thiazolidine-2,4-dione 7b (15 mmol) was added to a hot stirred solution of 3-(bromocoumaryl) coumarin derivatives 3a, b (15 mmol) in DMF (10 ml), K2CO3 (15 mmol), then the resulting mixture was stirred under reflux for 8 h. The precipitates were collected by filtration, washed with water, dried and recrystallized from hexane/ethanol to yield the final target compounds 11a-d.

1.1.5.1. 3-(2-Oxo-2-oxo-2H-chromen-3-yl)-5-(thiophen-2-ylmethylene)thiazolidine-2,4-dione 11a. Yellow crystals (yield, 83%); m. p. = 206–210 °C; 1H NMR (400 MHz, DMSO-d6) δ 8.13 (s, 1H, ArH), 7.93 (d, J = 3.6 Hz, 1H, ArH), 7.83 (m, 3H, ArH), 7.68–7.68 (m, 3H, ArH), 7.21–7.21 (m, 2H, ArH), 6.68 (d, J = 4.0 Hz, 1H, ArH), 5.40 (s, 2H, CH2, –N–CH2–CO–), 3.75 (s, 3H, OCH3); 13C NMR (101 MHz, DMSO-deuterated) δ 162.46, 162.02, 161.74, 161.59, 161.15, 154.65, 152.12, 134.08, 122.06, 120.32, 118.49, 115.74, 115.68, 113.18, 112.86, 110.05, 56.32, 56.38. Anal. Calcld. for C20H12BrNO5S (530.35): C, 52.09; H, 3.04; N, 3.64; Found: C, 51.89; H, 3.07; N, 3.63.

1.1.5.2. 5-(5-Methylfuran-2-ylmethylene)-3-(2-oxo-2-oxo-2H-chromen-3-yl)-5-(thiophen-2-ylmethylene)thiazolidine-2,4-dione 11b. Yellow crystals (yield, 70%); m. p. = 227–229 °C; 1H NMR (400 MHz, DMSO-d6) δ 9.12 (s, 1H, ArH), 7.96–7.96 (m, 3H, ArH, –CH═ and ArH), 7.62–7.62 (m, 2H, ArH), 7.09 (d, J = 4.0 Hz, 1H, ArH), 6.40 (d, J = 4.0 Hz, 1H, ArH), 5.60 (s, 2H, CH2, –N–CH2–CO–), 2.54 (s, 3H, CH3); 13C NMR (101 MHz, DMSO-deuterated) δ 162.45, 162.02, 161.57, 161.14, 153.33, 137.00, 134.41, 134.01, 131.08, 126.83, 126.38, 119.93, 118.48, 115.67, 112.85, 110.04, 50.74. Anal. Calcld. for C19H12BrNO5S (397.42): C, 57.42; H, 2.79; N, 3.52; Found: C, 57.26; H, 2.78; N, 3.55.

1.1.5.3. 3-(2-(6-Bromo-2-oxo-2H-chromen-3-yl)-5-(thiophen-2-ylmethylene)thiazolidine-2,4-dione 11c. Yellow crystals (yield, 76%); m. p. = 255–257 °C; 1H NMR (400 MHz, DMSO-d6) δ 8.60 (s, 1H, ArH), 8.27 (d, J = 2.4 Hz, 1H, ArH), 8.04 (s, 1H, ArH, –CH═), 7.99 (d, J = 5.2 Hz, 1H, ArH), 7.66 (d, J = 8.0 Hz, 1H, ArH), 7.48 (d, J = 3.6 Hz, 1H, ArH), 7.27 (t, J = 3.6 Hz, 1H, ArH), 5.20 (s, 2H, –N–CH2–CO–); 13C NMR (101 MHz, DMSO) δ 189.40, 167.97, 167.84, 165.35, 158.79, 147.90, 146.11, 137.84, 137.41, 136.00, 134.92, 133.42, 129.39, 127.63, 125.40, 125.37, 123.00, 132.66, 132.42, 130.36, 128.68, 127.03, 119.33, 118.51, 115.69, 112.88, 110.07. Anal. Calcld. for C21H12BrNO5 (504.74): C, 49.07; H, 2.20; N, 2.78; Found: C, 50.13; H, 2.21; N, 2.80.

1.1.5.4. 3-(2-(6-Bromo-2-oxo-2H-chromen-3-yl)-5-(thiophen-2-ylmethylene)thiazolidine-2,4-dione 11d. Yellow crystals (yield, 72%); m. p. = 208–210 °C; 1H NMR (400 MHz, DMSO-d6) δ 8.61 (s, 1H, ArH), 8.22 (s, 1H, ArH), 7.87 (d, J = 8.8 Hz, 1H, ArH), 7.79 (s, 1H, –CH═), 7.51–7.59 (m, 3H, ArH), 7.43 (d, J = 8.8 Hz, 1H, ArH), 7.34 (t, J = 8.0 Hz, 1H, ArH), 5.14 (2H, –N–CH2–CO–); 13C NMR (101 MHz, DMSO) δ 170.46, 158.94, 154.43, 154.09, 146.11, 137.06, 134.21, 134.16, 133.92, 133.01, 131.75, 129.88, 129.61, 129.39, 129.23, 128.93, 120.54, 118.88, 116.83, 30.50. Anal. Calcld. for C21H12BrNO5 (549.19): C, 45.93; H, 2.02; N, 2.55; Found: C, 46.09; H, 2.03; N, 2.53.

1.2.4.8. 5-Benzylidene-3-[(2-(6-bromo-2-oxo-2H-chromen-3-yl)-2-oxoethyl)thiazolidine-2,4-dione 10h. Yellow crystals (yield, 78%); m. p. = 252–254 °C; 1H NMR (400 MHz, DMSO-d6) δ 8.60 (s, 1H, ArH), 8.21 (d, J = 2.4 Hz, 1H, ArH), 7.87 (d, J = 7.6 Hz, 1H, ArH), 7.78 (s, 1H, –CH═), 7.43–7.61 (m, 6H, ArH), 5.14 (2H, CH2, –N–CH2–CO–); 13C NMR (101 MHz, DMSO) δ 168.61, 168.30, 146.11, 137.06, 134.29, 133.60, 133.42, 133.01, 131.94, 130.81, 130.45, 129.78, 125.88, 124.64, 120.54, 118.86, 117.04, 116.83, 50.83 (–N–CH2–); Anal. Calcld. for C21H12BrNO5S (429.20): C, 53.63; H, 2.57; N, 2.98; Found: C, 53.81; H, 2.56; N, 3.00.
3.1. Chemistry

The proposed synthetic routes to obtain the target coumarins are depicted in Scheme 1 and Scheme 2. First, condensation of 2-hydroxybenzaldehydes 1a-b with ethyl 3-oxobutanoate in refluxing absolute ethanol in the presence of a few drops of piperidine yielded 3-acetylcoumarins 2a-b. These were subjected to bromination via reaction with Br2 in glacial acetic acid to yield the key 3-(bromoacetyl)coumarin intermediates 3a-b, which were subsequently treated with thiazolidine-2,4-dione 4 in refluxing DMF using anhydrous K2CO3 as base and KI as a nucleophilic catalyst to afford 3- (2-oxo-2H-chromen-3-yl)ethyl)thiazolidine-2,4-dione 5a-b (Scheme 1).

Synthesis of compounds 8a-g and 9a-b (Scheme 2) was achieved via refluxing of thiazolidine-2,4-dione 4 with benzaldehyde derivatives 6a-g and 7a-b in glacial acetic acid and anhydrous sodium acetate. Treatment of 8a-g and 9a-b with the key intermediates 3a-b in refluxing DMF using anhydrous K2CO3 and KI furnished the corresponding final targets 5-benzylidene-3-(2-oxo-2-(2-oxo-2H-chromen-3-yl)ethyl)thiazolidine-2,4-dione 10a-n and 11a-d, respectively. Proposed structures for the synthesised coumarins were in agreement with their various spectroscopic and analytical data.

3.2. Carbonic anhydrase inhibition

The inhibitory influence of all the synthesised coumarins 5a-b, 10a-n and 11a-d was investigated against hCA I, II IX and XII isoforms using a stopped flow CO2 hydrase assay and a well-known hCA I, acetazolamide (AAZ) as control [36]. From the resulting inhibition constants (KIs) shown in Table 1, certain structure activity relationship (SAR) can be inferred.

Coumarins 5a-b, 10a-n and 11a-d are devoid of significant inhibition towards the off-target ubiquitous hCA I and the physiologically dominant hCA II (Ks > 100 μM) isoforms, Table 1. In contrast, these coumarins inhibited the cancer-related hCA IX with inhibition constants spanning a range between 0.12 and 18.2 μM, (Table 1). Regarding the unsubstituted thiazolidinedione-bearing coumarins 5a-b, the absence of substitution at 6-position of coumarin scaffold furnished the most effective hCA IX inhibitor in this work displaying Ki of 0.12 μM, whereas, the 6-bromination decreased the hCA IX inhibitory power 2-folds (Ki = 0.24 μM).

In the context of hCA IX inhibition constants of the benzylidene counterparts 10a-n, it was found that 6-unsubstituted coumarins 10a-g showed effective inhibition (Ks ranged from 0.82 to 12.3 μM) compared to the 6-bromo analogues 10h-n (KiS spanned between 0.93 and 18.2 μM). In the term of coumarins with the benzylidene moiety, 10a-g, it is worth stressing that appending an unsubstituted aryl ring to the thiazolidinedione moiety 10a provided the most effective hCA IX inhibitor within this series (Ki = 0.82 μM). Indeed, the incorporation of ortho-chloro or ortho-bromophenyl (10f and 10g, respectively) decreased the inhibition constants to low micromolar values (Ks = 2.2 and 2.3 μM, respectively). Regrettably, the remaining phenyl substitution pattern were similarly poorer hCA IX inhibitors with Ks equalling 4.3 μM (p-methyl, 10b), 5.8 μM (p-methoxy, 10c), 8.4 μM (2,5-dimethoxy, 10d), 12.3 μM (p-nitro, 10e).

In a similar fashion, the appending of unsubstituted aryl ring to the 6-bromocoumarins 10h-n produced the most potent hCA
IX inhibitor within this series (10h; $K_I = 0.93 \mu M$), whereas ortho chlorination or bromination reduced the inhibition constants to low micromolar values (10m and 10n; $K_S = 2.9$ and $3.4 \mu M$, respectively). Similar to the 6-unsubstituted coumarins 10a–g, it was noted that in the 6-bromocoumarin series, inclusion of other phenyl substituents (compounds 10h–n) lowered the hCA IX inhibition constants showing $K_S$ in the range 6.2–18.2 $\mu M$. Superiorly, the replacement of six-membered phenyl with five-membered 2-thienyl ring potentially elevated the inhibition constants for both 6-unsubstituted and 6-bromocoumarins (11a and 11c; $K_S = 0.48$ and 0.59 $\mu M$, respectively) compared to their phenyl counterparts (10a and 10h; $K_S = 0.82$ and 0.93 $\mu M$, respectively). Notably, utilising 2-furyl functionality in place of phenyl/thienyl group did not result in significant change in potency (11b and 11d; $K_S = 0.79$ and 0.91 $\mu M$, respectively).

Collectively, the deduced SAR for hCA IX inhibition suggests that applying of unsubstituted thiazolidinedione (5a–b) is more favoured than their substituted analogues (10a–n and 11a–d) affording the most potent hCA IX inhibitors in this study. Furthermore, the lack of substitution at 6-position of coumarin (5a, 10a–g and 11a–b) is more advantageous for such type of activity relative to 6-bromo counterparts (5b, 10h–n and 11c–d). Additionally, appending of unsubstituted phenyl ring (10a, 10h) is the most beneficial pattern within all tested benzylidene counterparts (10a–n), while replacement of phenyl with 2-thienyl moiety gave the most potent hCA IX inhibitors (11a and 11c) within all arylidene derivatives (10a–n and 11a–d).

Finally, the inhibition profiles (Table 1) revealed that the cancer-related hCA XII isozyme was inhibited by the coumarins 5a–b, 10a–n and 11a–d displaying a range of inhibition constants from submicromolar level to low micromolar values ($K_S$ ranged from 0.15 to 10.4 $\mu M$). The unsubstituted thiazolidinedione-bearing coumarins 5a–b emerged as the most effective hCA XII inhibitors, with submicromolar inhibition constants (5a; $K_I = 0.15 \mu M$ and 5b; $K_I = 0.31 \mu M$).

It should be pointed out that bromination at 6-position of coumarin 5b led to 2-fold diminished inhibition for hCA XII relative to the unsubstituted analogue 5a, in a similar manner observed in the SAR for hCA IX inhibition, Table 1. Concerning the benzylidene derivatives 10a–n, it was observed that appending a phenyl to thiazolidinedione moiety resulted in the most potent hCA XII inhibitors (at submicromolar level) within this series (10a; $K_I = 0.75 \mu M$ and 10h; $K_I = 0.87 \mu M$). The incorporation of different substituents to the phenyl group reduced the inhibitory potential affording $K_S$ spanning between 2.3 and 10.4 $\mu M$. Furthermore, the absence of substitution at 6-position of coumarin is beneficial for inhibition (10a; $K_I = 0.75 \mu M$), whereas 6-bromination decreased the activity (10h; $K_I = 0.87 \mu M$), Table 1. It was noted that replacement of the phenyl group (10a; $K_I = 0.75 \mu M$ and 10h; $K_I = 0.87 \mu M$) with 2-thienyl or 2-furyl functionalities reduced $K_S$ for the 6-unsubstituted coumarins (11a; $K_I = 0.83 \mu M$ and 11b; $K_I = 1.1 \mu M$, respectively), while raised $K_S$ for the 6-bromocoumarins (11c; $K_I = 0.44 \mu M$ and 11d; $K_I = 0.82 \mu M$, respectively). This is unlike the pattern in hCA IX inhibition profile and points to a potential future avenue of exploration towards selectivity of hCA XII over hCA IX.

To summarise, the elicited SAR highlighted that unsubstituted thiazolidinedione derivatives (5a–b) exerted more superior potency relative to their substituted counterparts (10a–n and 11a–d) resulting in the most potent hCA XII inhibitors in this work (5a–b). Moreover, within all benzylidene derivatives 10a–n, the unsubstituted phenyl counterparts exerted the best inhibition

### Scheme 2. Reagents and conditions: (i) glacial acetic acid, reflux 3 h.; (ii) DMF, potassium carbonate, potassium iodide, reflux 8 h.
Table 1. Inhibition data for hCA I, II, IX and XII isoforms with 2,4-thiazolidinedione-tethered coumarins (5a–b, 10a–n and 11a–d) and AAZ.

| Cmpd  | R1 | R2 | Kᵢ (µM)ᵃᵇ |
|-------|----|----|------------|
|       |    |    | CA I      | CA II     | CA IX     | CA XII    |
| 5a    | H  | –  | >100       | >100       | 0.12      | 0.15      |
| 5b    | Br | –  | >100       | >100       | 0.24      | 0.31      |
| 10a   | H  | –  | >100       | >100       | 0.82      | 0.75      |
| 10b   | H  | –  | >100       | >100       | 4.3       | 4.0       |
| 10c   | H  | –  | >100       | >100       | 5.8       | 4.5       |
| 10d   | H  | –  | >100       | >100       | 8.4       | 6.2       |
| 10e   | H  | –  | >100       | >100       | 12.3      | 8.0       |
| 10f   | H  | –  | >100       | >100       | 2.2       | 3.8       |
| 10g   | H  | –  | >100       | >100       | 2.3       | 4.1       |
| 10h   | Br | –  | >100       | >100       | 0.93      | 0.87      |
| 10i   | Br | –  | >100       | >100       | 6.2       | 2.3       |
| 10j   | Br | –  | >100       | >100       | 8.9       | 4.9       |
| 10k   | Br | –  | >100       | >100       | 16.4      | 6.6       |
| 10l   | Br | –  | >100       | >100       | 18.2      | 10.4      |
| 10m   | Br | –  | >100       | >100       | 2.9       | 3.2       |
| 10n   | Br | –  | >100       | >100       | 3.4       | 2.8       |
| 11a   | H  | –  | >100       | >100       | 0.48      | 0.83      |
| 11b   | H  | –  | >100       | >100       | 0.79      | 1.1       |
| 11c   | Br | –  | >100       | >100       | 0.59      | 0.44      |
| 11d   | Br | –  | >100       | >100       | 0.91      | 0.82      |
| AAZ   | –  | –  | 250        | 12.5       | 25.0      | 5.7       |

ᵃMean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5–10% of the reported values);ᵇincubation time of 6 h.

Profiles (10a and 10h), however the replacement of phenyl with 2-thienyl or 2-furyl along with 6-bromination at coumarin scaffold potentiated the inhibitory impact of compounds (11c and 11d). Overall, the herein reported coumarins emerge as selective inhibitors towards the tumour-related hCA IX and XII over the off-target hCA I and II that suggests their use as promising candidates for the development of more potent, selective hCA IX and XII inhibitors as anticancer agents.
3.3. Anticancer activity

3.3.1. In vitro antiproliferative activity against MCF-7 breast cancer cell line

The antiproliferative action of the most potent and selective hCA IX/XII inhibitors 10a, 10h and 11a–d was assessed against MCF-7 breast cancer cell line, since the overexpression of hCA IX is well-reported to be associated with poor prognosis of breast cancer [37] and the cell line has been previously used as a model in CA medicinal chemistry investigations. The antiproliferative potential was investigated using MTT assay [38] under hypoxic conditions employing staurosporine as a reference anticancer drug. The results are presented in Table 2 as median inhibitory concentration (IC50) which denotes the concentration of the tested drug required to produce 50% growth inhibition of the cancer cell compared to the negative control.

Investigation of the antiproliferative effects towards MCF-7 breast cancer cell line confirmed that the tested coumarins 10a, 10h and 11a–c exhibited moderate to excellent growth inhibitory influence (IC50 ranged between 0.48 and 11.1 μM). Of special interest, the 2-thienyl-bearing 6-unsubstituted coumarin 11a, that displayed potent hCA IX/XII inhibition at submicromolar level, exerted excellent antiproliferative action at submicromolar value (IC50 equals 0.48 μM). Likewise, the other tested coumarins 10a, 10h, 11b–d demonstrated moderate growth inhibitory action with IC50 values equal 3.13, 11.1, 4.14, 9.56 and 1.65 μM, respectively compared to staurosporine as reference drug (IC50 = 2.44 μM), Table 2.

Table 2. Anti-proliferative activities of 2,4-thiazolidinedione-tethered coumarins 10a, 10h and 11a–c against MCF-7 cell line.

| Compound | IC50 (μM)a (MCF-7) |
|----------|--------------------|
| 10a      | 3.13 ± 0.18        |
| 10h      | 11.1 ± 0.65        |
| 11a      | 0.48 ± 0.03        |
| 11b      | 4.14 ± 0.24        |
| 11c      | 9.56 ± 0.56        |
| 11d      | 1.65 ± 0.1         |
| Staurosporine | 2.44 ± 0.1           |

IC50 values are the mean ± SD of three experiments.

3.3.2. Cell cycle analysis

The influence of 2-thienyl-bearing 6-unsubstituted coumarin 11a on the cell cycle progression was investigated by flow cytometric in MCF-7 breast cancer cells, at 24h following treatment at its IC50 value (0.48 ± 0.03 μM), Figure 2.

As illustrated in Figure 2, the flow cytometric results showed that the exposure of MCF-7 breast cancer cells to compound 11a gave rise to a significant rise in the cell populations at Sub-G1, which increased by 19.7 folds with concomitant decrease in G2-M phase by 2.6 folds compared to the control, in addition to decline in cell populations within S and G0-G1 phases. This observation strongly suggests coumarin 11a induces apoptosis in MCF-7 cells.

3.3.3. Annexin V-FITC/propidium iodide (AV/PI) apoptosis assay

Annexin V-FITC/propidium iodide (AnxV/PI) dual staining assay was employed to confirm the potential apoptotic impact of coumarin 11a on early and late apoptosis percentages in MCF-7 breast cancer cells (Figure 3 and Table 3). This flow cytometric analysis highlighted that compound 11a was able to induce apoptosis in MCF-7 cells as indicated by the significant elevation in the percentage of annexin V-FITC-stained apoptotic cells including early apoptosis (Figure 3, lower right) from 0.37 to 4.23% and late apoptosis, Figure 3, upper right) from 0.15 to 25.7%. This represents 57 folds total increase relative to the control in apoptotic cells.

Figure 2. Impact of the tested coumarin 11a on the progression of cell cycle of MCF-7 cells.

Figure 3. Effect of coumarin 11a on the percentage of AV positive staining in breast MCF-7 cells.
Table 3. Distribution of AV-FITC/PI positive stained apoptotic MCF-7 cells.

| Comp. | Total | Early | Late | Necrosis |
|-------|-------|-------|------|----------|
| 11a   | 29.93 | 4.23  | 25.7 | 17.39    |
| Control | 0.52  | 0.37  | 0.15 | 1.14     |

4. Conclusions

In this study, different 2,4-thiazolidinedione-tethered coumarins 5a-b, 10a-a and 11a-d have been synthesised and evaluated for their inhibitory action against the cancer-associated hCAs IX and XII, in addition to the physiologically dominant hCAs I and II, in order to explore their selectivity. Interestingly, none of the coumarins had any inhibitory effect on off-target hCA I and II isoforms. Unsubstituted phenyl-bearing coumarins 10a, 10h, and 2-thienyl/furfuryl-bearing coumarins 11a-c exhibited the best hCA IX (Ks between 0.48 and 0.93 μM) and hCA XII (Ks between 0.44 and 1.1 μM) inhibitory actions. Coumarins 10a, 10h and 11a-c were subjected to an in vitro antiproliferative assay, and then the most potent antiproliferative agent 11a was tested to explore its impact on the cell cycle phases and apoptosis in MCF-7 breast cancer cells furnishing more insights on the potential anticancer activity of such compounds.

Disclosure statement

No potential conflict of interest was reported by the author(s). CT Supuran is Editor-in-Chief of the Journal of Enzyme Inhibition and Medicinal Chemistry. He was not involved in the assessment, peer review, or decision-making process of this paper. The authors have no relevant affiliations of financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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