Cytotoxic Activity of Some Pyrazolo[4,3-*e*][1,2,4]Triazines Against Human Cancer Cell Lines[1]

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Summary: The aim of this study is to investigate the potential cytotoxic effect of some synthesized pyrazolo[4,3-*e*][1,2,4]triazine derivatives against four different tumor cell lines: PC-3 (prostate cancer), MCF-7 (breast cancer), H460 (non-small cell lung cancer cells), and Colo205 (colorectal adenocarcinoma). Cytotoxic effect was measured using MTT reduction assay. Several compounds demonstrated significant broad cytotoxic activity in low micromolar range. The screened pyrazolo[4,3-*e*][1,2,4]triazines were obtained in direct condensation of 5-acyl-1,2,4-triazines with hydrazine or its derivatives under acidic conditions in good yield. The mode of cyclocondensation is considerably dependent on the electronic nature of a phenyl ring substituent of the aromatic hydrazones: electron-donating substituents favor the cyclization in shorter time contrary to electron-withdrowing substituents which work favorable for the formation of pyrazolo[4,3-*e*][1,2,4]triazines in longer time. Nucleophilic substitution of methylsulfonyl group in the position 5 takes place with *O*-, *N*- and *C*-nucleophiles to yield related substitution products in high yields. 5-Hydrazino derivative **6** (Nu = NH-NH_2, R^2 = CH_3, R^3 = Ph) is useful for the preparation of **7**, **8** and **9**. Alkylation of the *N*-1 unsubstituted **4** (R^1 = SCH_3, R^2 = CH_3, R^3 = H) provided mixture of **10** and **11**.

Introduction

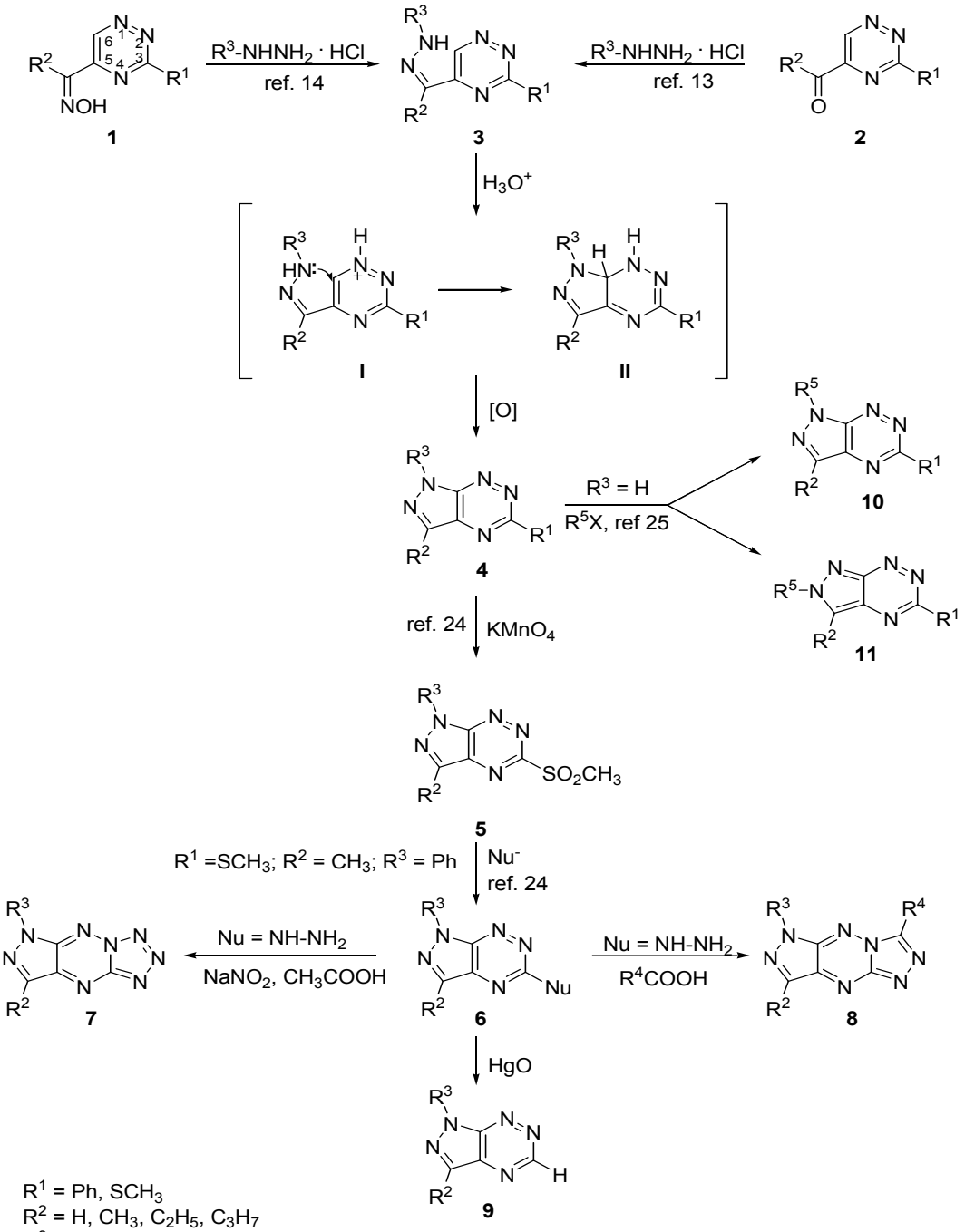
Fused 1,2,4-triazines are important as the basic framework for a variety of pharmaceuticals and agrochemicals [2]. For example, the pyrazolo-1,2,4-triazines have received considerable attention due to their pharmacological applications as antiviral [3], antitumour [4], antifungal [5], analgesic [6], anti-inflammatory [7], and antipyretic agents [6]. 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 pyrazolo-triazines. In the past few decades, scientists from Germany, Japan and Russia have reported the isolation and structural characterization of seven naturally occurring pyrazolo[4,3-*e*][1,2,4]triazines: pseudoiodinine [8], nostocine A [9], and fluviols A-E [10]. These natural compounds with wide antibiotic and antitumour activities were found as extracellular metabolites of some microorganism of the class *Pseudomonas fluorescens* var. *pseudoiodinine* and *Nostoc spongiaeforme*. Few different methods are available in the literature for the construction of 1,3,5-trisubstituted pyrazolo[4,3-*e*][1,2,4]triazines [11,12], most of them engage a multi-step procedure where the key step involve intramolecular cyclization of the respective hydrazones using phosphorous oxychloride [13] or acid-promoted ring closure [14,15]. In literature, the reported procedures illustrate that the pyrazole ring was constructed onto the 1,2,4-triazine core. Although, there are two reports concerned with a new approach for the construction of the 1,2,4-triazine nucleus on a pyrazolo derivatives [16,17]. Moreover, it should be noted that Kelly *et al.* published the synthesis of some naturally occurring derivatives of pyrazolo[4,3-*e*][1,2,4]triazine *e.g.* nostocine A, fluviol A, and pseudoiodinine [18].

Recently, Gucky *et al.* reported that 1,3,5-trisubstituted pyrazolo[4,3-*e*][1,2,4]triazines with 3,4,5-trimethoxyphenyl substituent in position 3 of the pyrazole ring also have cytotoxic activity on human cancer cell [19]. Some 1,5-diaryl-3-(3,4,5-trimethoxyphenyl)pyrazolo[4,3-*e*][1,2,4]triazines have exhibited significant and relative selective activity against A549 (lung adenocarcinoma) cell line, while they were generally less active against leukemia cell line, including otherwise highly chemosensitive CEM lymphoblasts [19]. The importance of the tested pyrazolo[4,3-*e*][1,2,4]triazine group for biological activity has been explored as has the positioning of the aryl groups on the system which could elevate the cytotoxicity activity since a large number of molecules bearing the 3,4,5-trimethoxyphenyl group shown various biological activity.

In our previous work we have described the synthesis and structural investigations of the series of 3,5,7-trisubstituted pyrazolo[4,3-*e*][1,2,4]triazines [14, 15] and some annulated derivatives [20, 21] without their cytotoxic activity. With the respect to this fact, we now report cytotoxic activity for some selected derivatives of pyrazolo[4,3-*e*][1,2,4]triazine ring system synthesized in our laboratory.

Chemistry

The most novel derivatives of the pyrazolo[4,3-*e*][1,2,4]triazine were prepared by one-pot reaction of hydrazine or its alkyl- and aryl-derivatives with 5-acyl-1,2,4-triazines (**2**) or suitable oximes of 5-formyl- and 5-acyl-1,2,4-triazines (**1**) under acidic conditions as depicted in Scheme-1.



R^1 = Ph, SCH_3
R^2 = H, CH_3, C_2H_5, C_3H_7
R^3 = H, CH_3, Ph, substituted phenyl
Nu = OH, OCH_3, OC_2H_5, $OCH_2CH{=}CH_2$, $OCH_2CH_2CH{=}CH_2$, *o*-cyano-phenol, NH_2, NH-C_4H_9, NH-CH_2-Ph, NH-Ph, S-Ph, CH(COOEt), NH-NH_2
R^4 = H, CH_3
R^5 = CH_3, C_2H_5, C_3H_7, C_4H_9, CH_2Ph

Scheme 1: Synthetic route of the screened compounds.

Briefly, all used oximes **1** and 5-acyl-1,2,4-triazines **2** were prepared by the literature procedure [22, 23]. Both oximes **1** and ketones **2** upon treatment with ethanolic solution of hydrazine or its alkyl- or arylderivatives and catalytic amount of acid provided in the first step suitable intermediates-hydrazones **3** which in the next step undergo ring closure involving the bicyclic structure **II**, which undergoes an air oxidations to give target **4** [14]. This new approach to the synthesis of the functionalized derivatives **4,** seems to be general and very efficient methods for the construction of variously substituted pyrazolo[4,3-*e*][1,2,4]triazine **4**. It should be noted, that we investigated also both thermally-induced transformation of the hydrazones **3** [15] and under microwave irradiations [24]. Moreover, the presence of the methylsulfanyl substituent in 1,2,4-triazine core offers the possibility for the functionalization of the bicycle system in the position 5 by nucleophilic *ipso*-substitution [25]. However, methylsulfanyl group appeared to be not reactive towards nucleophilic reagents. Thus, sulfone **5** was obtained upon treatment of sulfide **4** (R^1 = SCH_3, R^2 = CH_3, R^3 = Ph) with potassium manganate (VII) and acetic acid under phase transfer catalytic conditions. The dealing of the sulfone **5** with several *O*-, *N*-, *S*-, and *C*-nucleophiles yielded some new 5-substituted 3-methyl-1-phenyl-1*H*-pyrazolo[4,3-*e*][1,2,4]-triazines [25]. The hydrazine substituent in the position 5 of the pyrazolo-triazine system shown to be useful for the preparation of **9** and new annulated derivatives **7** and **8** [20,21,25]. All novel derivatives **10** and **11** were prepared in the reaction of 1-unsubstituted-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine **4** (R^1=SCH_3, R^2=CH_3, R^3=H) with several alkyl reagents [26]. The yield and ratio of isomeric structure **10** and **11** depend on the reaction conditions. The derivatives of the pyrazolo[4,3-*e*][1,2,4]triazine were obtained in satisfactory yields. The structures of the synthesized and screened compounds were confirmed by MS, ^{1}H-NMR, IR spectra, and elemental analysis. Analytical and spectral data were in good agreement with the composition of the compounds and were already published [14, 15, 25-27].

Pharmacology

All compounds were examined under *in vitro* conditions using MTT assays towards four tumor cell lines: PC-3 (prostate cancer), MCF-7 (breast cancer), H460 (non-small cell lung cancer cells), and Colo205 (colorectal adenocarcinoma). The present data represent mean values from three independent experiments; the standards deviation did not usually exceed 15% on the mean.

Cells and Cell Culture

All cell lines used in our study derived from human cancers and were purchased from ATCC (Manassa, VA, USA). Chosen for this study cancer cell lines: H460 (non-small cell lung cancer cells), MCF-7 (breast cancer), PC-3 (prostate cancer), and Colo-205 (colorectal adenocarcinoma) grew as monolayer cultures.

Culture Conditions

Cell lines were maintained in the following media Colo-205: RPMI-1640, PC-3: Ham's F12K, MCF-7: MEM, H460: RPMI-1460. Cell media were also supplemented with 10% fetal bovine serum (for H460) or 10% fetal calf serum (for Colo-205, PC-3, MCF-7), sodium puruvate (for Colo-205 and MCF-7), insulin (for MCF-7), glutamine (2 mM Glutamax-I), non-essential amino acids, glucose, and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin for H460), all obtained from GibcoBRL, Grand Island, NY. All cell lines were cultured at 37 $^{\circ}$C in a humidified atmosphere of 5% CO_2 in air. Cells growing as monolayer culture were harvested by trypsinisation (0.25% trypsin + 0.02% EDTA). Cells were counted using an electronic particle analyzer (Coulter® Particle Analyzer Z™ Series) and suspended in fresh medium to obtain the concentration of H460: 2.5 x 10^5 cell/mL, Colo-205: 10^5 cell/mL, PC-3 and MCF-7: 8 x 10^4 cell/mL. Then the cell suspensions were plated out into 12-well culture plates. The cell lines were preincubated for 24 h to allow the cells to attach to the plates. Then the drug solution was added and cells were incubated for additional 48 h.

Results and Discussion

All compounds were investigated for cytotoxic activity in 4 human cancer lines and exhibited differential biological activity. Table-1 reports the IC_{50} values of the evaluated compounds. Evaluated pyrazolo[4,3-*e*][1,2,4]triazine derivatives can be divided into three groups *e.g.* very active, active and non active compounds at the concentration below 100 µM. The values of IC_{50} for compounds of the first and the second group are presented in Fig. 1 and Fig. 2. The compounds of the first group were found to be much more active than the rest ones. The annulated pyrazolo[4,3-*e*][1,2,4]triazine **7** was established to be one of the most active antiproliferative pyrazolotriazines (IC_{50} of **7** values range between 0.4 and 0.5 µM, Table-1). Therefore, we attempted the synthesis of analogues with triazole ring system. Replacing the tetrazole ring with an unsubstituted triazole decreases cytotoxic potency while

replacement with a methyltriazole ring dramatically reduced anticancer potency (compare **7** with **8a** and **8b,** Table-1). Biological activity of the sulfones (**5a-5c**, **11a**) depends on the kind and position of the substituents at the pyrazole ring. Thus, placement of alkyl group instead of phenyl ring at the position *N*-1 of the pyrazolotriazine system reduces cytotoxic potency while introduce of the methyl group at the position *N*-2 brought decrease biological activity. Low biological effect was also observed while methylsulfonyl group was substituted with the amine moiety (**6a-6d**).

Table-1: Results of MTT cytotoxic activity tests (IC_{50} in μM/L).

Compound	R^1	R^2	R^3	R^4	R^5	Nu	PC3	MCF-7	H460	Colo205
4a[ref.28]	SCH_3	C_2H_5	4-NO_2- Ph				98	78	36	75
4b[ref.29]	Ph	CH_3	Ph				98	NA	NA	NA
4c[ref.15]	SCH_3	CH_3	Ph				NA	NA	NA	NA
4d[ref.14]	SCH_3	C_2H_5	4-CH_3-Ph				NA	NA	NA	NA
4e[ref.14]	Ph	C_2H_5	3-Cl-Ph				NA	NA	NA	NA
4f[ref.14]	Ph	CH_3	2,4-diCl-Ph				NA	NA	NA	NA
4g[ref.26]	SCH_3	CH_3	H				NA	NA	NA	NA
4h[ref.26]	SCH_3	C_3H_7	H				NA	NA	NA	NA
5a[ref.25]		CH_3	Ph				25	50	25	25
5b[ref.27]		CH_3	CH_3				NA	NA	NA	NA
5c[ref.27]		CH_3	*n*-C_4H_9				NA	NA	NA	NA
6a[ref.25]		CH_3	Ph			NH-Ph	81	90	86	4
6b[ref.25]		CH_3	Ph			NH-Bu	66	77	NA	25
6c[ref.25]		CH_3	Ph			NH-CH_2-Ph	NA	NA	NA	50
6d[ref.25]		CH_3	Ph			NH_2	NA	NA	NA	72
6e[ref.25]		CH_3	Ph			OCH_3	NA	NA	NA	91
6f[ref.25]		CH_3	Ph			OC_2H_5	NA	NA	NA	NA
6g[ref.25]		CH_3	Ph			$CH(COOEt)_2$	NA	NA	NA	NA
7[ref.25]		CH_3	CH_3				0.4	0.5	0.4	0.4
8a[ref.25]		CH_3	Ph	H			3	4	3	2
8b[ref.25]		CH_3	Ph	CH_3			NA	NA	NA	NA
9[ref.25]		CH_3	CH_3				NA	NA	NA	NA
10a[ref.26]	SCH_3	CH_3			CH_3		NA	NA	NA	NA
10b[ref.26]	SCH_3	CH_3			C_2H_5		NA	NA	NA	NA
10c[ref.26]	SCH_3	CH_3			*n*-C_3H_7		NA	NA	NA	NA
10d[ref.26]	SCH_3	CH_3			CH_2Ph		NA	NA	NA	NA
10e[ref.26]	SCH_3	CH_3			CH_2COOMe		NA	NA	NA	NA
11a[ref.30]	SO_2CH_3	CH_3			CH_3		60	74	74	NA
11b[ref.26]	SCH_3	CH_3			CH_3		NA	NA	NA	NA
11c[ref.26]	SCH_3	CH_3			CH_2Ph		NA	NA	NA	68

NA - not active at tested concentration (<100 μM/L).

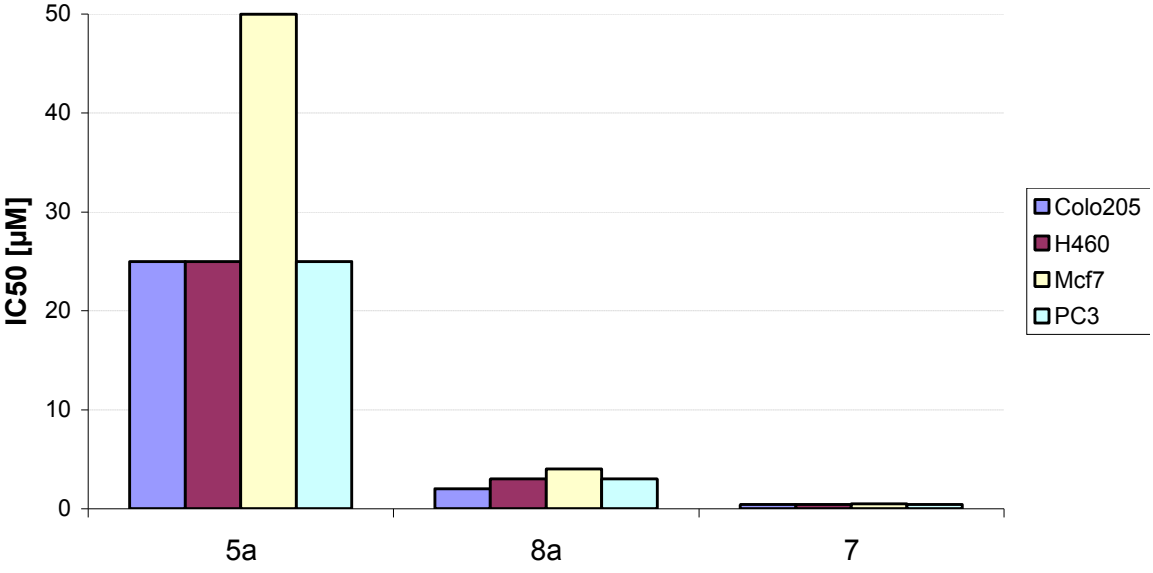


Fig. 1: *In vitro* cytotoxic effects of the most active pyrazolo[4,3-*e*][1,2,4]triazines against four human cancer cell lines.

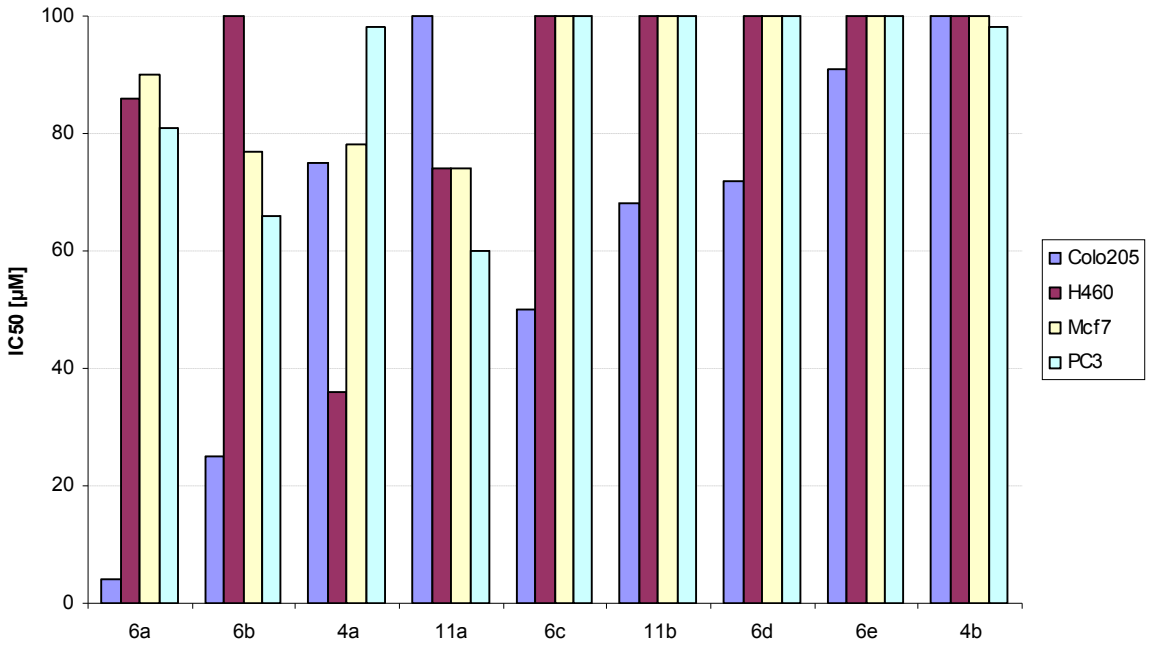


Fig. 2: *In vitro* cytotoxic effects of pyrazolo[4,3-*e*][1,2,4]triazines against four human cancer cell lines.

Cytotoxic MTT Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed on cells cultured in 96-well plates [21]. The cells were seeded at density $5x10^3$ cells/well. After 24 h preincubation period needed for cells to attach to the bottom and achieve exponential growth, the cells were treated with different concentrations of the freshly prepared test compounds in complete medium (three wells per concentration) for 48 h. Control wells contained 0.2% of DMSO as vehicle control which had no influence on the growth of the cells comparing with cells cultured in normal complete medium. After 48 h, the solutions were removed from all plates and the cells were assayed using MTT solution (0.5 mg/mL MTT in RPMI-1640 medium without serum and phenol red). Aliquots (50 µL) of the MTT stock solution were pipetted into each well. Plates were incubated for 4 h following which the formed purple formazan crystals were dissolved in 10% SDS (sodium dodecyl sulfate). The complete dissolving required overnight incubation at room temperature. The absorbance was measured with a spectrophotometric microplate reader PowerWave XS (Bio-Tek, Winooski, VT, USA) at a test wavelength of 570 nm and a reference wavelength of 690 nm. The optical density (OD), proportional to the number of viable cells inside the well, was calculated as the difference between the absorbance at the test wavelength and that at the reference wavelength. The percentage of viable cells within the well as compared with the control wells was calculated as (OD of drug-treated sample – OD of blank/OD of control – OD of blank) x 100. The dose response curves were constructed and fitted in Sigma-Plot software using non-linear regression three parametric Hill function (R^2 >0.9). IC_{50} values (concentrations required to reduce the viability of cells by 50% as compared with the control cells) were computed by using the fitted Hill equation. They are presented as mean ± SD from at least three independent experiments. To compare the means, the data from the MTT assay were analyzed using one-way analysis of variance (ANOVA) followed by posthoc Tukey's test (Statistica, StatSoft, USA). $P < 0.05$ was considered significant.

Conclusion

This work was undertaken as the initial stage of biological evaluation of newly synthesized derivatives of pyrazolo[4,3-*e*][1,2,4]triazine ring system. These compounds present a new class of condensed pyrazolo-triazines endowed with promising activity against human cancer cell lines. However, it is too early to speculate on the structure-activity relationships of these compounds.

Acknowledge

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28. 3-Ethyl-5-(methylsulfanyl)-1-(4-nitrophenyl)-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**4a**): mp. 138 ^{o}C; ^{1}H-NMR ($CDCl_3$) δ: 1.53 (t, 3H, J=7.6 Hz), 2.78 (s, 3H), 3.17 (q, 2H, J=7.6 Hz), 8.40-8.45 (m, 2H), 8.66-8.71 (m, 2H); IR (KBr) cm^{-1}: 2930, 1600, 1520, 1340, 860; MS [$C_{13}H_{12}N_6O_2S$] (*m/z*, *%*): 316 (9) [M^+], 286 (11), 255 (9), 166 (36), 151 (16), 70 (100); Anal. Calcd. for $C_{13}H_{12}N_6O_2S$: C, 49.36; H, 3.82; N, 26.57. Found: C, 49.43; H, 3.93; N, 26.54.
29. 1,5-Diphenyl-3-methyl-1*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**4b**): mp.186 ^{o}C; ^{1}H-NMR ($CDCl_3$) *δ*: 2.83 (s, 3H), 7.32-7.41 (m, 1H), 7.54-7.63 (m, 5H), 8.40-8.47 (m, 2H), 8.64-8.69 (m, 2H); IR (KBr) cm^{-1}: 2960, 1530, 1460, 760, 700; MS (EI 70eV, *m/z*, *%*): 287 (13) [M^+], 259 (100), 218(76), 115 (60), 77 (49); *Anal*.Calcd. for $C_{17}H_{13}N_5$: C, 71.07; H, 4.56; N, 24.37. Found: C, 71.17; H, 4.43; N, 24.25.
30. 2,3-Dimethyl-5-methylsulfonyl-2*H*-pyrazolo[4,3-*e*][1,2,4]triazine (**11a**): mp. 164 ^{o}C; ^{1}H-NMR ($CDCl_3$) *δ*: 2.87 (, 3H), 3.58 (s, 3H), 4.42 (s, 3H); IR (KBr) cm^{-1}: 1320, 1130; MS (EI 70eV, *m/z*, *%*): 227 (13) [M^+], 199 (8), 136 (7), 79 (15), 56 (100); Anal.Calcd. for $C_7H_9N_5SO_2$: C, 37.00; H, 3.96; N, 30.83. Found: C, 37.11; H, 3.82; N, 31.05. For the synthesis procedure see ref. 27.