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Pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides as carbonic anhydrase inhibitors with antitumor activity

Mariusz Mojzych [a,*], Anna Bielawska [b], Krzysztof Bielawski [b], Mariangela Ceruso [c], Claudiu T. Supuran [c,d,*]

[a] Department of Chemistry, Siedlce University of Natural Sciences and Humanities, 3-go Maja 54, 08-110 Siedlce, Poland
[b] Department of Medicinal Chemistry and Drug Technology, Medical University of Bialystok, Bialystok, Poland
[c] Università degli Studi di Firenze, Polo Scientifico, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, 50019 Sesto Fiorentino, Florence, Italy
[d] Università degli Studi di Firenze, NEUROFARBA Department, Sezione di Scienze Farmaceutiche e Nutraceutiche, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy

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ABSTRACT

A series of sildenafil analogues and aniline substituted pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides were prepared and evaluated as carbonic anhydrase (CA, EC 4.2.1.1) inhibitors and for their anticancer activity against two human breast cancer cell lines (MCF-7, MDA-MB-231). The new compounds were ineffective as CA I inhibitors, poorly inhibited CA II, but were more effective against the tumor-associated isoforms CA IX and XII, with some compounds acting as low nanomolar inhibitors. Evaluation of the cytotoxicity by using an MTT assay, the inhibition of [^{3}H]thymidine incorporation into DNA as well as collagen synthesis inhibition, demonstrated that these sulfonamides exhibit cytotoxic effects on breast cancer cell lines ex vivo.

1. Introduction

The pyrazolo-1,2,4-triazines have received considerable attention due to their pharmacological activities as antiviral,[1] antitumour,[2] antifungal,[3] analgesic,[4] anti-inflammatory,[4] and antipyretic agents.[5] However, despite of the wide range of biological activity the pyrazolo[4,3-*e*][1,2,4]triazines are a less known class in the group of condensed pyrazolotriazines. In the past decades, the isolation and structural characterization of naturally occurring pyrazolo[4,3-*e*][1,2,4]triazines, pseudoiodinine, nostocine A and fluviols A–E, was reported.[5–7] In addition, these compounds were reported to have cytotoxic activity on human cancer cell, such as A549 (lung adenocarcinoma).[8]

The sulfonamides represent an important class of pharmaceutical compounds with a wide spectrum of biological activities[9] such as anticancer,[10] carbonic anhydrase (CA) inhibitory,[11] antibacterial,[12] antimalarial,[13] antitumor,[14] antihypertensive,[15] anti-inflammatory,[16] and antiprotozoal activities among others.[17]

Considering our interest in the development of new anticancer agents belonging to the carbonic anhydrase (CA, EC 4.2.1.1) inhibitor class, in this work we report the synthesis of new series of pyrazolo[4,3-*e*][1,2,4]triazines derivatives belonging two groups, that is, analogues of sildenafil and aniline substituted pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides. The new compounds were evaluated as CA inhibitors and for their cytotoxic effects on several breast cancer cell lines.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of 4a–j

The synthesis of the aza-sildenafil analogues **4a–j** was achieved starting from 1,3-dimethyl-5-methylsulfanyl-1*H*-pyrazolo[4,3-*e*]-[1,2,4]triazine (**1**)[18] which was reacted[19] with 2-ethoxyphenylboronic acid in the presence of copper(I) 3-methylsalicylate, leading to derivative **2** in an excellent yield. Chlorosulfonylation of **2** with chlorosulfonic acid at 0 °C proceeded smoothly and selectively at the 5′-position of the phenyl ring to give compound **3** in excellent yield. The chlorosulfonyl derivative **3** was then coupled with the

* Corresponding authors. Tel.: +48 025 643 1105; fax: +48 025 6442045 (M.M.); tel.: +39 055 457 3005; fax: +39 055 457 3385 (C.T.S.).
E-mail addresses: mmojzych@yahoo.com (M. Mojzych), claudiu.supuran@unifi.it (C.T. Supuran).

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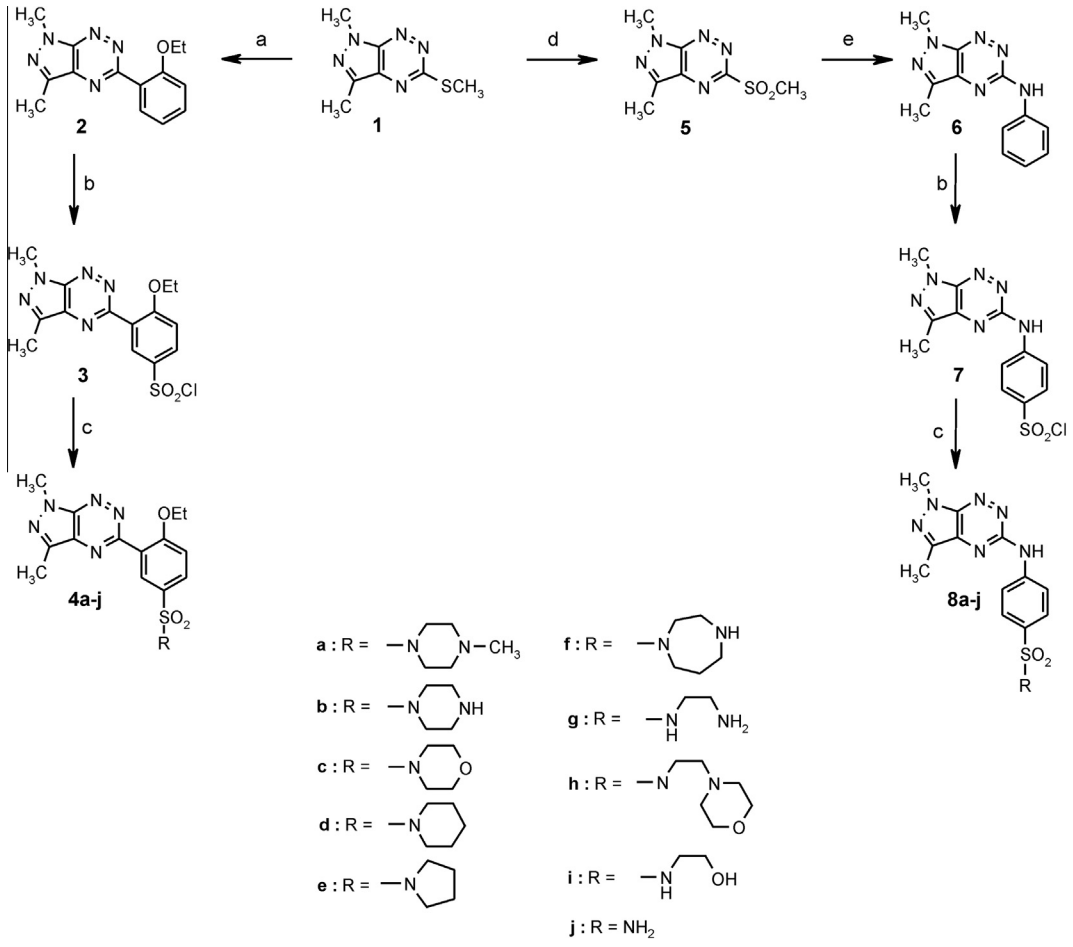

Scheme 1. Reagents and conditions: (a) ethoxyphenylboronic acid, $Pd(PPh_3)_4$, CuMeSal, THF, Ar, reflux, overnight; (b) $ClSO_3H$, 0 °C to rt, 2 h; (c) NH_3/H_2O or amine, MeCN, rt; (d) $KMnO_4$, Bu_4NBr, CH_3COOH, benzene-H_2O, rt, 1 h and (e) aniline, seal tube, 9 days, 150 °C.

appropriate amine in acetonitrile at room temperature to give sulfonamides **4a–j** as new aza-analogues of sildenafil (Scheme 1).

2.1.2. Synthesis of 8a–j

Derivative **1** was treated with potassium manganate(VII) under phase transfer catalytic conditions at room temperature for 1 h to give sulfone **5** in nearly quantitative yield.[20] Compound **5** was then reacted with anhydrous aniline in a sealed tube at 150 °C for 9 days. Chlorosulfonylation of the aniline substituted pyrazolotriazine **5** with chlorosulfonic acid at 0 °C proceeded selectively at the 4′-position of the phenyl ring, and gave compound **6** in an excellent yield. The chlorosulfonyl derivative **6** was then coupled with amines to produce the target sulfonamides **8a–j** as shown in Scheme 1.

Structures and purity of the newly synthesized compounds were characterized using the ^1H and ^{13}C NMR, as well as MS methods together with elemental analysis. The spectral data was published earlier[21,22] and they fully confirmed the structures of all obtained sulfonamides.

2.2. Pharmacology

2.2.1. Cytotoxic activity of 4a–4i

The viability of MCF-7 and MDA-MB-231 breast cancer cells was measured by the method of Carmichael using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (Table 1).[23] Although growth inhibition was concentration-dependent in either cell line, it was more pronounced at shorter times, in MCF-7 than MDA-MB-231 (Table 1). In terms of reduction in cell viability, the compounds rank in both MCF-7 and MDA-MB-231 cells in the order **chlorambucil** > **4d** > **4a** > **4e** > **4f** > **4h** > **4b** > **4c** > **4g** > **4i**.

We studied the effect of compounds **4a–4i** and chlorambucil on DNA synthesis in both MDA-MB-231 and MCF-7 breast cancer cells (Table 1). All of the tested compounds showed concentration dependent activity, yet with different potency. The concentrations of **4g**, **4i** needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) in MDA-MB-231 and MCF-7 cells was above 200 μM suggesting low cytotoxic potency compared to chlorambucil (IC_{50} = 49 ± 2 μM and 56 ± 2 μM, respectively). The concentrations of **4d**, **4f**, **4e**, **4a**, **4h**, **4c**, **4b** needed to 50% reduction in [^{3}H]thymidine incorporation into DNA in breast cancer MCF-7 (IC_{50}) obtained in the range 112 ± 1 to 150 ± 2 μM.

2.2.2. Cytotoxic activity of 8a–8i

The cytotoxicity was determined as described above for compounds **4a–i** (Table 2).[23] In terms of reduction in cell viability, the compounds ranked in both MCF-7 and MDA-MB-231 cells in the order: chlorambucil > **8a** > **8g** > **8f** > **8h** > **8b** > **8d**,**8c**,**8i**,**8e**. Among these derivatives, compound **8a** in both MDA-MB-231 and MCF-7 proved to be only slightly less potent than chlorambucil, with IC_{50} values of 99 ± 2 and 102 ± 2 μM, respectively, compared to 93 ± 2 and 97 ± 2 μM for chlorambucil.

We studied the effect of compounds **8a–8i** and chlorambucil on DNA synthesis in both MDA-MB-231 and MCF-7 breast cancer cells (Table 2). All of the tested compounds showed concentration dependent activity, yet with different potency. The concentrations

Table 1
Cytotoxic and cytostatic activities of new sulfonamides derivatives of pyrazolo[4,3-*e*][1,2,4]triazines **4a–4i** after 24 h incubation

Compd	MTT assay, IC_{50}[a] (μM)		[^{3}H]thymidine incorporation, IC_{50}[a] (μM)	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
4a	110 ± 2	115 ± 2	140 ± 1	152 ± 2
4b	172 ± 2	182 ± 2	150 ± 2	164 ± 2
4c	181 ± 2	189 ± 2	148 ± 1	160 ± 1
4d	105 ± 3	98 ± 2	112 ± 1	126 ± 2
4e	112 ± 2	120 ± 2	130 ± 2	159 ± 1
4f	123 ± 2	130 ± 1	129 ± 2	115 ± 1
4g	190 ± 1	>200	>200	200 ± 1
4h	132 ± 2	136 ± 2	145 ± 1	156 ± 2
4i	>200	>200	>200	>200
Chlorambucil	97 ± 2	93 ± 2	56 ± 2	49 ± 2

[a] Data represent the mean ± S.D. of each compound from four independent experiments.

Table 2
Cytotoxic and cytostatic activities of new sulfonamides derivatives of pyrazolo[4,3-*e*][1,2,4]triazines **8a–8i** after 24 h incubation

Compd	MTT assay, IC_{50}[a] (μM)		[^{3}H]thymidine incorporation, IC_{50}[a] (μM)	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
8a	102 ± 2	99 ± 2	87 ± 2	80 ± 2
8b	150 ± 2	130 ± 2	170 ± 2	103 ± 2
8c	>200	>200	>200	>200
8d	200 ± 2	140 ± 1	150 ± 2	135 ± 1
8e	>200	>200	>200	>200
8f	140 ± 3	155 ± 2	123 ± 1	150 ± 2
8g	126 ± 1	120 ± 1	85 ± 1	90 ± 1
8h	146 ± 1	125 ± 2	99 ± 1	120 ± 2
8i	>200	>200	>200	>200
Chlorambucil	97 ± 2	93 ± 2	56 ± 2	49 ± 2

[a] Data represent the mean ± S.D. of each compound from four independent experiments.

of **8c**, **8e**, **8i** needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) in MDA-MB-231 and MCF-7 cells was above 200 μM suggesting low cytotoxic potency compared to chlorambucil (IC_{50} = 49 ± 2 μM and 56 ± 2 μM, respectively). The concentrations of **8b**, **8d**, **8f**, **8g**, **8h**, needed to 50% reduction in [^{3}H]thymidine incorporation into DNA in breast cancer MCF-7 (IC_{50}) obtained in the range 85 ± 1 to 170 ± 2 μM. Among the derivatives, compound **8a** in both MDA-MB-231 and MCF-7 proved to be slightly less potent than chlorambucil, with IC_{50} values of 80 ± 2 and 87 ± 2 μM, respectively.

Collagen biosynthesis was measured in MCF-7 and MDA-MB231 breast cancer cells treated with various concentrations of compounds **8a–8i** (Table 3) and chlorambucil for 24 h. In both cell lines compound **8a** was found to be more effective inhibitor of collagen biosynthesis than chlorambucil. IC_{50} for **8a** and chlorambucil (in MDA-MB231: 47 μM and 52 μM, in MCF-7: 58 μM and 72 μM, respectively) showed specific inhibitory effect of compound **8a** on collagen biosynthesis.

Table 3
Collagen synthesis, measured by 5-[^{3}H]-proline incorporation into proteins susceptible to the action of bacterial collagenase, in MCF-7 and MDA-MB-231 breast cancer cells in the presence of compounds **8a–8i** and chlorambucil

Compd	IC_{50}[a] (μM)	
	MCF-7	MDA-MB-231
8a	47 ± 1	58 ± 2
8b	175 ± 1	180 ± 2
8c	>200	>200
8d	163 ± 2	145 ± 1
8e	>200	>200
8f	133 ± 1	135 ± 2
8g	112 ± 1	95 ± 1
8h	140 ± 1	137 ± 2
8i	>200	>200
Chlorambucil	52 ± 1	72 ± 2

[a] Mean values from three independent experiments done in duplicates ± standard deviation (S.D.) are presented.

Our experimental studies have demonstrated that sildenafil analogue **4a–i** and aniline substituted pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides **8a–i** treatment prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells. The structure–activity correlation of the obtained results revealed that the presence of the NH spacer between the pyrazolotriazine moiety and phenyl ring is 'essential' for biological activity. The compound **8a**, which possess a *N*-metylpiperazine function, is the most cytotoxic compound among a series of aniline substituted pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides we have synthesized to date. Further investigations on the mechanisms of the cytotoxicity, and preparation of new pyrazolo[4,3-*e*][1,2,4]triazine derivatives are now in progress.

2.2.3. CA activity of 8a–8j

As sulfonamides are the preferred pharmacophore for the inhibition of carbonic anhyrases (CAs, EC 4.2.1.1)[25] we investigated whether the new compounds reported here may show such an activity. We investigated inhibition of the cytosolic ubiquitous isoform hCA I and II (h = human isoform) as well as the transmembrane, tumor-associated ones hCA IX and XII with compounds **5–8** reported in the paper (Table 4).

It may be observed that hCA I was not inhibited by all derivatives except **8j** (the primary sulfonamide) which acted as a weak inhibitor, comparable to the clinically used acetazolamide.

The physiologically dominant isoform hCA II was potently inhibited (K_Is of 6.3–8.0 nM) only by **8e** and **8j**, incorporating the five-membered heterocyclic ring and the SO_2NH_2 group, respectively, whereas the remaining derivatives showed quite weak activity against this isoform, with inhibition constants ranging between

Table 4
CA I, II, IX and XII inhibition data for compounds **5**, **6** and sulphonamides **8a–8i**[24]

Compd	K_I^* (nM)			
	hCA I	hCA II	hCA IX	hCA XII
5	>50,000	774	89	6.6
6	>50,000	826	82	9.0
8a	>50,000	667	>50,000	5.3
8b	>50,000	703	>50,000	5.7
8c	nt	nt	nt	nt
8d	>50,000	492	>50,000	6.2
8e	>50,000	6.3	652	8.1
8f	>50,000	918	>50,000	5.9
8g	>50,000	62.1	23.7	7.1
8h	>50,000	719	824	6.2
8i	>50,000	772	26.5	7.5
8j	270	8.0	43.8	7.9
Acetazolamide	250	12	25	5.8

Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was used as standard drug in the assay.
nt = not tested.
* Mean from 3 different assay, by a CO_2 hydration stopped-flow assay method.

62.1–918 nM. It is difficult to explain the striking difference of activity between **8e** (an effective CAI) and the structurally congeners related **8d** and **8f**, differing by few atoms compared to **8e**, and acting as ineffective hCA II inhibitors (Table 4).

The tumor-associated isoforms hCA IX and XII,[26] were on the other hand better inhibited by some of the derivatives investigated here. Thus, hCA IX was not at all inhibited by four of the new derivatives (**8a**, **8b**, **8d** and **8f**), was weakly inhibited by two of them (**8e** and **8h**), whereas **5**, **6**, **8g**, **8i** and **8j** were more effective as hCA IX inhibitors, with K_Is in the range of 23.7–89 nM.

On the contrary, hCA XII was potently inhibited (K_Is <10 nM) by all the reported compounds from the ms (Table 4).

3. Experimental

3.1. Pharmacology

3.1.1. Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02%EDTA in calcium-free phosphate buffered saline, counted in hemocytometers and plated at 5×10^5 cells per well of 6-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

3.1.2. DNA synthesis assay

MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and were incubated with varying concentrations of **6a–6l** or chlorambucil and 0.5 μCi of [^{3}H]-thymidine for 24 h at 37 °C. The cells were then harvested by trypsinization, washed with cold phosphate-buffered saline and centrifuged for 10 min at 1500 g several times (4–5) until the dpm in the washes were similar to the reagent control. Radioactivity was determined by liquid scintillation counting. [^{3}H]-thymidine uptake was expressed as dpm/well.

3.1.3. Cell viability assay

The assay was performed according to the method of Carmichael using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[23] Confluent cells, cultured for 24 h with various concentrations of studied compounds in 6-well plates were washed three times with PBS and then incubated for4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCI in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer cells cultured in the presence of studied compounds was calculated as a per cent of control cells. The experiments were performed in triplicates. After treatment of the cells with drug, the ratio of survived to dead cells in tested and control (untreated) cells was calculated for each drug concentration. Cell number was plotted versus drug concentration, and IC_{50} values were calculated from dose-response curves as the concentration of drugs that reduce the number of viable cells to 50% of control using an Origin 7.5 software.

3.1.4. Collagen synthesis assay

Incorporation of a radioactive precursor into proteins was measured after labeling the cells with 5-[^{3}H]proline (5 μCi/mL, 28 Ci/mmol) for 24 h in growth medium with varying concentrations of PAMAM-CH conjugate or chlorambucil.[27] Incorporation of a tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky.[28] Results were shown as combined values for cell plus medium fractions.

3.1.5. CA activity and inhibition measurements

An applied photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 for maintaining constant the ionic strength, following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s.[24] 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 uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (0.01 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[29] and represent the mean from at least three different determinations.

4. Conclusion

In conclusion, we report a practical, high yielding, and scalable method for the preparation of two groups of new 1*H*-pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides from simple, available starting materials. The preliminary antitumor studies revealed that these derivatives exhibited moderate antitumor activity in breast cancer cells ex vivo. These compounds were ineffectively as hCA I and II inhibitors, but inhibited appreciably the tumor-associated isoforms hCA IX and XII.

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