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Design and synthesis of mono-and di-pyrazolyl-*s*-triazine derivatives, their anticancer profile in human cancer cell lines, and *in vivo* toxicity in zebrafish embryos

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ABSTRACT

s-Triazine is considered a privileged structure, as it is found in several FDA-approved drugs. In the framework of our ongoing medicinal chemistry project based on the use of s-triazine as a scaffold, we synthesized a series of mono- and di-pyrazolyl-s-triazine derivatives and tested them against four human cancer cell lines, namely Human breast carcinoma (MCF 7 and MDA-MB-231), hepatocellular carcinoma (HepG2), colorectal carcinoma (LoVo), and leukemia (K562). The cell viability assay revealed that most of the *s*-triazine compounds induced cytotoxicity in all four types of human cancer cell lines, however, compounds **4a**, and **6g**, both of them have a piperidine moiety in their structure were most effective. These two compounds affected the cell viability of cancer cells, with IC_{50} values within the range between 5 to 9 μM. The cell cycle analysis showed that **4a** and **6g** induced S and G2/M phase cell cycle arrest in K562 cells. This could be the mechanism by which these molecules induced cytotoxicity in tested cancer cells. The prepared compounds were tested in zebrafish embryos to evaluate *in vivo* and developmental toxicity of the pyrazolyl-*s*-triazine derivatives in animals. None of the derivatives were lethal in the concentration range tested.

1. Introduction

The search for new agents with therapeutic efficiency is one of the major concern in medicinal chemistry. However, the potential development of resistance or tolerance to these compounds over time, particularly in the context of the treatment of recurrent diseases such as cancer, seriously limits their clinical use [1].

1,3,5-Triazine (s-triazine) can be considered a privileged structure since several drugs approved by the corresponding agencies are based on its structure. An example of such a drug is Enasidenib (Idhifa) (Fig. 1), which was approved by the FDA for the treatment of the IDH2-positive acute myeloid leukemia in 2017 [2].

2,4,6-Tris-1-aziridinyl-s-triazine (Tetramine, Fig. 1) was the first *s*-triazine derivative to demonstrate anticancer activity, which can be easily synthesized from 2,4,6-trichlorotriazine (TCT, Fig. 1) [3]. In this regard, the broad availability of TCT, as well as its high reactivity, has led many anticancer medicinal chemistry programs to replace the Cl (aziridinio in tetramine) by (alkyl) amino, alkoxy, aryl or sulfonamide [4].

On the other hand, the attachment of pyrazolyl rings to the *s*-triazine moiety has been extensively studied and reported to exert anticancer activity [5]. For example, Brzozowski and F. Sączewski [6]

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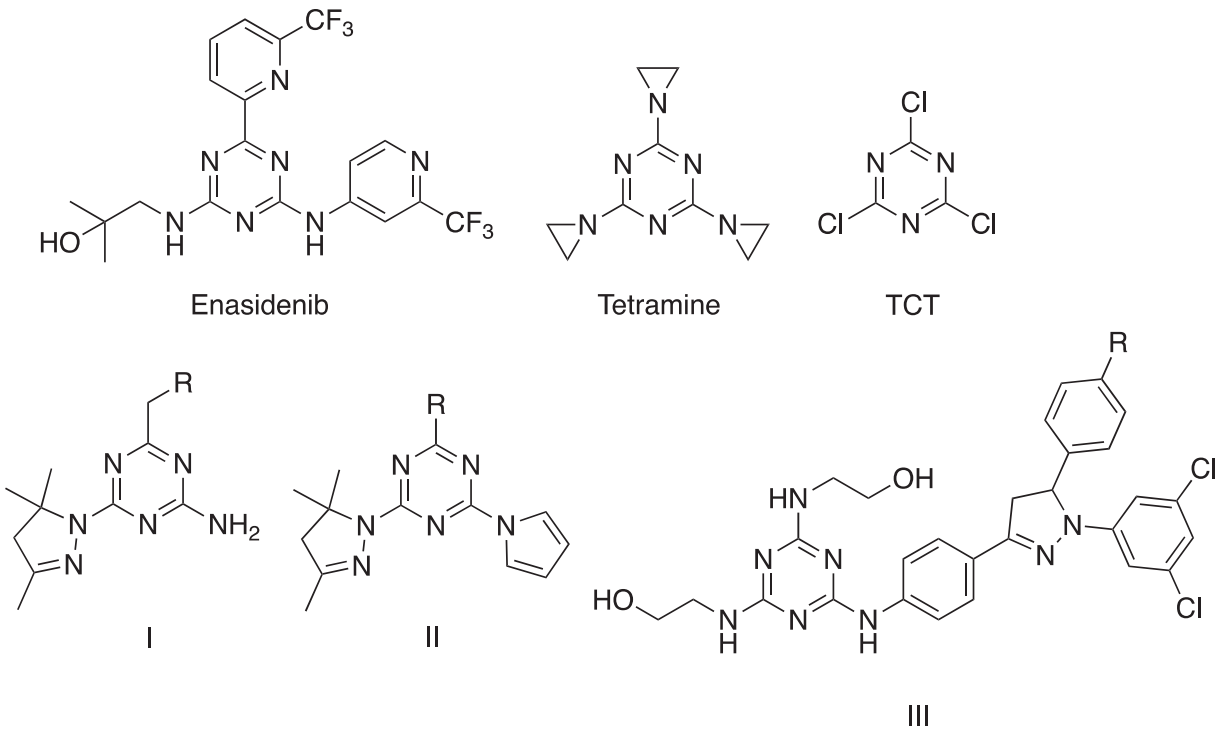


Fig. 1. Selected triazine derivatives.

reported the syntheses and antitumor activities of 4-(3,5,5-trimethyl-2-pyrazolino)-1,3,5-triazine derivatives **I** and **II** against a panel of 60 tumor cell lines at the National Cancer Institute (NCI). More recently, Insuasty and co-workers have reported a family of 4,6-bis(2-hydroxyethyl)amino)-1,3,5-triazin-2-yl derivatives **III** with a good anticancer profile (Fig. 1) [5e].

In the framework of our ongoing medicinal chemistry project based on the use of *s*-triazine as a scaffold [7] for anticancer drug development, series of mono- and di-pyrazolyl-*s*-triazine derivatives were synthesized and tested against four types of common human cancer cells, namely breast carcinoma (MCF-7 and MDA-MB-231 cells), liver carcinoma (HepG2 cells), colorectal carcinoma (LoVo cells) and leukemia (K562 cells). The cell cycle analysis of pyrazolyl-*s*-triazine derivatives treated K562 cells was carried out to determine the potential mechanism of action of these compounds in human cancer cells.

To assess the potential *in vivo* toxicity of synthetic compounds which are intended to be use for the treatment of humans or animals, they must first be tested in a suitable animal model prior to clinical trials. Similarly, the developmental toxicity of the compounds must also be tested to evaluate any harmful effect on fetal growth. In this context, Zebrafish provide an ideal *in vivo* system which not only serve to evaluate the *in vivo* toxicity of the synthesized compounds but also helpful to predict the developmental toxicity [8]. In this regard, pyrazolyl-*s*-triazine derivatives were screened using zebrafish embryos to evaluate their potential *in vivo* and developmental toxicity.

2. Results and discussion

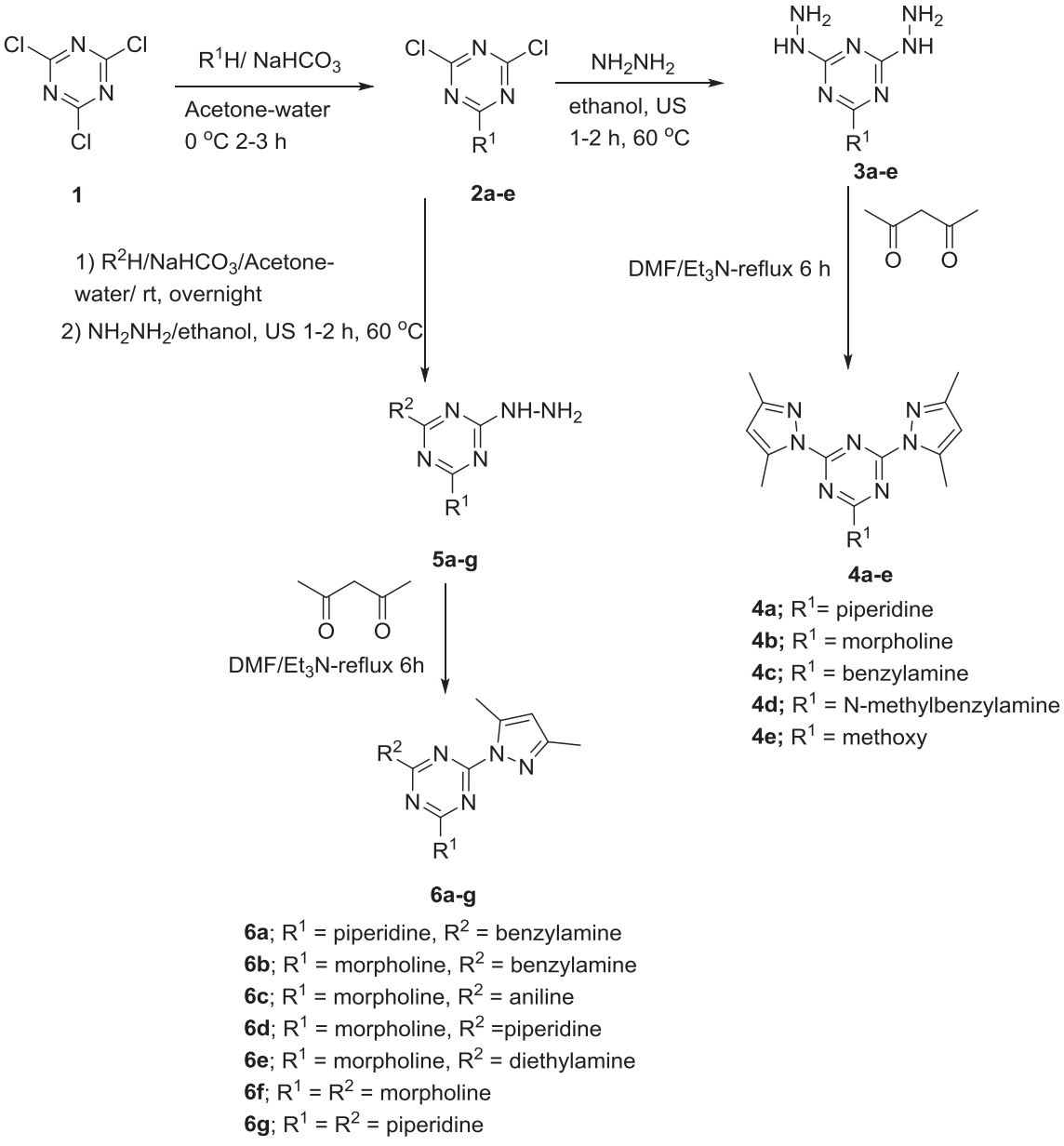
2.1. Chemistry

The mono- and di-pyrazolyl-*s*-triazine derivatives were synthesized following the reported method, where the hydrazinyl-*s*-triazine was reacted with acetylacetone in the presence of triethylamine and DMF as solvent [7d,9]. The initial nucleophilic substitution of the first chlorine atom of cyanuric chloride **1** was achieved with great regioselectivity as the reaction is a temperature-controlled process. Thus, the first chlorine atom was replaced by amine (morpholine, piperidine, benzylamine, *N*-methylbenzylamine or methoxy) at 0–5 °C to afford the products **2a-e** (2,4-dichloro-6-substituted-*s*-triazine derivatives). The second chlorine atom was replaced by a second amine at room temperature for 24 h, yielding 2-chloro-4,6-disubstitued-*s*-triazine derivatives. The di-chloro and mono-chloro derivatives were then treated with hydrazine hydrate (80%) in ethanol for 2 h under ultrasonic irradiation (US) [10] to afford the hydrazine derivatives **3a-e** and **5a-g**, respectively. Compounds **3a-e** and **5a-g** were then reacted with acetylacetone following the reported methods [7d,9] to render the target products **4a-e** and **6a-g**, respectively, as shown in Scheme 1.

2.2. Triazine compounds induced cytotoxicity in human cancer cells

The anticancer activity of the synthesized triazine derivatives (Fig. 2) were studied in human breast cancer (MCF-7 and MDA-MB-231), human liver carcinoma (HepG2), human colorectal carcinoma (LoVo), and human leukemia (K562) cell lines.

Most of the triazine compounds affected the cell viability of the four cancer cell lines, as determined by the MTT cell viability assay (Table 1 and Fig. 3). The most effective molecules were **4a**, and **6g**, which affected the cell viability of tested cancer cell lines with IC_{50}, values within the range between 5 and 9 μM. Of note, both compounds contain piperidinyl moiety in their structure. Interestingly, and although piperidine and morpholine can be considered to be from the same family in some aspects of their synthetic chemistry, the presence of the morpholine moiety was detrimental for anticancer activity. Thus, compounds containing morpholine in the absence of piperidine were inactive (**4b**, **6c**, **6f**). This observation was confirmed by the very low activity of compound **6d** in breast cancer cells (IC_{50} values of 48.7 μM in MDA-MB-231 and 33 μM in MCF7 cells) and the counteraction of the negative effect of morpholine by the presence of piperidine. The presence of benzylamine was not as beneficial to anticancer activity as that of piperidine (**4c** vs **4a**), but its presence helps to keep some activity in the presence of piperidine (**6a**), which is as expected slightly superior to **6b** (benzylamine and morpholine). The methoxy derivative showed no anticancer activity (**4e**).



Scheme 1. Synthetic route for the synthesis of triazine derivatives.

2.3. Cell cycle distribution activity of compounds *4a* and *6g*

To gain insight into the mechanism underlying anti-proliferation capacity, the effect of the most active compounds on cell cycle distribution was investigated by fluorescence-activated cell sorting (FACS) analysis. K562 cells were exposed to 10 and 6 μM of the compounds **4a** and **6g** respectively for 24 h. The analysis showed the accumulation of S phase, from 20% to 27% and 26.98% respectively. This accumulation was also accompanied by a compensatory decrease in G1 to phase cells form from 47.22% to 23% and 33.55%, respectively (Fig. 4). Compounds **4a** and **6g** caused a minor accumulation of cells at G2/M phase arrest, from 24% in untreated cells to 29.98% and 31%, respectively. These results suggested that these two compounds inhibited cell proliferation *via* S and G2/M phase arrest.

2.4. In vivo toxicity testing in zebrafish embryos

To evaluate the safety profile of these newly synthesized triazine compounds in animals, drug toxicity assays in zebrafish (*Danio rerio*) were performed. As shown in Table 2 and Fig. 5, most of them induced developmental arrest in treated zebrafish embryos. The activity of these compounds in zebrafish embryos was analogous to their activity in human cancer cells. Compounds **4a** and **6g** were the most active in zebrafish embryos and they caused a significant arrest in the development of the embryos at a minimum concentration between 0.5 and 1 μM (Table 2), with **6g** being the most effective. Control (DMSO 0.5% V/V) embryos developed normally as observed at 24 hpf; however, the development of embryos treated with compounds **4a** and **6g** was significantly delayed and arrested (Fig. 5; B and C). The treated embryos exhibited severe developmental delay after continuous exposure of **4a** and **6g** for another 24 h when compared to control (Fig. 5 E and F). The compounds were not lethal to the embryos in the concentration range tested, and when the treated embryos were moved to compound-free water, they grew normally without any sign of developmental arrest.

In general, it is believe that the chemically synthesize compounds contain some level of toxicity as compared to natural products, and these kind of compounds, even though produce very good results obtained from in vitro systems (cell lines), fail in clinical trials if they are

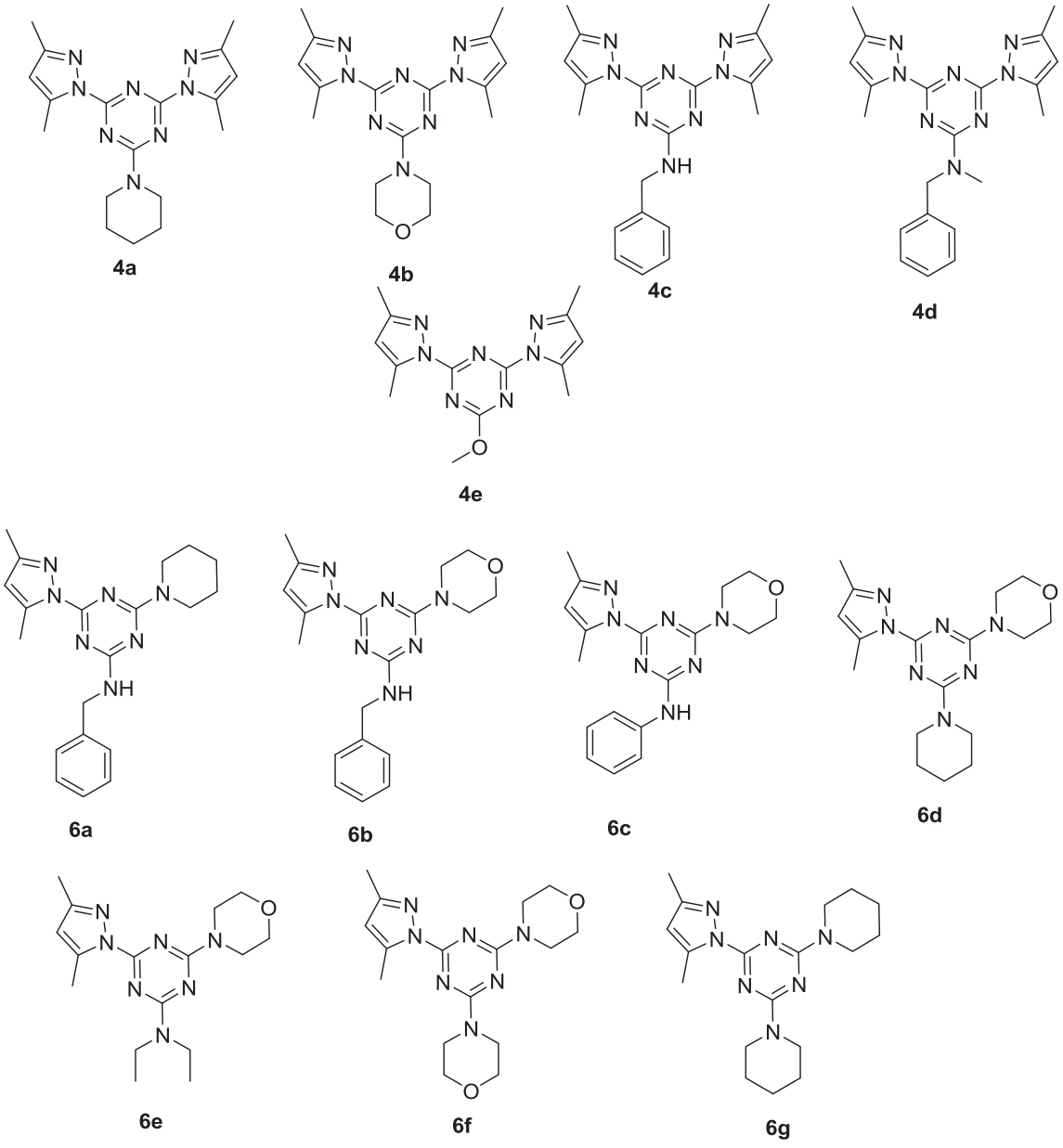


Fig. 2. Structure of the target compounds.

Table 1
The cytotoxicity profile of pyrazole compounds in human cancer cell lines.

Compounds	Anti-cancer activity in human carcinoma cells lines IC_{50} values (μM)				
	MCF7	MDA-MB-231	HepG2	LoVo	K-562
4a	7.5	14	17.5	6.1	9.8
4b	NA*	NA	18.5	41.1	15.2
4c	19	27	13.6	11.8	16.2
4d	12.5	50	8.2	5.6	NA
4e	NA	NA	NA	NA	NA
6a	23.5	30	10.5	14.9	14.5
6b	38	43	8.8	5.9	37.3
6c	54	NA	11.9	40.8	NA
6d	33	48.7	9.4	9.6	31.8
6e	NA	NA	NA	NA	NA
6f	NA	NA	10.8	NA	NA
6g	5	15	21.2	8.4	5.9

* NA = Not Active.

not tested in suitable animal model system and hence discarded or approved as potential drugs. Testing new compounds in suitable animal models is therefore important to save efforts and capital. The zebrafish has emerged as a highly effective tool to test the synthesized compounds for potential toxicity. Moreover, zebrafish assays can also indicate possible developmental toxicity for the foetus.

The developmental arrest observed upon treatment of zebrafish embryos with pyrazole derivatives described herein could be due to cell cycle arrest. Previous studies analysing the cell cycle content during normal embryonic development in zebrafish has revealed a prolonged S phase during the first cleavage (1.5 h) and then G1, G2 and M phase accumulation during the transition from blastula (4–5 h) to shield (6 h) stage [11] Any intervention in embryonic development at this time would mean that a transition from S phase to G2 or M has been retarded. A similar kind of developmental delay has also been reported for other types of triazine derivatives in zebrafish screening assays [12].

The pyrazole derivatives, particularly **4a** and **6g**, caused significant developmental arrest in zebrafish embryos, given this observation, we propose, that the embryonic developmental delay in zebrafish upon

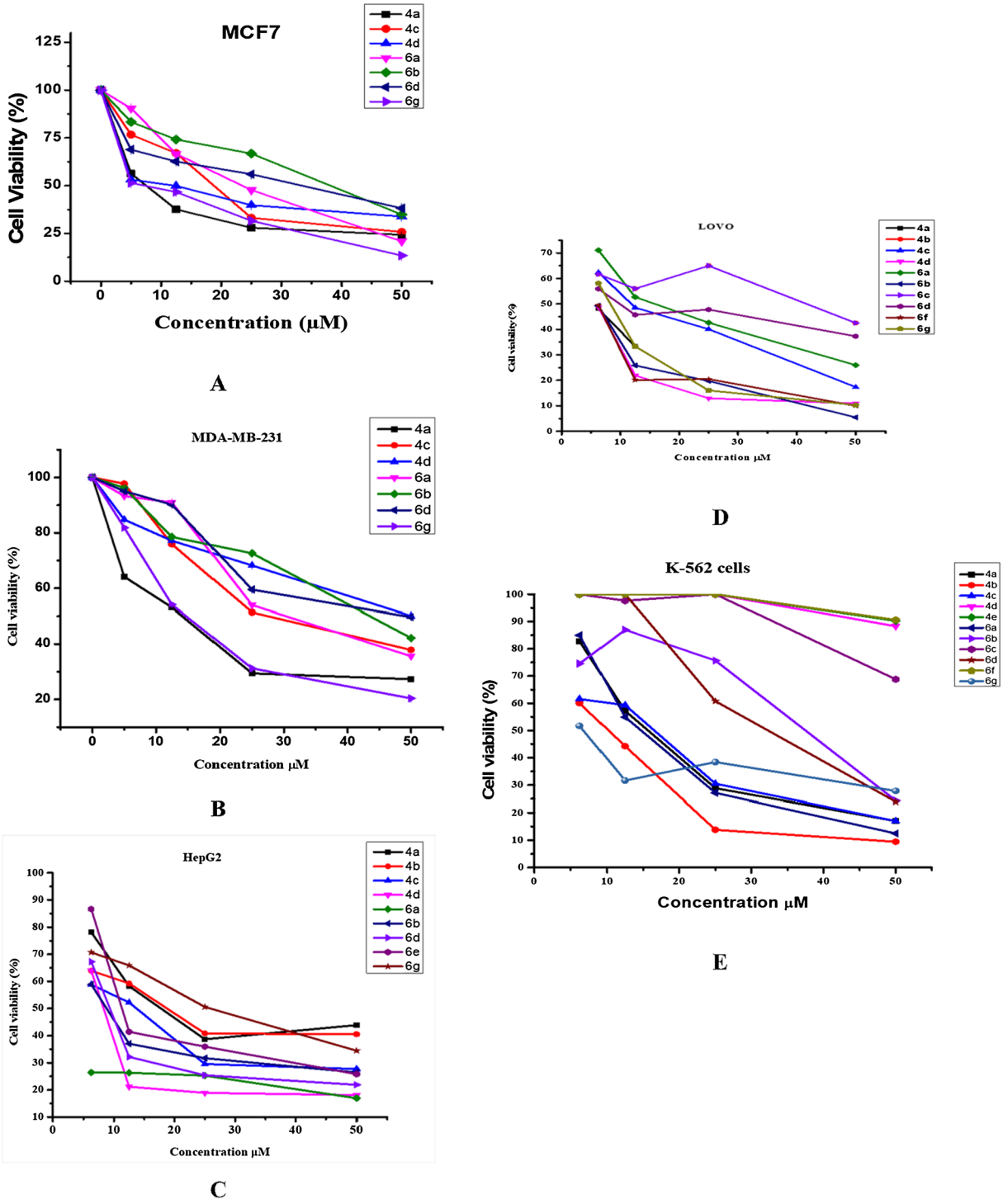


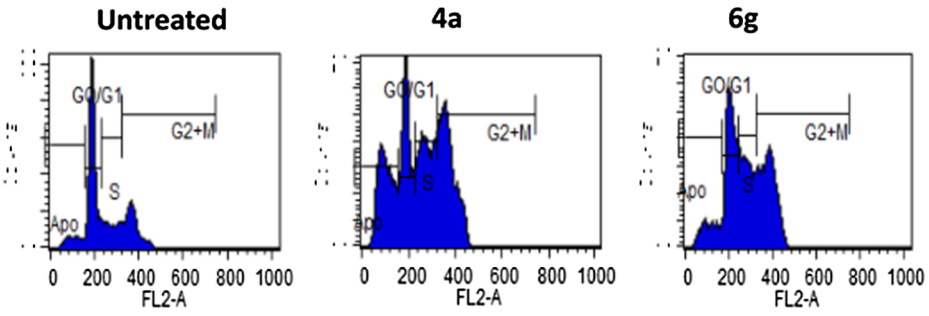
Fig. 3. Line graph showing the anticancer profile of triazine compounds in the following cell lines: (A) MCF-7; (B) MDA-MB-231; (C) HepG2; (D) LoVo; and (E) K562.

treatment with pyrazole derivatives is most likely due to cell cycle arrest and this notion is also supported by the observation that these pyrazole derivatives exerted their effect on cancer cells via S and G2/M phase arrest. Therefore, cell cycle arrest emerges as the possible mode of action of these compounds both in *in vitro* (cancer cells) and *in vivo* (zebrafish embryos).

3. Conclusions

The pyrazole derivatives described herein showed moderate to strong cytotoxicity in four types of human cancer cell lines. Among these compounds, **4a** and **6g** (Fig. 6) were the most effective at inhibiting the cell survival of breast carcinoma, hepatocellular carcinoma, colorectal carcinoma, and leukemia cell lines, with an IC_{50} within the range of 5–9 μM. These two compounds also caused a significant delay in the embryonic development of zebrafish. The cell cycle analysis in K562 cells suggested that these pyrazole derivatives exert their action by causing S and G2/M phase cell cycle arrest. The activity of these compounds could be attributed to the presence of piperidinyl moieties in their structure, since the compounds with piperidinyl moieties (**4a** and **6g**) were the most active (Fig. 6).

Given the demonstrated activity of the pyrazole derivatives against



Marker	% Gated
All	100.00
Apo	8.56
GO/G1	47.22
S	20.69
G2+M	24.08

Marker	% Gated
All	100.00
Apo	20.27
GO/G1	23.05
S	27.04
G2+M	29.98

Marker	% Gated
All	100.00
Apo	8.55
GO/G1	33.55
S	26.98
G2+M	31.00

Fig. 4. K562 cells were treated with compounds **4a** and **6g** and analysed at 0 h, and 24 h by DNA flow cytometry. Histograms show the number of cells per channel (vertical axis) vs. DNA content (horizontal axis). The values indicate the percentage of cells in the relevant phases of the cell cycle.

Table 2
The comparative *in vivo* toxicity profile of triazine compounds in zebrafish embryos.

Compound	Concentration (μM)*	Level of developmental arrest in zebrafish embryos**
4a	1	+++
4b	NA	–
4c	1.5	++
4d	NA	+
4e	NA	–
6a	2.5	+
6b	2.0	+
6c	12	+
6d	NA	–
6e	NA	+
6f	NA	–
6g	0.5	+++

NA = Not Active.
* Concentration at which developmental arrest was induced.
** The observation is based on at least three biological replications and using clutches of embryos from different parents.
\+ Mild developmental delay.
\++ Moderate developmental delay (few hours).
\+++ Severe developmental delay (at least 15–18 h).

four types of human cancer cells and minimal lethality in zebrafish embryos, we can conclude that the capacity of these compounds as potent anticancer agents deserves further attention.

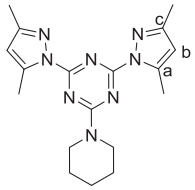
4. Experimental section

4.1. Chemistry

Materials and method: Solvents were of analytical reagent grade and were used without further purification. NMR (^{1}H and ^{13}C) spectra were recorded on a JEOL 400 MHz spectrometer at room temperature in $CDCl_3$ and/or DMSO-d_6 using internal standard δ = 0 ppm. Elemental analysis was performed on Perkin-Elmer 2400 elemental analyzer. Melting points were determined on a Mel-Temp apparatus in an open capillary tube and are uncorrected. Fourier transform infrared spectroscopy (FTIR) spectra were recorded on Shimadzu model IRAffinity-1 Spectrometer from KBr disks. Silica gel-coated TLC plates (Type 60 GF254, Merck) were used to monitor the reaction and check the purity of the compounds. A mixture of methanol-chloroform (1:9) or ethyl acetate-hexane (4:6) was used as eluent.

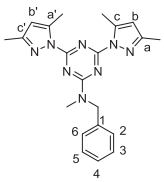
The substituted *s*-triazine compounds (**2a-e**, **3a-e**, **4a-e**, **5a-g** and **6a-g**) were synthesized following the strategies and methods already reported by our group [7d] and others [13], as shown in Scheme 1. The characterization of some of the compounds (**2a-e**, **3a-e**, **4a-c**, and **6a-e**) studied herein has already been reported by our group [7d] while the characterization of the remaining compounds is described below.

*4.1.1. 2,4-Bis(3,5-dimethyl-1H-pyrazol-1-yl)-6-(piperidin-1-yl)-1,3,5-triazine, **4a** [7d]*



White crystals, mp = 141–142 °C, in 78%; IR (KBr, cm^{-1}) 1660, 1614 (C=N), 1595 (C=N, C=C); ^{1}H NMR ($CDCl_3$): δ 1.60 (m, 6H, $3CH_2$), 2.27 (s, 6H, $2CH_3$), 2.62(s, 6H, $2CH_3$), 3.78 (t, 4H, , J = 4.4 Hz, $2CH_2$-NCH_2), 5.97 (s,2H, 2CH) ppm; ^{13}C NMR ($CDCl_3$): δ 13.5(CH_2-$\underline{C}H_2$-CH_2), 15.8($\underline{C}H_2$-CH_2-$\underline{C}H_2$), 24.3(CH_3), 25.5(CH_3), 45.1(CH_2-N-CH_2), 110.8(C_b, pyrazole), 143.3(C_c, pyrazole), 151.7(C_a, pyrazole), 163.5(C=N, triazine), 164.7(C=N, triazine) ppm.

*4.1.2. N-Benzyl-4,6-bis(3,5-dimethyl-1H-pyrazol-1-yl)-N-methyl-1,3,5-triazin-2-amine, **4d***



White crystals, mp 248–250 °C, yield 86%; IR (KBr, cm^{-1}): 1634 (C=N), 1595, 1541 (C=N, C=C); ^{1}H NMR ($CDCl_3$): δ 2.54 (s, 3H, CH_3), 2.57 (s, 3H, CH_3), 2.68 (s, 3H, CH_3), 2.87 (s, 3H, CH_3), 3.28 (s, 3H, CH_3), 4.97 (s, 2H, phCH_2), 6.31 (s, 1H, CH), 6.37 (s, H, CH), 7.32–7.39 (m, 5H, C_6H_5) ppm; ^{13}C NMR ($CDCl_3$): δ 13.7(CH_3), 15.7(CH_3), 40.1(N-CH_3), 45.2(ph-CH_2), 111.2 (C_b, pyrazole),

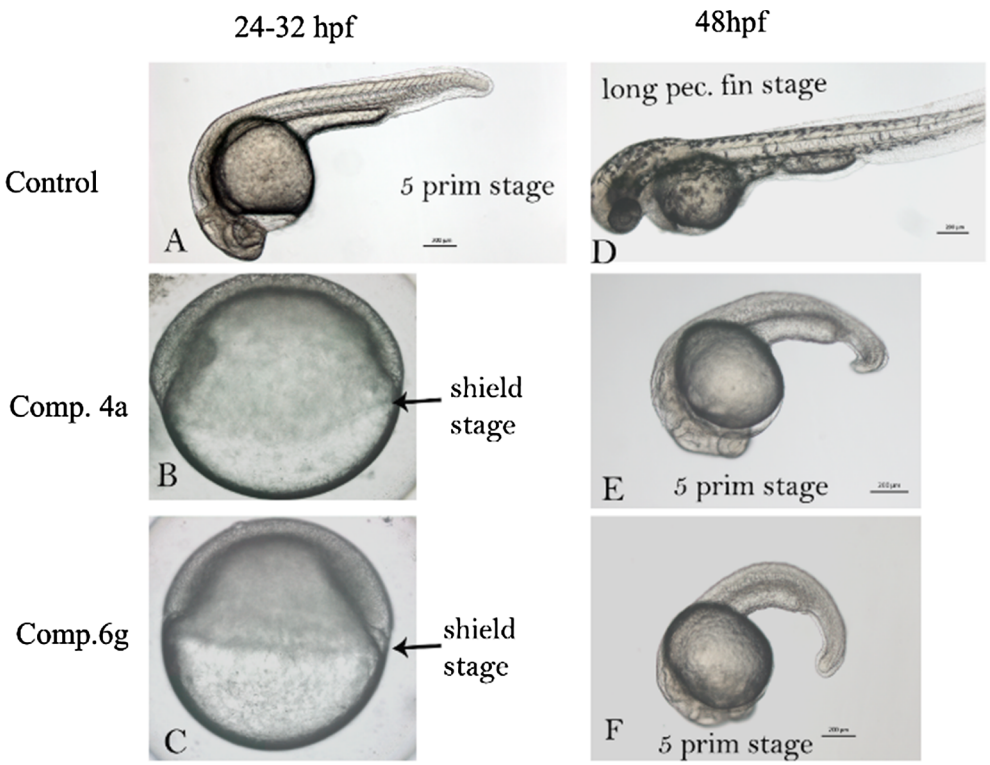


Fig. 5. Pyrazolyl-s-Triazine derivatives induced developmental delay in zebrafish embryos. Representative micrograph of zebrafish embryos at 24–32 hpf and 48 hpf of control; A & D, and treated with compound **4a;** B & E, and **6g;** C & F. Control (mock treated) embryos were around 5 prim stage (24–32 hpf), while the treated embryos either with **4a** or **6g** were at 50% epiboly (shield stage which is usually observed after 6 hpf during normal embryonic development in zebrafish embryos). The treated embryos showed marked delay in development upon continuous exposure of compounds for another 24 h (compare D with E &F). Control embryos were at long pectoral fin stage (48hpf: D), while those embryos treated with **4a** (E) or **6g** (F) showed significant level of developmental delay and were almost 24 h delay in development (by developmental stage criteria) as compared to control. Abbreviation used hpf: hours post fertilization.

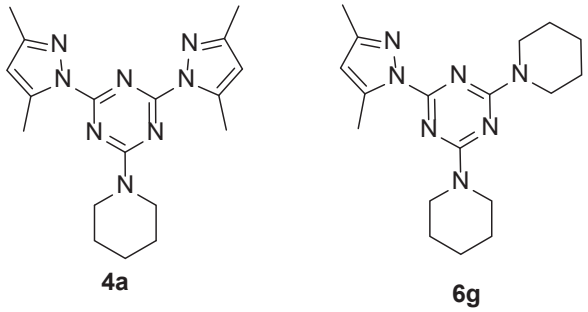
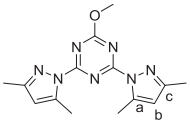


Fig. 6. Structure of the most reactive compounds.

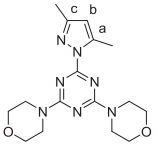
127.1(C_4), 128.6($C_{3,5}$), 137.4 ($C_{2,6}$), 143.9(C_1), 144.4(*Ca,*pyrazole), 152.3(*Cc,* pyrazole), 159.8 (C=N, triazine), 165.6(C=N, triazine) ppm. Anal. Calc. for $C_{21}H_{24}N_8$ (388.48): C, 64.93; H, 6.23; N, 28.84; Found: C, 64.81; H, 6.43; N, 28.99.

4.1.3. 2,4-Bis(3,5-dimethyl-1H-pyrazol-1-yl)-6-methoxy-1,3,5-triazine, ***4e***



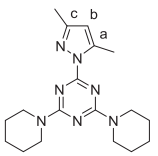
White crystals, mp 175–176 °C, yield 87%; IR (KBr, cm^{-1}): 3376 (NH), 1635 (C=N), 1595, 1542 (C=N, C=C); ^{1}H NMR ($CDCl_3$): δ 2.30 (s, 6H, $2CH_3$), 2.72 (s, 6H, $2CH_3$), 4.11 (s, 3H, OCH_3), 6.05 (s, 2H, 2CH) ppm; ^{13}C NMR ($CDCl_3$): δ 13.8(CH_3),15.8(CH_3), 55.7(OCH_3), 111.9(C_b, pyrazole), 144.4(C_a, pyrazole), 153.3(C_c, pyrazole), 164.8 (*N*-C=N, triazine), 172.3(−O−C=N, triazine) ppm. Anal. Calc. for $C_{14}H_{17}N_7$ (299.34): C, 56.18; H, 5.72; N, 32.76; Found: C, 56.33; H, 5.91; N, 32.99.

4.1.4. 4,4′-(6-(3,5-Dimethyl-1H-pyrazol-1-yl)-1,3,5-triazine-2,4-diyl) dimorpholine, ***6f***



Off-white solid, mp 152–153 °C, yield 81%; IR (KBr, cm^{-1}): 1643, 1622 (C=N), 1553 (C=N, C=C); ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 3.17 (t, 8H, J = 2.4 Hz, $4CH_2$), 3.82 (brs, 8H, $4CH_2$), 5.97 (s, 1H, CH) ppm; ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3), 16.0(CH_3), 43.6 (C−N−C, morpholine), 43.9 (C−N−C, morpholine), 66.7 (C−O−C, morpholine), 66.8 (C−O−C, morpholine), 110.4(C_b, pyrazole), 143.1(C_a, pyrazole), 151.3(C_c, pyrazole), 163.2(C=N, triazine),165.4(C=N, triazine) ppm; Anal. Calc. for $C_{16}H_{23}N_7O_2$ (345.41): C, 55.64; H, 6.71; N, 28.39; Found: C, 55.84; H, 6.90; N, 28.55.

4.1.5. 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-4,6-di(piperidin-1-yl)-1,3,5-triazine, ***6g***



Off-white solid, mp 178–179 °C, in yield 83%; IR (KBr, cm^{-1}): 1643, 1654 (C=N), 1563 (C=N, C=C); ^{1}H NMR ($CDCl_3$): δ 1.67 (brs, 12H, $6CH_2$), 2.47 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 3.87 (brs, 8H, $4CH_2$), 6.21 (s, 1H, CH) ppm; ^{13}C NMR ($CDCl_3$): δ 12.7 (CH_3), 15.3(CH_3), 24.2(CH_2-CH_2-CH_2), 25.5(CH_2-CH_2-CH_2), 45.6(CH_2-N-CH_2), 112.7(C_b, pyrazole), 145.6(C_a, pyrazole), 151.9(C_c, pyrazole), 155.4 (C=N, triazine), 159.7(C=N, triazine) ppm; Anal. Calc. for $C_{18}H_{27}N_7$ (341.23): C, 63.32; H, 7.97; N, 28.71; Found: C, 63.13; H, 8.01; N, 28.93.

4.2. Biological evaluation

4.2.1. Material and method

4.2.1.1. Human cancer cell lines. The cancer cell lines used in this study were purchased from the American Type Culture Collection (American Type Culture Collection (ATCC) Manassas, VA 20,108 USA). The celll lines are: (1) K562 human chronic myeloid leukemia cells (ATCC® CCL-243™); (2) MCF7 human breast adenocarcinoma cells (ATCC® HTB-22™); (3) MDA-MB-231 human breast adenocarcinoma cells (ATCC® HTB-26™); (4) HepG2 human hepatocellular carcinoma cells (ATCC® HB-8065™); and (5) LoVo human colorectal adenocarcinoma cells (ATCC® CCL-229™).

K562 cells were maintained in Roswell Park Memorial Institute medium, commonly known as RPMI 1640 (R8758 Sigma Aldrich), while all the other cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (11965-092 Thermo Fisher). Media were supplemented with 10% FCS (Cambrex Bio Science), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (Sigma) supplemented with 10% fetal bovine serum (Lonza) and 1% ABM (GIBCO).

4.2.1.2. Cell culture and cell viability assay. Cytotoxicity was assessed by the colorimetric MTT cell viability assay, which was done essentially as previously described [8c,d].

4.2.2. Flow cytometric analysis of cellular DNA content

2×10^6 cells were fixed in 1 mL ethanol (70%) for 60 min at room temperature. Harvested cells were re-suspended in 1 mL Na citrate (50 mM) containing 250 µg RNase A, and incubated at 50 °C for 60 min. Next, the cells were re-suspended in the same buffer containing 4 µg propidium iodine (PI) and incubated for 30 min before being analysed by flow cytometry (Becton Dickinson, San Jose, CA, USA). The percentage of cells in various cell cycle phases was determined using Cell Quest Pro software (Becton Dickinson).

4.2.3. Zebrafish toxicity assays

4.2.3.1. Animals. The wild type (AB Tubingen) was obtained from the Zebrafish International Resource Center, University of Oregon, Oregon USA. Adult zebrafish were raised and maintained following the guidelines described in the literature [14] The zebrafish embryos used in this study were less than 120 h post fertilization (hpf), and, therefore, according to the new EU Directive 2010/63/EU, did not require permission from the Institutional Animal Care and Use Committee (IACUC) [15].

4.2.3.2. Embryo treatment. The toxicity test for zebrafish embryo was based on a 12 h exposure of freshly fertilized eggs to a serial dilution of each s-triazine compound. The wild type (AB Tubingen) zebrafish embryos were obtained by natural pairwise mating. The development and growth of embryos were monitored after every 6 h until 24 hpf and then after every 24 h until 96 hpf. The embryo medium was replaced daily, including the compounds at the desired concentrations.

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