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SHORT COMMUNICATION

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Sulfocoumarins as dual inhibitors of human carbonic anhydrase isoforms IX/XII and of human thioredoxin reductase

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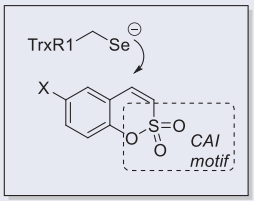
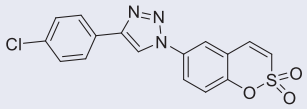
ABSTRACT

The hypothesis that sulfocoumarin acting as inhibitors of human carbonic anhydrase (CA, EC 4.2.1.1) cancer-associated isoforms *h*CA IX and – *h*CA XII is being able to also inhibit thioredoxin reductase was verified and confirmed. The dual targeting of two cancer cell defence mechanisms, i.e. hypoxia and oxidative stress, may both contribute to the observed antiproliferative profile of these compounds against many cancer cell lines. This unprecedented dual anticancer mechanism may lead to a new approach for designing innovative therapeutic agents.





	IC_{50}, μM		K_i, nM
TrxR activity	154	*h*CA I	>10,000
HT-1080	11.0	*h*CA II	>10,000
SHSY5Y	22.0	*h*CA IX	7.8
MCF-7	1.8	*h*CA XII	17.7

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Anticancer agents; carbonic anhydrase inhibition; thioredoxin reductase inhibition; hypoxia; oxidative stress

1. Introduction

Earlier, we reported 6-substituted sulfocoumarins **1**[1] (designed as isosteres of the structurally related coumarins[2–6]) as potent and remarkably isoform-selective inhibitors of the metallo-enzyme carbonic anhydrase (CA, EC 4.2.1.1)[7,8]. The ability of sulfocoumarins to selectively inhibit membrane-bound *h*CA IX and XII isoforms were attributed to the unique mechanism of action of these compounds whereby they act as prodrugs activated by CA-mediated hydrolysis[1–6]. This makes these inhibitors fundamentally different from the classical carbonic anhydrase inhibitors (CAIs) – e.g. those of sulphonamide type which act by binding to the CA prosthetic zinc ion present in all isoforms, which makes designing isoform-selective sulphonamide CAIs particularly difficult. On the contrary, CA-mediated hydrolysis of sulfocoumarins **1** (as well as their progenitors coumarins) leads to the *in situ* formation of the *Z*-configured stiryl sulphonic acid (*Z*)-**2** which is likely to isomerise to (*E*)-**2**, the active inhibitor form whose binding to CA was confirmed by X-ray crystallography[1]. This inhibitor activation and binding apparently occurs only in the protein environment of the two membrane-bound isoforms (*h*CA IX and XII) which makes these mechanistically distinct inhibitors ideal tools for targeting hypoxia survival mechanism in tumour cells providing which overexpression of precisely these two isoforms is considered responsible for[9]. Indeed, selective targeting of *h*CA IX and XII has been confirmed to lead to retardation of tumour growth and, ultimately, reduction of tumour size[10].

Another principal mechanism of tumour survival which we have been recently tackling[11,12] as a target for anticancer agent design, is that providing tumour cell defence against oxidative stress (reactive oxygen species or ROS). In particular, tumour cells have been shown to overexpress thioredoxin reductase (TrxR, EC 1.8.1.9) which contributes to their resistant phenotype characterised by higher levels of ROS[13]. Thus, targeting TrxR1 (the most widespread cytosolic isoform of human TrxR) has been investigated as an emerging approach to selective killing of cancer cells[14]. This selenocysteine (Sec) enzyme, along with NADPH and thioredoxin (Trx) is part of the Trx system and responsible for maintaining Trx in its reduced bis-sulfhydryl state. Among several classes of inhibitors of varying degree of electrophilicity towards the catalytic Sec residue (recently reviewed by Bellelli[15] and Fang[16]), we found covalent Michael acceptor inhibitors (such as Ugi-type adducts **3** which we dubbed "Ugi Michael Acceptors" or UMAs) to be particularly efficacious[12]. The mechanism of inhibitory action of UMAs towards TrxR1 likely involves the irreversible covalent trapping of the selenide group of the catalytic Sec residue (which exists in the ionised form at physiological pH[17]) by the electrophilic β-benzoylacrylamide moiety present in **3**.

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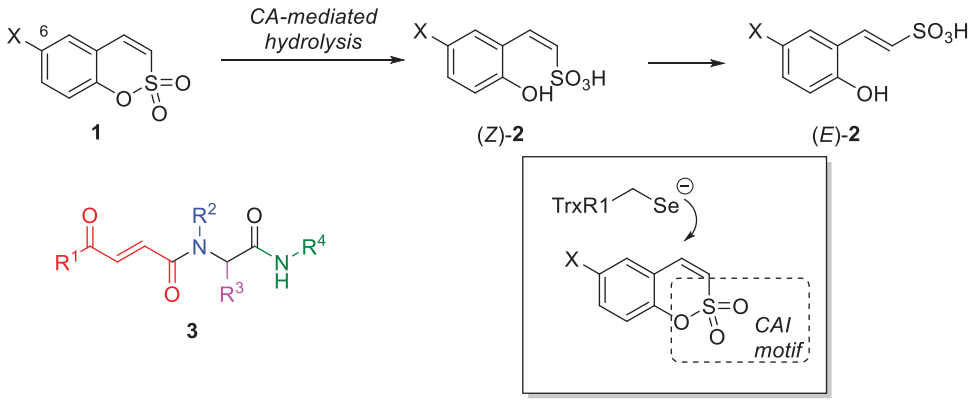


Figure 1. Sulfocoumarins 1 and their CA inhibition mechanism, the previously reported Ugi Michael acceptor TrxR inhibitors (fragments originating from the four components of the Ugi reaction are colour-coded) and the hypothesis for dual CA/TrxR targeting verified in this work.

Considering the presence of a potential Michael acceptor moiety in sulfocoumarins **1**, we hypothesised that in addition to their inhibitory activity towards *h*CAs, these compounds could potentially act as Michael acceptor-type TrxR inhibitors (Figure 1), thus acting as dual inhibitors which target two cancer cell defence mechanisms at a time. Herein, we present our preliminary results obtained in the course of verifying this hypothesis.

2. Materials and methods

2.1. Chemical syntheses – general

Reagents and starting materials were obtained from commercial sources (Sigma-Aldrich, St. Louis, MO) and used as received. The solvents were purified and dried by standard procedures prior to use; petroleum ether of boiling range 40–60° C was used. Flash chromatography was carried out using Merck silica gel (230–400 mesh). Thin-layer chromatography was performed on silica gel, spots were visualised with UV light (254 and 365 nM). Melting points were determined on an OptiMelt automated melting point system. IR spectra were measured on a Shimadzu FTIR IR Prestige-21 spectrometer. NMR spectra were recorded on Varian Mercury (400 MHz) spectrometer with chemical shifts values (*d*) in ppm relative to TMS using the residual DMSO-d_6 signal as an internal standard. Elemental analyses were performed on a Carlo Erba CHNSeO EA-1108 apparatus. Starting material sulfocoumarins (**4**[18] and **5**[19]) were prepared as described previously. Alkynes employed in the synthesis of **1a**–**b** are commercially available. Tetrazoles employed in the synthesis of **1c**–**d** were prepared according to the literature protocols[20,21]. All reagents for biological assays were purchased from Sigma (St. Louis, MO).

2.2. General procedure 1: preparation of sulfocoumarins 1a–b (GP1)

To a solution of **4** (1.0 equiv.) in dry THF (1 mL per mmol of **4**) *N,N*-diisopropylethylamine (DIPEA) (50 equiv.), the appropriate alkyne (1.1, 2.0, or 5.0 equiv.), and CuI (2 equiv.) were added. The resulting mixture was stirred at room temperature under an argon atmosphere for 20 h. Saturated NH_4Cl was added and extracted with EtOAc, washed with brine and dried over Na_2SO_4, and evaporated.

2.2.1. 4-(4-Chlorophenyl)-1-(2,2-dioxido-1,2-benzoxathiin-6-yl)-1H-1,2,3-triazole (1a)

Prepared from **4** (0.15 g, 0.67 mmol), 4-chlorophenylacetylene (0.18 g, 1.34 mmol), CuI (0.26 g, 1.34 mmol), and DIPEA (5.85 mL, 33.6 mmol) according to GP1. Crystallisation from ethanol afforded **1a** as yellow crystalline solid (0.19 g, 77%). Mp 236–237 °C. IR (KBr, cm^{-1}) ν_{max}: 1369 (S–O), 1179 (S–O), and 1169 (S–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.55–7.60 (m, 2H), 7.70 (d, J= 10.4 Hz, 1H), 7.75 (d, J= 8.9 Hz, 1H), 7.84 (d, J= 10.4 Hz, 1H), 7.92–7.97 (m, 2H), 8.12 (dd, J= 8.9, 2.7 Hz, 1H), 8.39 (d, J= 2.7 Hz, 1H), and 9.38 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 119.9, 120.2, 120.3, 121.4, 123.7, 124.0, 127.0, 128.9, 129.2, 132.9, 134.2, 135.8, 146.4, and 150.1. Anal. Calcd. for $C_{16}H_{10}N_3O_3SCl$ (359.79): C, 53.41; H, 2.80; N, 11.68. Found: C, 53.22; H, 2.79; N, 11.32.

2.2.2. 1-(2,2-Dioxido-1,2-benzoxathiin-6-yl)-4-(4-fluorophenyl)-1H-1,2,3-triazole (1b)

Prepared from **4** (0.15 g, 0.67 mmol), 4-fluorophenylacetylene (0.16 g, 1.34 mmol), CuI (0.26 g, 1.34 mmol), and DIPEA (5.85 mL, 33.6 mmol) according to GP1. Yellow crystalline solid (0.19 g, 80%). Mp 224–225 °C. IR (KBr, cm^{-1}) ν_{max}: 1359 (S–O) and 1179 (S–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.32–7.39 (m, 2H), 7.71 (d, J= 10.4 Hz, 1H), 7.75 (d, J= 8.9 Hz, 1H), 7.84 (d, J= 10.4 Hz, 1H), 7.94–8.00 (m, 2H), 8.12 (dd, J= 8.9, 2.6 Hz, 1H), 8.39 (d, J= 2.6 Hz, 1H), and 9.33 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 116.1 (d, J= 21.9 Hz), 119.8, 119.9, 120.2, 121.4, 123.7, 124.0, 126.6 (d, J= 3.2 Hz), 127.4 (d, J= 8.3 Hz), 134.2, 135.9, 146.6, 150.1, and 162.4 (d, J= 245.3 Hz). Anal. Calcd. for $C_{16}H_{10}N_3O_3SF$ (343.33): C, 55.97; H, 2.94; N, 12.24. Found: C, 55.78; H, 2.94; N, 12.24.

2.2.3. 5-(2,2-Dioxido-1,2-benzoxathiin-6-yl)-1-phenyl-1H-tetrazole (1c)

Compound **5** (0.200 g, 0.649 mmol), 1-phenyl-1,2,3,4-tetrazole[20] (0.190 g, 1.30 mmol), Cs_2CO_3 (0.233 g, 0.714 mmol), CuI (0.124 g, 0.649 mmol), $Pd(OAc)_2$ (0.0146 g, 0.0649 mmol), and tris(2-furyl) phosphine (0.030 g, 0.130 mmol) were suspended in dry toluene (3 mL). The mixture was stirred at 40 °C under argon for 20 h, then EtOAc (20 mL) was added and the mixture was filtered through celite. Celite was washed with EtOAc (50 mL). The filtrate and washings were combined and concentrated under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/EtOAc 2:1) and additionally crystallised from EtOH to give **1c** as yellow crystalline solid (0.076 g, 36%). Mp 189–190 °C. IR (KBr, cm^{-1}) ν_{max}: 1370 (S–O) and 1178 (S–O). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.49–7.56 (m, 2H), 7.58–7.68 (m, 6H), 7.78 (d, 1H, J= 10.4 Hz), and 8.09–8.12 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6)

δ: 119.2, 119.3, 121.6, 123.6, 126.0, 130.0, 130.8, 131.0, 132.5, 133.8, 135.9, 152.2, and 152.4. Anal. Calcd. for $C_{15}H_{10}N_4O_3S$ (326.33): C,55.21; H, 3.09; N, 17.17. Found: C, 55.25; H, 3.09; N, 17.08.

2.2.4. 1-(2,2-Dioxido-1,2-benzoxathiin-6-yl)-5-(4-fluorophenyl)-1H-1,2,3-triazole (1d)

To a solution of **5** (0.25 g, 1.12 mmol) and 4-fluorophenylacetylene (0.27 g, 2.24 mmol) in dry DMF (0.7 mL), $Cp^*Ru(PPh_3)_2Cl$ (0.01 mmol) was added and the resulting mixture was stirred at 100 °C under an argon atmosphere for 20 h. The solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (petroleum ether/EtOAc 2:1) to give **1d** as yellow crystalline solid (0.11 g, 28%). Mp 157–158 °C. IR (neat, cm^{-1}) ν_{max}: 1373 (S–O) and 1176 (S–O). ^1H NMR (400 MHz, DMSO-d_6) δ: 7.25–7.33 (m, 2H), 7.37–7.43 (m, 2H), 7.56 (dd, *J*= 8.8, 2.5 Hz, 1H), 7.61 (d, *J*= 8.8 Hz, 1H), 7.67 (d, *J*= 10.4 Hz, 1H), 7.75 (d, *J*= 10.4 Hz, 1H), 7.97 (d, *J*= 2.5 Hz, 1H), and 8.17 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 116.1 (d, *J*= 22.1 Hz), 119.6, 119.8, 122.4 (d, *J*= 3.2 Hz), 123.7, 127.1, 129.3, 131.1 (d, *J*= 8.8 Hz), 133.4, 133.6, 135.7, 137.0, 150.8, and 162.6 (d, *J*= 247.7 Hz). Anal. Calcd. for $C_{16}H_{10}N_3O_3SF$ (343.33): C, 55.97; H, 2.94; N, 12.24. Found: C, 56.17; H, 2.93; N, 11.93.

2.3. Carbonic anhydrase inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity[22]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Tris (pH 8.3) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionised water and dilutions up to 0.005 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house[23–26].

2.4. TrxR activity by DTNB reduction assay

Determination of TrxR activity in SHSY5Y cell lysate. TrxR activity in cell lysate was measured in 96-well plates using previously described methods[27,28]. For TrxR activity measurement, compounds of different concentrations were incubated with 50 μg of cell lysate and 200 μM NADPH in a volume of 100 μL of 50 mM Tris–HCl and 1 mM EDTA, pH 7.5 (TE buffer), for different time points in 96-well plates at room temperature. Then, 100 μL of TE buffer containing DTNB and NADPH was added (final concentration: 2.5 mM and 200 μM, respectively), and the linear increase in absorbance at 412 nm during the initial 2 min was measured with a Tecan Infinite M1000 multifunctional microplate reader. TrxR activity was calculated as a percentage of enzyme activity of that of DMSO vehicle treated sample.

2.5. Cytotoxicity assay

Thus, monolayer tumour cell lines HT-1080 (human fibrosarcoma), SHSY5Y (human neuroblastoma), and MCF-7 (breast adenocarcinoma) were cultured in standard medium DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum. About 2000–4000 cells per well (depending on line nature) were placed in 96-well plates and after 24 h compounds were added to the wells. Untreated cells were used as a control. The plates were incubated for 48 h, 37 °C, and 5% CO_2. The number of surviving cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT). MTT-test: after incubating culture medium was removed and 200 μL fresh medium with 20 μL MTT (2 mg/mL in HBSS) was added in each well of the plate. After incubation (3 h, 37 °C, 5% CO_2), the medium with MTT was removed and 200 μL DMSO were added at once to each sample. The samples were tested at 540 nm on Thermo Scientific Multiskan EX microplate photometer. The half-maximal inhibitory concentration (IC_{50}) of each compound was calculated using Graph Pad Prism® 3.0 (GraphPad Software, La Jolla, CA).

3. Results and discussion

3.1. Chemistry

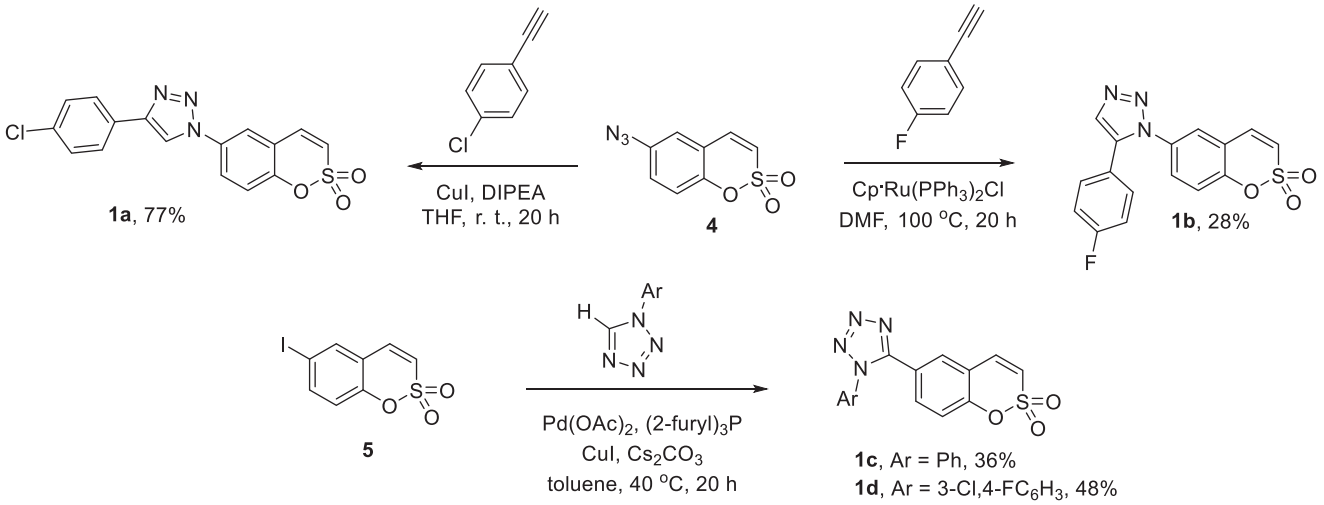
Compounds **1a** and **1b** were synthesised from azide **4** as described previously[18]. CuI-catalysed Huisgen azide-alkyne cycloaddition gave 1,4-disubstituted 1,2,3-triazole **1a** while employing Ru^{II}-catalysed protocol gave 1,5-disusbstituted 1,2,3-triazole **1b**. For the synthesis of 1,5-disubstituted tetrazoles **1c**–**d**, the previously described[19] Pd-catalysed arylation of 1-aryl tetrazoles with aryl iodide **5** was employed (Scheme 1).

3.2. Biological evaluation

To our utmost delight, when the previously established[18,19] potent and selective inhibitory profile of compounds **1a**–**d** towards cancer-related *h*CA IX and *h*CA XII isoforms was confirmed in reference to known CAI acetazolamide (**AAZ**), we have also found these compounds to display dose-dependent inhibition of TrxR activity in SHSY5Y cell lysate with IC_{50} values confidently residing in the $10^{-5} \ldots 10^{-4}$ M range. Adding to the satisfaction over having our initial hypothesis regarding the dual CA/TrxR inhibitory effects of compounds **1**, rather potent antiproliferative activity was established as evaluated against cultures of cancer cells such as HT-1080 (human fibrosarcoma), SHSY5Y (human neuroblastoma), and MCF-7 (breast adenocarcinoma). These findings are summarised in Table 1.

4. Conclusions

The previously described sulfocoumarins that were shown to potently and selectively inhibit cancer-related *h*CA IX and *h*CAXII isoforms (whose overexpression is a well-established mechanism of tumour cell defence against hypoxia) also display noticeable, dose-dependent inhibition of TrxR activity in cancer cell lysates. As overexpression of TrxR in cancer cells is a defence mechanism against oxidative stress, the established dual inhibition pattern constitutes a significant starting point for the design and discovery of new anticancer agents based on the dual targeting of the



Scheme 1. Synthesis of compounds **1a–d**.

Table 1. Inhibitory profile towards four *h*CA isoforms, TrxR activity in SHSY5Y cell lysate and cytotoxicity towards cancer cell lines determined for compounds **1a–d** (nd: not determined).

	IC_{50}, μM				K_i, nM			
Compound	HT-1080	SHSY5Y	MCF-7	TrxR IC_{50}, μM	*h*CA I	*h*CA II	*h*CA IX	*h*CA XII
1a	11.0	22.0	1.8	154	>10,000	>10,000	7.8	17.7
1b	8.7	29.0	79.0	40	>10,000	>10,000	8.3	7.8
1c	12.3	18.4	101	156	>10,000	>10,000	8.5	7.1
1d	7.9	18.5	50.0	125	>10,000	>10,000	6.9	5.4
AAZ	nd	nd	nd	nd	250	12	25	5.7

two defence mechanisms crucial for cancer cell survival. This communication opens a new line of research in our laboratories aimed at investigating the practical aspects of the new dual inhibitor design and establishing a well-understood link between inhibition of these two enzyme groups and the dual inhibitors' antitumor activity. The results of this research will be reported in due course.

Disclosure statement

No potential conflict of interest was reported by the authors.

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