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ABSTRACT

Based on the encouraging results found for 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4-(3H)-one **7** previously tested by us, as well as the consideration that heterocycle fused tetrazepinones bearing the 2-chloroethyl substituent show a better cytotoxic profile than temozolomide and mitozolomide against human cancer cell lines which express the DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT), in this paper we report the multistep synthesis and the biological study of 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4-(3H)-one **10**. Like compound **7**, it was active on P-glycoprotein expressing cells (MDR) HL60 and on K562 cell line that are resistant to apoptosis induced by different stimuli, showing GI₅₀ values of 14 and 18 μM respectively. As an antiproliferative agent against the above cells compound **10** was about 2.2 times more active than compound **7**. Compound **10** was also tested against WiDR cells which are overexpressing the DNA repair protein MGMT, showing a GI₅₀ value of 2.3 μM. Finally, concerning the effect on cell cycle we observed an evident difference between compounds **7** and **10**. In fact, compound **7** induces a block of cell cycle in G₀-G₁, therefore acting as phase-specific drug, in contrast, compound **10** is a not phase-specific agent. Both the compounds are able to increase the apoptotic sub G₀-G₁ peak of cell cycle.

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1. Introduction

Temozolomide, or 8-carbamoyl-3-methylimidazo[5,1-d][1,2,3,5]tetrazin-4-(3H)-one **1**, active principle of Temodar[®] and Temodal[®], is a chemotherapeutic drug used in patients with recurrent and refractory high-grade glioblastoma, gliomas, lymphomas and melanoma [1–6]. Temozolomide **1** is a prodrug affording under physiological conditions a methyl-diazonium cation which alkylate the O⁶-position of guanine in DNA to form O⁶-methylguanine-DNA [7,8].

However, clinical efficacy of temozolomide **1** is limited by drug resistance which is the principal reason for the failure of anti-cancer therapies. In particular, chemoresistance to alkylating agents may result from the cells' capacity to repair cytotoxic lesions. O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein, is known to remove alkyl groups from O⁶-guanine, thus regenerating intact DNA [9,10]. So, cell lines SF-188 (Mer+) and WiDR (Mer+), expressing elevated levels of MGMT, show significant resistance to the action of alkylating agents like temozolomide **1** than the MGMT-deficient tumor cells SF-126 (Mer-) and BE (Mer-) [11].

The effort to overcome drug resistance to temozolomide **1**, led to the discovery of some fused 1,2,3,5-tetrazepin-4-(3H)-ones **2**, **3** and **4** which, like temozolomide **1**, contain the atomic sequence N=

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N–N(R)CO–N (Fig. 1) but their cytotoxic effects is independent of the cell phenotypes, showing similar activity on SF-188 (Mer+) and WiDR (Mer+) as well as SF-126 (Mer-) and BE (Mer-) cell lines [11].

Tetrazepinones act with a mechanism of action, not yet fully understood, that is different than the one of temozolomide **1**. In fact, they are weak alkylating agents but are able to induce significant levels of DNA single strand breaks in cancer cells. Tetrazepinones do not appreciably hydrolyze to produce an alkylating monomethyltriazeno as temozolomide does, indeed they produce a major benzimidazole derivative which has a weak effect on the cells. As a consequence, the cytotoxic activity is due to tetrazepinone molecule itself or probably to a short-lived species afforded during its degradation [11–15].

Due to the significant activity of 1,2,3,5-tetrazepin-4(3H)-ones on Mer+ cell lines, expressing elevated levels of MGMT, and the low number of example in the literature of tetrazepinones fused with an heterocyclic ring [11,14], we reported in earliest papers [16,17] the synthesis of the two pyrazolo[3,4-f][1,2,3,5]tetrazepinones **6** and **7** (Fig. 2), as well as the antiproliferative activity and the antiapoptotic effects against K562, K562R (imatinib mesilate resistant), HL60 and multidrug resistant (MDR) HL60 cell lines of **7**.

Compound **7** showed antiproliferative and apoptotic effects against K562, K562R (imatinib mesilate resistant), HL60 and multidrug resistant (MDR) HL60 cell lines. In particular, the proapoptotic activity against HL60 and K562 resistant cell lines was markedly higher than etoposide and busulfan respectively. Finally, flow cytometry studies carried out on K562 cells allowed to establish that **7** induces G0-G1 phase arrest followed by apoptosis.

Considering the encouraging results found for compounds **7** [17], it was thought interesting to investigate a different substitution at the 3-position of the tetrazepinone ring of **7**. At this point we selected the 2-chloroethyl group as the 3-substituent of a new tetrazepinone derivative. The choice was based on the fact that heterocycle fused tetrazepinones such as **8** and **9** (Fig. 3) bearing the 2-chloroethyl substituent, show a better cytotoxic profile than temozolomide and mitozolomide [11] when they are tested against human cancer cell lines which express the DNA repair protein MGMT. In this paper we report the multi-step synthesis and the biological study of 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one **10** (Fig. 4).

2. Chemistry

The synthesis of the novel 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]

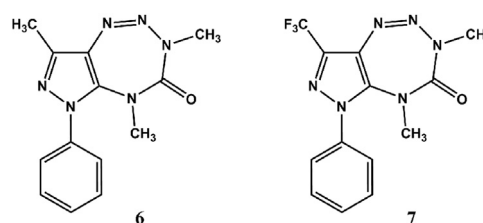


Fig. 2. Structure formulas of 3,5,8-trimethyl-6-phenyl-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one **6** and 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one **7**.

tetrazepin-4(3H)-one **10** was carried out as outlined in Scheme 1.

The starting pyrazole-5-amine derivative **11** was N-formylated by formic acid to give the formamide derivative **12** which in turn was reduced to secondary amine **13**. This compound was transformed to the diamine derivative **14** by nitrosation of the pyrazole nucleus of **13** and subsequent reduction of the nitrous group [17]. The 4-amino group of **14** was first protected with the *tert*-butoxycarbonyl group, by treatment with di-*tert*-butyl dicarbonate (Boc)₂O, affording **15**. Compound **15** was then reacted with chloroethyl isocyanate at room temperature for 20 days to give compound **16**. Attempts to obtain **16** by heating the reaction mixture were unsuccessful. At this point the compound was deprotected by treatment with trifluoroacetic acid in dichloromethane to give 1-[4-amino-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-3-(2-chloroethyl)-1-methylurea **17**. Finally, diazotization of derivative **17** with sodium nitrite in HCl 6 N and subsequent adjustment to pH 8, afforded the 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one **10**. This last transformed rapidly to pyrazolotriazole derivative **18** when it was heated in chloroform according to the decomposition mechanism previously reported [17].

All the above new compounds were identified on the basis of satisfactory elemental and spectroscopic data. The ¹H NMR of **12** indicated that the compound exists as a Z/E mixture at room temperature. When the spectrum was registered at 100 °C three distinct singlets (broad) were showed for NH, CHO and pyrazole H-4. Compounds **10**, **15**, **16** and **17** showed signals attributable to the methyl group and to aromatic protons in the range 2.73–2.90 δ and 7.42–8.18 δ respectively. Moreover, compounds **15**, **16**, showed signals, exchangeable with D₂O, for two NH in the ranges 5.50–8.18 and 6.69–8.72 δ respectively. Compound **17** showed also signals, exchangeable with D₂O, for NH₂ and NH at 4.44 δ and 6.77 δ respectively. Finally, two triplet at 3.81 δ and 4.32 δ were showed for compound **10** attributable to the chloroethyl moiety.

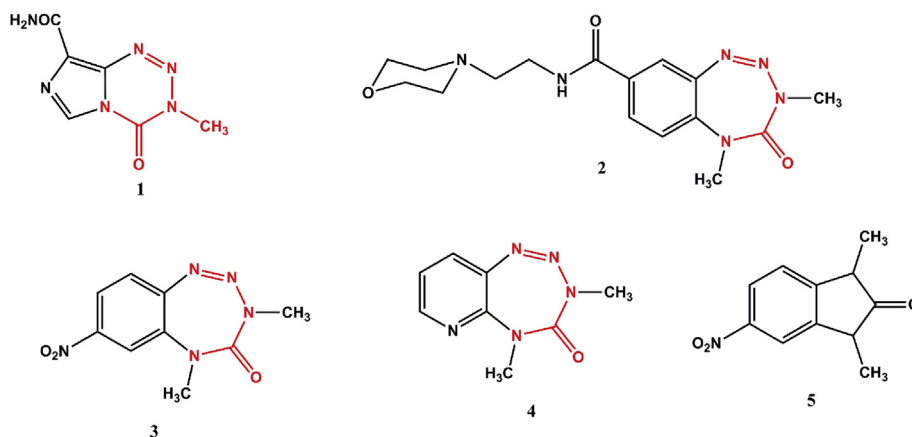


Fig. 1. Some examples of active 1,2,3,5-tetrazepin-4(3H)-ones from the literature bearing, like Temozolomide **1**, the sequence N=N–N(R)CO–N.

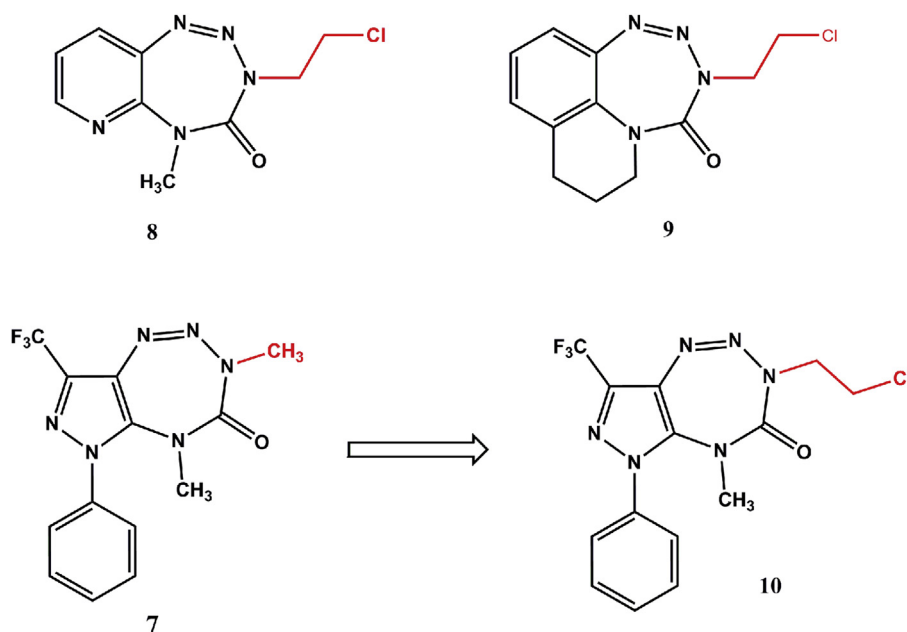


Fig. 3. Designed modification of compound **7** to improve the cytotoxic activity.

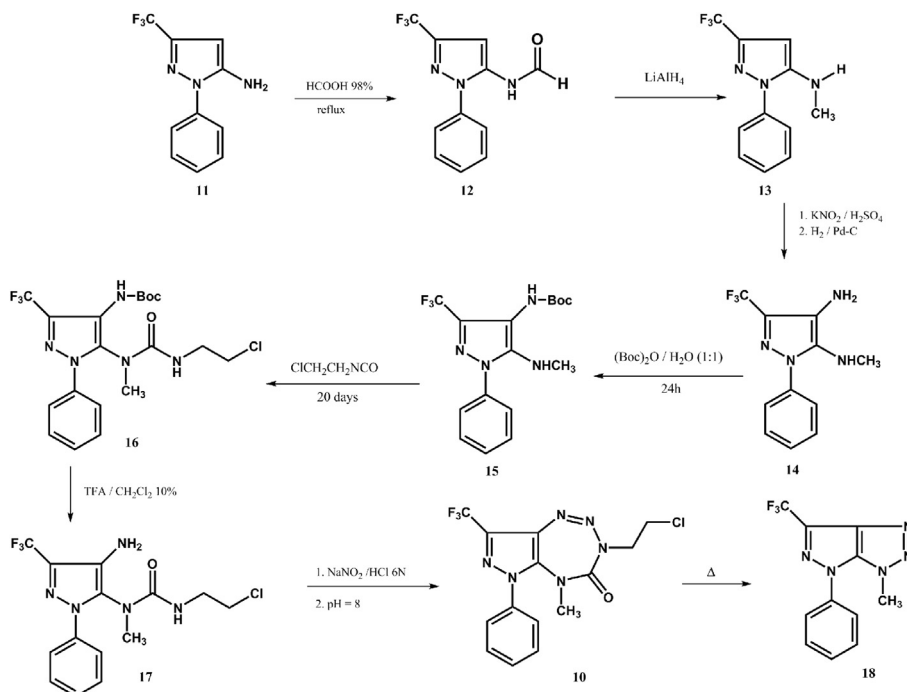
3. Pharmacological results and discussion

3.1. Cytotoxicity

Compound **10** was tested against sensitive HL60 cells (acute promyelocytic leukemia), P-glycoprotein expressing (MDR) HL60-R cells, K562 cells (a Bcr-Abl expressing leukemia) and HepG2 cells (human hepatocellular carcinoma). Cells were exposed to different concentrations of compound **10** and the number of living cells and the percentage of apoptotic cells were determined after 48 h as reported in section “Experimental protocols”. Antiproliferative and apoptosis-inducing activities were expressed as GI50

(concentration able to inhibit 50% of cell growth) and AC50 (concentration able to induce apoptosis in 50% of cells) respectively (Table 1).

As shown in Table 1, the antiproliferative and apoptotic activities of compound **10** were similar in sensitive HL60 and MDR HL60-R cell lines, respectively. This indicated that the presence of P-glycoprotein in cells does not modify the activity of compound **10**. Compared to compound **7**, that was also active in P-glycoprotein expressing cells, compound **10** was 2.3 and 2.1 times more active as antiproliferative agent and 1.9 and 1.7 times more active as apoptotic agent in HL60 and HL60-R cells respectively (Tables 1 and 2).



Scheme 1. Synthetic route to obtain the pyrazoletetrazepinone **10**.

Table 1GI₅₀(μ M) and AC₅₀ (μ M) values of compound **7** and **10** in HL60, HL60-R, K562 and HepG2 cell lines and GI₅₀(μ M) values of Daunorubicin and Etoposide in HL60 and HL60-R.

Compounds	HL60		HL60-R		K562		HepG2	
	IC ₅₀	AC ₅₀	IC ₅₀	AC ₅₀	IC ₅₀	AC ₅₀	IC ₅₀	AC ₅₀
7	21 \pm 3.3	36 \pm 5.5	30 \pm 4.8	38 \pm 2.8	40 \pm 6.2	57 \pm 8.2	n.t.	n.t.
10	9 \pm 1.6	19 \pm 3.1	14 \pm 2.2	22 \pm 2.8	18 \pm 2.8	37 \pm 5.2	26 \pm 4.0	43 \pm 6.7
Daunorubicin	0.1 \pm 0.024	n.t.	38 \pm 5.3	n.t.	n.t.	n.t.	n.t.	n.t.
Etoposide	0.4 \pm 0.03	n.t.	>400	n.t.	n.t.	n.t.	n.t.	n.t.

Table 2

Percentage of cells in G0–G1, S, G2–M after 24 h and 48 h exposure of K562 cells to compound **10**. These percentages were calculated on the cycling cells and do not include the subG0–G1 fraction. The percentage of subG0–G1 cells was calculated on the total cell number.

Time of drug exposure	G0–G1(%)	S (%)	G2–M (%)	SubG0–G1
0 (control)	44.65 \pm 6.8	43.19 \pm 7	12.16 \pm 2.2	3.21 \pm 0.4
24 h	43.96 \pm 7.2	43.59 \pm 6.3	12.45 \pm 3.1	15 \pm 2
48 h	40.97 \pm 5.3	44.24 \pm 3.9	14.79 \pm 1.8	38.35 \pm 5.2

When compared to other chemotherapeutic drugs commonly used in acute leukemias, such as daunorubicin or etoposide, compound **10** was substantially less active in sensitive HL60 cells but more active than daunorubicin and markedly more active than etoposide in HL60-R cells (Table 1). Of interest, compound **10** was also active in K562 cells, a cell line expressing the oncogene Bcr-Abl which confers resistance toward drug-induced apoptosis, and in human hepatocellular carcinoma HepG2 (Table 1).

Finally, compound **10** was tested on human colon carcinoma WiDr cell line which express high levels of O⁶-methylguanine-DNA methyltransferase (MGMT) and which is resistant toward alkylating agents such as temozolomide [11]. Our tests confirmed that temozolomide is ineffective as cytotoxic agents in WiDr cell line, showing an IC₅₀ of 1720(\pm 123) μ M. Interestingly, compound **10** was quite active in this cell line showing an IC₅₀ of 2.3(\pm 0.3) μ M.

3.2. Cell cycle

The effect of compound **10** on cell cycle distribution was analyzed in K562 cells. Cells were cultured in the presence of 30 μ M compound **10**, and flow cytometric analysis of cell cycle was carried out after 24 h and 48 h of treatment as described in section “Experimental protocols”. As shown in Fig. 4 and Table 2, compound **10** did not cause important modifications in the percentage of cell cycle distribution. A proportional decrease in G0–G1, S, and G2–M peaks and a proportional increase of the apoptotic sub-

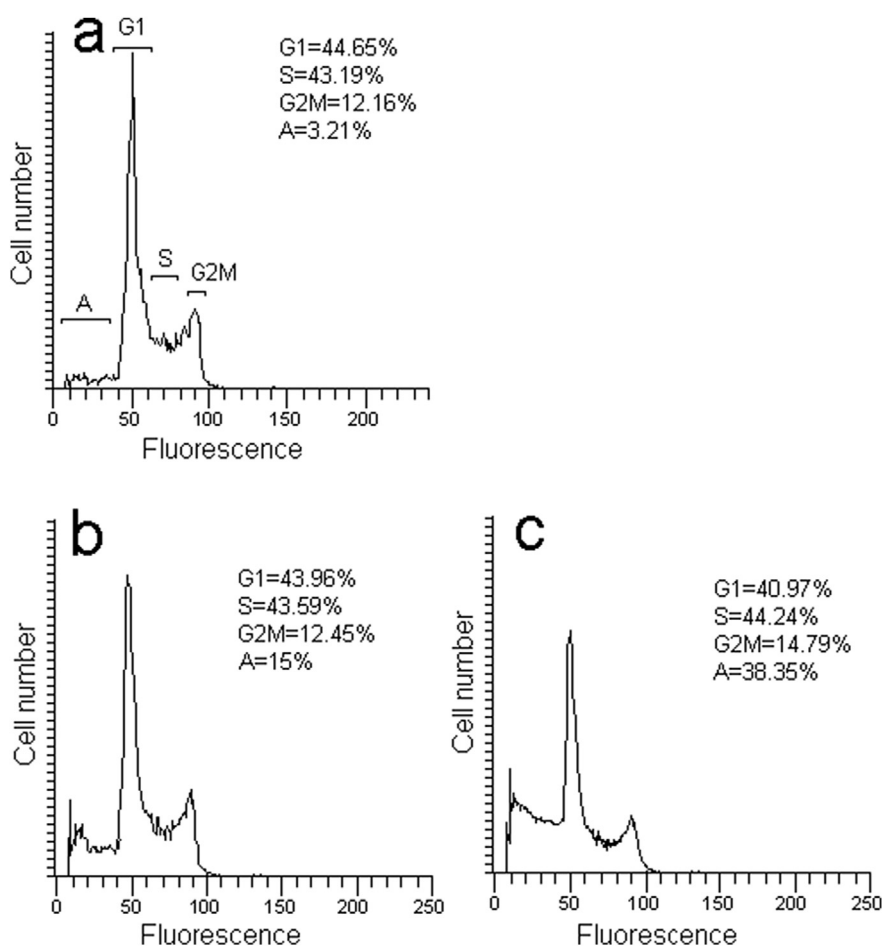


Fig. 4. Effects of compound **10** on DNA content/cell following treatment of K562 cells for 24 h (b) and 48 h (c). The cells were cultured without compound (control, panel a) or with 30 μ M compound **10** as reported in section “Experimental protocols”. SubG0–G1 (A), G0–G1, S and G2–M cells are indicated in panel a.

G0–G1 peak was correlated to the time of exposure (24 or 48 h). This indicate that compound **10** do not acts on a specific phase of cell cycle but is able to kill cells in G0–G1, S and G2–M (decrease in G0–G1, S, and G2–M peaks) causing a proportional increase in sub G0–G1 peak. Therefore, compound **10** could be effective in cancers with different kinetics. This is particularly interesting, because it could be useful alone or in combination with other anticancer agents to decrease the percentage of minimal residual disease caused by kinetic factors.

As regards the slight difference in the antiproliferative activity and cell cycle effects between **7** and **10**, it possibly could be explained considering the minor alkylating mechanism of tetrazepinones (see Fig. 5). The chloroethyldiazonium cation **20b** derived from **10** could afford alkylation as well as cross-linking in the DNA double strand, causing more damage than the mono alkylating methyldiazonium cation **20a**.

4. Conclusion

The data reported here show that the 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one **10** is an antiproliferative agent and inducer of apoptosis *in vitro* both on leukemia cells (HL60, HL60-R, K562) and on solid tumor (HepG2). It seems that the substitution of the methyl group at the 3 position of the pyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one system in compound **7** with the 2-chloroethyl moiety in compound **10** had a positive effect on activity. Compound **10** is found to be active on cells that express the MDR phenotype but with an antiproliferative as well as proapoptotic activity 2.1 and 1.7 times higher than compound **7** respectively. Of interest, compound **10** is active on WiDr, a cell line overexpressing MGMT and markedly resistant to temozolomide. Finally, the effects of compound **10** on cell cycle is different from compound **7**. In fact, while **7** acts as phase-specific drug blocking cells in the G0–G1 phase, compound **10** do not modify the percentage of cells in each phase of cell cycle but induce a proportional decrease of G1–S–G2M peaks with a concomitant increase of the subG0–G1 apoptotic peak. The slight difference in the antiproliferative activity and cell cycle effects between **7** and **10** might be explained considering the potential minor alkylating mechanism of tetrazepinones.

5. Experimental protocols

5.1. Chemistry

5.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F₂₅₄, 0.2 mm). Organic solutions were dried over Na₂SO₄. Evaporation refers to removal of solvent on a rotary

evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer Spectrum RXI FT-IR System spectrophotometer as solid in KBr disc or nujol mull supported on NaCl disks. ¹H and ¹³C NMR spectra (250 and 62.90 MHz respectively) were obtained using a Bruker AC-E 250 spectrometer (tetramethylsilane as an internal standard); chemical shifts are expressed in δ values (ppm). Mass spectra at 70 eV were obtained using an Autospec Ultima Orthogonal T.O.F.T. (Micromass) spectrometer or a GC–MS Varian Star 3400cx Saturn III spectrometer. Merck silica gel (Kieselgel 60/230–400 mesh) was used for flash chromatography columns. Microanalyses data (C, H, N) were obtained by a Elemental Vario EL III apparatus and are within $\pm 0.4\%$ of the theoretical values. Yields refer to purified products. Names of compounds were generated by ACD/chem-sketch (free ware) 2012 software, file version 14.01.

5.1.2. Preparation of N-[1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]formamide **12**

A solution of 1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine **13** [17] (1 g, 4.40 mmol) in 95% formic acid (5 mL) was refluxed for 1 h. The reaction mixture was cooled to room temperature and basified with 10% aqueous sodium hydroxide until pH = 8. The solid was filtered off and crystallized from diethyl ether/cyclohexane.

Yield: 80%; m.p.: 84–86 °C; MS (*m/z*) 255 (*M*⁺); ¹H NMR (CDCl₃) δ 6.49 and 7.00 (1H, 2 s, pyrazole H); 7.42–7.71 (6H, a set of signals, C₆H₅ and NH, exchange for 1H with D₂O); 8.25 and 8.41 (1H, 2 s, formyl proton). Singlets at 6.49/7.00 and 8.25/8.41 δ collapsed to two very broad singlets at 6.82 and 8.30 δ respectively when the spectra was registered at 100 °C. Anal Calcd for C₁₁H₈F₃N₃O: C, 51.77; H, 3.16; N, 16.47. Found C, 51.99; H, 3.36; N, 16.21.

5.1.3. Preparation of N-methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine **13**

To a solution of N-[1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]formamide **12** (0.2 g, 0.78 mmol) in anhydrous diethyl ether (2.3 mL) a suspension of aluminum lithium hydride (90 mg, 2.4 mmol, in 4.5 mL of anhydrous diethyl ether) was added. After 24 h of reflux under stirring, the reaction mixture was cooled to room temperature and quenched with methanol and water. Then the solvent was evaporated, the aqueous residue was extracted with diethyl ether and the organic layer was evaporated to give an oily product that was identical in all respect with an authentic specimen of N-methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine **13** (IR, ¹H NMR, TLC) [16]. Yield: 95%.

5.1.4. The N⁵-methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazole-4,5-diamine **14**

It was obtained starting from **13** and following a literature method [17].

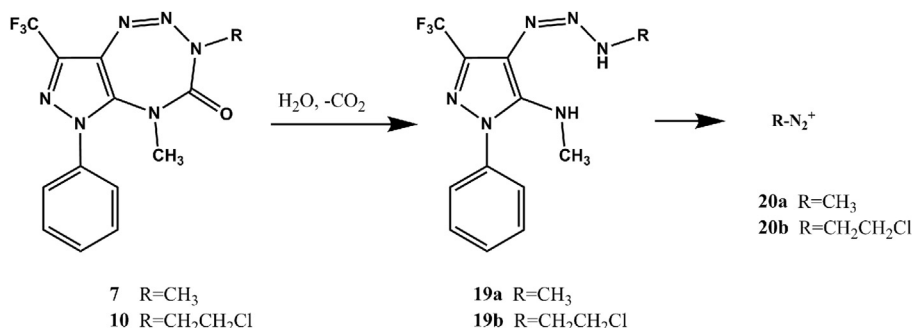


Fig. 5. Potential minor mechanism of action of tetrazepinones **7** and **10**: formation of DNA-alkylating diazonium ions **20a,b**.

5.1.5. Preparation of tert-butyl [5-(methylamino)-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]carbamate **15**

To a magnetically stirred mixture of 0.46 g (1.8 mmol) of N⁵-methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazole-4,5-diamine **14** and 1.8 mL of water, 0.454 mL (0.432 g, 1.98 mmol) of di-tert-butyl-dicarbonate were added at room temperature. Stirring was continued overnight and then the mixture was diluted with cold water (15 mL) and stirred until solidification. The solid separated was filtered off and then recrystallized to afford tert-butyl (5-(methylamino)-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl)carbamate **15**.

15: yield 85%; mp 98–100 °C (ethyl acetate/petroleum ether, b.p. 40–60 °C); MS (*m/z*) 300 ($M^+ - CH_2 = C(CH_3)_2$); IR (KBr) (cm^{-1}) 3428, 3395, 3319 (2 × NH), 1720, 1702 (CO); ¹H NMR (DMSO-*d*₆) δ 1.43 (9H, s, *t*-butyl); 2.73 (3H, d, *J* = 5.1 Hz, NH–CH₃); 5.50 (1H, q, *J* = 5.1 Hz, exchangeable slowly with D₂O, NH–CH₃); 7.45–7.58 (5H, a set of signals, C₆H₅); 8.18 (1H, s, exchangeable with D₂O, NH). Anal Calcd for C₁₆H₁₉F₃N₄O₂: C, 53.93; H, 5.37; N, 15.72. Found C, 53.63; H, 5.57; N, 15.45.

5.1.6. Preparation of tert-butyl [5-[(2-chloroethyl)carbamoyl](methylamino)-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]carbamate **16**

To a solution of 0.950 g (2.7 mmol) of tert-butyl (5-(methylamino)-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl)carbamate **15** in 25 mL of anhydrous benzene, 2.3 mL (2.81 g, 26.7 mmol) of 2-chloroethyl isocyanate were added, and the mixture was stirred for 20 days at room temperature. Then the mixture was filtered and the filtrate was evaporated at 30 °C by an oil vacuum pump to remove the excess of chloroethyl isocyanate and solvent. A white crystalline residue was obtained which was recrystallized to give pure **16**.

16: yield 83%; mp 80–81 °C (benzene); MS (*m/z*) 461; IR (KBr) (cm^{-1}) 3440, 3323 (2 × NH), 1742, 1656 (2 × CO); ¹H NMR (DMSO-*d*₆) δ 1.43 (9H, s, *t*-butyl); 2.87 (3H, s, N–CH₃); 3.10–3.50 (4H, m, 2 × CH₂), 6.90 (1H, br s, exchangeable with D₂O, NH); 7.50–7.55 (5H, a set of signals, C₆H₅); 8.72 (1H, br s, exchangeable with D₂O, NH). Anal Calcd for C₁₉H₂₃ClF₃N₅O₃: C, 49.41; H, 5.02; N, 15.16. Found C, 49.30; H, 5.32; N, 14.90.

5.1.7. Preparation of 1-[4-amino-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-3-(2-chloroethyl)-1-methylurea **17**

1 g of tert-butyl [5-[(2-chloroethyl)carbamoyl](methylamino)-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]carbamate **16** was deprotected using 35 mL of a mixture of 10% trifluoroacetic acid/dichloromethane for 1 h under stirring at room temperature. The reaction mixture was evaporated, and the residue was solubilized with dichloromethane and washed with a saturated aqueous sodium hydrogen carbonate solution. The organic layer was dried over anhydrous sodium sulfate and then evaporated to afford a residue which was crystallized to give compound **17**.

17: yield 64%; mp 139–140 °C (diethyl ether); MS (*m/z*) 361; IR (KBr) (cm^{-1}) 3447–3265 (multiple bands, NH₂, NH), 1660 (CO); ¹H NMR (DMSO-*d*₆) δ 2.90 (3H, s, N–CH₃); 3.10–3.40 (4H, m, 2 × CH₂); 4.44 (2H, s, exchangeable with D₂O, NH₂); 6.77 (1H, br s, exchangeable slowly with D₂O, NH); 7.42–7.50 (5H, a set of signals, C₆H₅). Anal Calcd for C₁₄H₁₅ClF₃N₅O: C, 46.48; H, 4.18; N, 19.36. Found C, 46.65; H, 4.57; N, 19.60.

5.1.8. Preparation of 3-(2-chloroethyl)-5-methyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-*ff*][1,2,3,5]tetrazepin-4(3H)-one **10**

To a magnetically stirred cold solution (*T* = –5 °C) of 0.25 g (0.69 mmol) of 1-(4-amino-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl)-3-(2-chloroethyl)-1-methylurea **17** in 10 mL of 6 N aqueous hydrochloric acid solution, 0.25 mL of a 20% aqueous

sodium nitrite solution was added dropwise, keeping the temperature at 0 °C. Stirring was continued at the above temperature for 1 h and then the mixture was extracted with cold dichloromethane (0 °C) (2 × 5 mL). The aqueous layer was neutralized with a cold 20% aqueous potassium hydroxide solution (0 °C), keeping the temperature at 0 °C, and then the pH was adjusted to 8 with a cold saturated aqueous sodium hydrogen carbonate solution (0 °C). The resulting solution was extracted again with cold dichloromethane (5 × 10 mL). The combined extracts were dried over anhydrous sodium sulfate, filtrated and evaporated under reduced pressure at room temperature to give **10** as a yellow solid. The product was dissolved at room temperature in diethyl ether, and the solution was scratched until crystals were formed. The suspension was cooled at –20 °C for 30 min and then filtered off.

10: Yield 37.7%; mp 72–74 °C dec (diethyl ether); MS (*m/z*) 267 ($M^+ - ClCH_2CH_2NCO$); IR (KBr) (cm^{-1}) 1670 (CO); ¹H NMR (CDCl₃) δ 2.84 (3H, s, N–CH₃); 3.81 (2H, t, *J* = 6.60 Hz, CH₂CH₂Cl); 4.32 (2H, t, *J* = 6.60 Hz, CH₂CH₂Cl); 7.48–7.58 (5H, a set of signals, C₆H₅). Anal Calcd for C₁₄H₁₂ClF₃N₆O: C, 45.11; H, 3.25; N, 22.55. Found C, 45.50; H, 3.35; N, 22.90.

5.2. Pharmacology

5.2.1. Cytotoxicity assays [18].

To evaluate the number of live and dead neoplastic cells, the cells were stained with trypan blue and counted on a hemocytometer. To determine the growth inhibitory activity of the drug tested, 2 × 10⁵ cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 mL of complete medium and treated with different concentrations of the compound to test (freshly prepared DMSO solution, the concentration of DMSO in the medium never exceeded 0.3% v/v). After 48 h of incubation, the number of viable cells was determined and expressed as the percentage of control proliferation.

5.2.2. Morphological evaluation of apoptosis and necrosis [19].

Drug-induced apoptosis and necrosis was determined morphologically after labeling with acridine orange and ethidium bromide. Cells (2 × 10⁵) were centrifuged (300 × g) and the pellet was resuspended in 25 μL of the dye mixture. Ten microliters of the mixture were examined in oil immersion with a 100 × objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of the ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by the perinuclear condensation of chromatin, stained by acridine orange (100 μg/mL) or ethidium bromide (100 μg/mL), respectively, and by the formation of apoptotic bodies. The percentage of apoptotic cells was determined after counting at least 300 cells.

5.2.3. Flow cytometric analysis of cell cycle distribution and apoptosis [20].

The effects of compound **10** on cell cycle distribution were studied on K562 cells (myeloblastic leukemia) by flow cytometric analysis after staining with propidium iodide. Cells were exposed 24 h to compound **10**. After treatment cells were washed once in ice-cold phosphate buffered saline medium and resuspended at 10⁶/mL in a hypotonic fluorochromone solution of propidium iodide (50 μg/mL) and nonidet P-40 (Sigma) [0.03% (v/v)] in 0.1% sodium citrate. After 30 min of incubation, the fluorescence of each sample was analyzed as single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the cell cycle and the apoptotic subG₀-G₁ peak was analyzed with the ModFit LT3 program (Verity Software House, Inc.).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.ejmech.2015.04.004>. These data include MOL files and InChIKeys of the most important compounds described in this article.

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