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Biological Activity Evaluation of Pyrazolo[4,3-e][1,2,4] Triazine Sulfonamides

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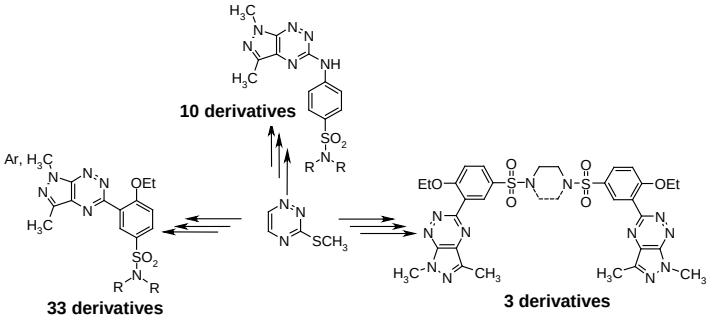
Graphical Abstract



Abstract

The aim of this study was to investigate the ability of synthesized pyrazolo[4,3-*e*][1,2,4]triazine sulfonamide compounds to affect development of Cytotoxic T Lymphocytes (CTL) *in vitro* in mixed leukocyte cultures. Compounds were dissolved in DMSO (1 mM) and added to cultures of mouse lymphocytes (BL/6) stimulated with irradiated allogeneic cells (BALB/c). Following 6 days of culture, cells were harvested from all cultures, washed, and assayed in 5 hr ^{51}Cr release assays with labeled P815 tumor target cells for analysis of CTL with BL/6 anti-BALB/c specificity. All assays were performed on three separate occasions, and data analyzed for evidence of compounds able to produce enhancement or suppression of CTL activity, compared with control cultures receiving DMSO only. As show below, several compounds with reproducible enhancement/suppressive activity were documented.

Keywords: Sulfonamides; Pyrazolo[4,3-*e*][1,2,4]triazine; Biological activity; Cytotoxic T Lymphocytes (CTL)

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Introduction

The pyrazolo[4,3-*e*][1,2,4]triazines constitute a less known class of condensed pyrazolo-1,2,4-triazines described in the literature. Naturally occurring derivatives of this heterocyclic ring system have been found as extracellular metabolites of cyanobacterium of the class *Pseudomonas fluorescens* var. *pseudoiodinine* and *Nostoc spongiaeforme* [1,2] The most important members in this family of naturally purine analogs are pseudoiodinine, nostocine A and fluviols A-E [3]. These compounds inhibit the growth of Gram-positive and Gram-negative bacteria and exhibit antitumor activity.

The combination of the natural pyrazolo[4,3-*e*][1,2,4]triazine system with pharmacophore groups has enabled the design of novel derivatives with potential biological activity. An important pharmacophore group is a sulfonamide moiety characteristic for many chemical compounds used in medicine [4,5]. Its importance stems from the fact of diverse biological activity of the substituted compounds, which includes antibacterial, antimalarial, hypotensive, diuretic, hypoglycemic, antithyroid, antiparasitic, and anti-inflammatory and antiglaucomatous properties [6]. Literature reports show that sulfonamides can act as inhibitors of enzymes such as Phosphodiesterase type 5 (PDE5) [7], carbonic anhydrase [8,9], tyrosinase [10,11] or Cyclin-Dependent Kinases (CDKs) [12,13]. Furthermore, studies have shown that sulfonamides may exhibit an antitumor effect by inhibiting the activity of Carbonic Anhydrase (CA; EC 4.2.1.1) [14-16]. It has been shown that two isoenzymes of carbonic anhydrase such as CA IX and CA XII are clearly associated with cancer and have over expression in many tumors [17,18] and they are involved in key processes associated with

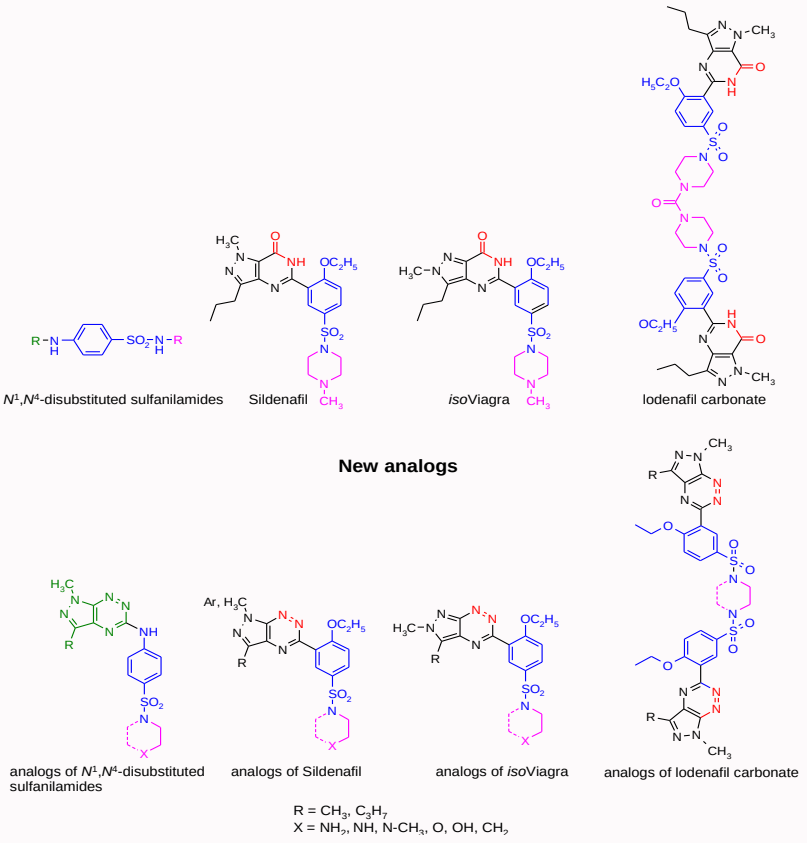

Figure 1: Biologically active sulfonamides and their analogs with pyrazolo[4,3-*e*][1,2,4]triazine core.

the tumor progression and response to treatment [19]. Therefore, the main subject of the research is related to biological evaluation of pyrazolo[4,3-*e*][1,2,4]triazine derivatives as new analogues of sulfonamides used in medicine i.e. sulfanilic acid amides, sildenafil, lodenafil carbonate and iso Viagra with documented in the literature biological activity (Figure 1).

In a search for the development of new anticancer agents, in this work we report analysis of biological activity in pyrazolo[4,3-*e*][1,2,4] triazine sulfonamides, based on their ability to cause suppression or enhancement of the development of CTL *in vitro* in mouse MLC cultures.

Materials and Methods

Mice

C57BL/6 and BALB/c mice (females: 8 weeks of age) were purchased from Jackson labs, Bar Harbour, Maine, USA. All mice were maintained in standard conditions in a CCAC approved animal facility with food and water ad libitum. All studies were performed under a protocol approved by a UHN animal care committee.

Cell culture and CTL assays

Splenocytes were harvested from a minimum of 3 BL/6 or BALB/c mice and single cell suspensions prepared in alpha medium with 10^{-6} μM 2-mercaptoethanol and 10% fetal calf serum (αF10). MLC cultures contained 4×10^6 BL/6 responder cells and 2×10^6 irradiated (2000 Rads) BALB/c stimulator cells in 2 ml αF10. Sulfonamides were added to a final concentration of 75 μM, with control cultures containing a similar concentration of DMSO-all groups were set up in triplicate. Cells were pooled and harvested from replicate cultures at 6 days, washed, and incubated in 250 μl cultures in triplicate at different effector: target ratios with 5×10^3 ^{51}Cr labeled P815 tumor target cells (maintained in αF10 throughout). Supernatants were harvest at 5hr and assayed for ^{51}Cr release. % specific killing in each group was assessed in standard fashion.

Results and Discussion

Chemistry

The synthesis of target sulfonamides was achieved by a convenient multiple procedure starting from 3-methylsulfanyl-1,2,4-triazine **1** (Figure 2). The key starting derivative **2** was obtained using our previously discovered procedure [20]. In the beginning pyrazolotriazine **2** was smoothly converted into the corresponding isomeric structures **3** and **4** upon the reaction with methyl iodide in the presence of potassium carbonate in an EtOH/H_2O mixture (1:1, v/v) at r.t. Derivative **2** can be prepared also by cyclization of the methylhydrazone of 5-acyl-1,2,4-triazine under acidic conditions, according to our previously published procedure [20]. In the next step, 5-methylsulfanyl-pyrazolo[4,3-*e*][1,2,4]triazines **3** and **4** were reacted with 2-ethoxyphenylboronic acid in the presence of copper (I) 3-methylsalicylate to obtain appropriate derivatives **5** and **6** [21]. Compound **5** was collected in excellent yield (90%), but derivative **6** was produced in 30% yield only. Chlorosulfonylation of compounds **5** and **6** in neat chlorosulfonic acid at 0°C proceeded smoothly and selectively at 5'-position of the phenyl ring to give the desired products **7** and **8** in excellent yield. However, it should be noted that chlorosulfonyl derivative **7** is not stable and after isolation was immediately reacted with amine. The final sulfonamides **9a-u** and *aza-iso* Viagra **10** were prepared by stirring chlorosulfonyl derivatives

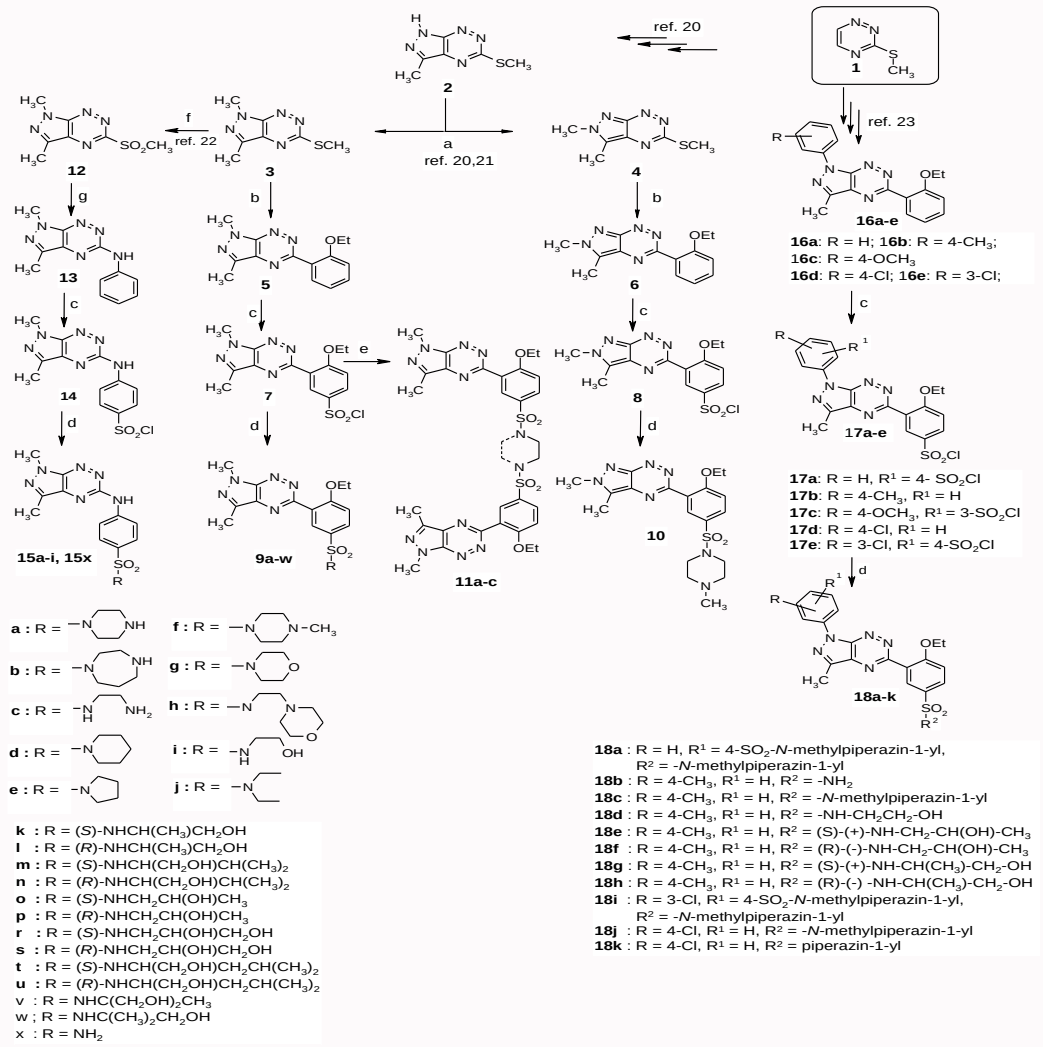

Figure 2: Synthetic pathway to the pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides. Reagents and conditions: (a) CH_3I, EtOH/H_2O/K_2CO_3; (b) ethoxyphenylboronic acid, $Pd(PPh_3)_4$, CuMeSal, THF, under argon atmosphere, reflux, overnight; (c) $ClSO_3H$, 0°C to rt, 2h; (d) ammonium water solution or appropriate amine, anhydrous MeCN, rt, overnight; (e) appropriate diamine [ratio of the diamine and 7 (2:1)], anhydrous MeCN, rt, overnight; (f) $KMnO_4$, Bu_4NBr, CH_3COOH, benzene-H_2O, rt, 2 h; (g) aniline, seal tube, 9 days, 150°C.

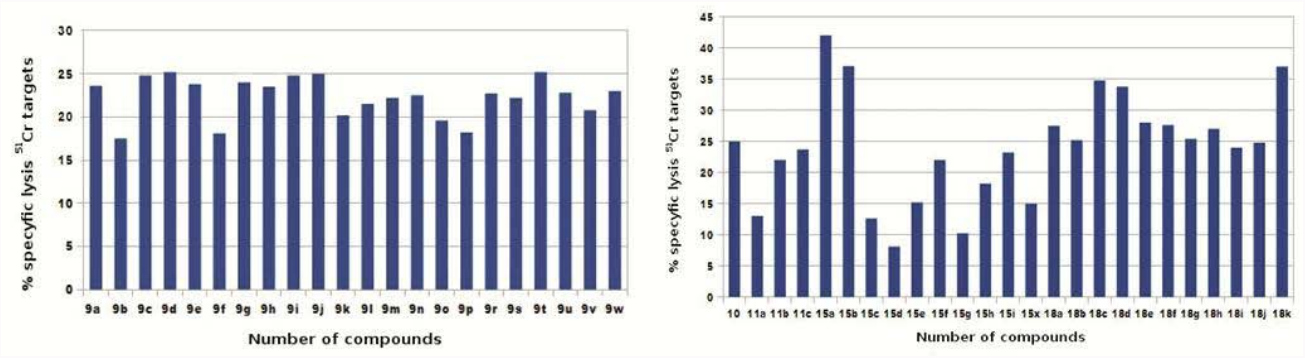

Figure 3: Data show mean lysis (5 hr ^{51}Cr) of 5 × 10^3 P815 tumor targets by effector cells (20:1 effector: target ratio) from 6d MLCs of BL/6 anti-BALB/c splenocytes. All cultures included different sulfonamide compounds (see abscissa), used at 75 mM final concentration. The average value of lysis in the control cultures with DMSO (vehicle) is only about 25%.

7 and **8** with three equivalents of appropriate amines in acetonitrile at room temperature overnight [21]. To obtain dimeric sulfonamides **11a-c** two equivalents of chlorosulfonyl derivative **7** were reacted with one equivalent of bifunctional amine (piperazine, homopiperazine and etylenediamine) in anhydrous acetonitrile at room temperature [21].

To prepare derivatives **15a-i**, pyrazolo triazine **3** was treated with potassium manganate (VII) under phase transfer catalytic conditions at room temperature for 1 hr to give sulfone **12** in nearly quantitative

yield [22]. Next, compound **12** was reacted with anhydrous aniline in a sealed tube at 150°C for 9 days. Obtained aniline substituted pyrazolo triazine **13** was treated with chlorosulfonic acid at 0°C to give the desired compound **14** in excellent yield. The chlorosulfonyl derivative **14** was readily coupled with amines to produce the target sulfonamides **15a-i** as shown in Figure 2.

The general method which was employed to prepare the compounds **18a-k** was presented previously [23]. Obtained under intramolecular nucleophilic substitution reaction of hydrogen [20]. 1H-pyrazolo[4,3-*e*][1,2,4]triazine derivatives **16a-e** were converted to compounds **17a-e** in neat chlorosulfonic acid at 0°C. It should be noted that ^{1}H-NMR spectra for the obtained derivatives **17b** and **17d** fully confirmed the presence of a chlorosulfonyl moiety at the C-5' position on ethoxyphenyl group. However, ^{1}H-NMR spectra for the other chlorosulfonyl derivatives **17a**, **17c** and **17e** show the presence of two chlorosulfonyl groups, one at expected position C-5' on ethoxyphenyl ring and the other on the aryl substituent linked to the pyrazole ring. The place of attachment of the chlorosulfonyl group to the aromatic ring linked to the pyrazole depends on the kind and position of the substituent present in the phenyl ring. In the case of derivative **16a** attachment of chlorosulfonyl group was observed in the *para* position relative to the pyrazole ring, while the presence of the methoxy group in derivative **16c** resulted chlorosulfonylation in the *ortho* position relative to the electro-donating group - OCH_3. It should be noted that in this case we have observed significant influence of the methoxy group on the chlorosulfonylation process. The product of this reaction was unstable and has been isolated in low yield (19%). The chlorosulfonation of **16e** given also compound with two chlorosulfonyl groups, one at C-5' position on the ethoxyphenyl ring and the other one in the *para* position relative to the N-1 nitrogen atom of the pyrazole ring. Chlorosulfonyl derivatives (**17a-b** and **17d-e**) were used as the precursors for the synthesis of the final sulfonamides **18a-k** and were readily coupled with ammonium or appropriate amine in anhydrous acetonitrile at room temperature.

The pyrazolo[4,3-*e*][1,2,4]triazine sulfonamides were obtained in satisfactory yields. Structures and purity of the all obtained derivatives were characterized using the ^{1}H and ^{13}C NMR, and MS methods together with elemental analysis. Analytical and spectral data were in good agreement with the composition of the compounds and were already published [21-25].

Biological activity

Subsequently all compounds were dissolved in DMSO and used in MLC cultures (75 μM and 150 μM concentrations) to suppress development of CTL in BL/6 cultures stimulated for 6 days in *vitro* with irradiated BALB/c stimulator cells. Cells from all cultures were assayed after this time by analysis of ^{51}Cr release from 5×10^3 P815 tumor target cells over 5 hrs. Typical data for one such assay (75 μM for all compounds) are shown in Figure 3.

In repeat studies similar data was seen, though there was marked variability in the functional activity of the various compounds under investigation. Data in Table 1 show a mean suppression/enhancement for selected compounds with reproducible activity in 3 independent assays. It is apparent from these data (Table 1) that different compounds can indeed be selected using this MLC screening assay which have the ability to either attenuate (**11a**, **13**, **15d**, **15g** and **15c**) or enhance (**15b**, **18c**, **18d** and **18k**) the biological responses studied.

Table 1: Mean suppression/enhancement of CTL induction by sulfonamide compounds.

Compounds under test[a]	% mean CTL response relative to control[b]
11a	15 ± 8.5
13	63 ± 16
15b	145 ± 23
15c	24 ± 8.8
15d	62 ± 17
15g	29 ± 9.2
18c	168 ± 38
18d	158 ± 15
18k	191 ± 43

[a] Sulfonamide compound tested in MLCs (75 μM final concentration-see e.g. Figure 3); [b] % of control response (mean ± SD) from cultures containing DMSO measured in 3 independent investigations-Figure 3 represents one such study for the different sulfonamides shown

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