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Original article

Synthesis and induction of G0–G1 phase arrest with apoptosis of 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo [3,4-*f*][1,2,3,5]tetrazepin-4(3*H*)-one

Benedetta Maggio [a], Demetrio Raffa [a], Maria Valeria Raimondi [a], Stella Cascioferro [a], Fabiana Plescia [a], Manlio Tolomeo [b], Eleonora Barbusca [b], Giuliana Cannizzo [b], Salvatrice Mancuso [b], Giuseppe Daidone [a,*]

[a] *Dipartimento di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy*
[b] *Divisione di Ematologia e Servizio AIDS, Università degli Studi di Palermo, Policlinico, via del Vespro 129, 90127 Palermo, Italy*

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Abstract

The multistep synthesis of 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-*f*][1,2,3,5]tetrazepin-4(3*H*)-one **15** has been carried out. The compound showed antiproliferative and apoptotic effects against K562, K562-R (imatinib mesilate resistant), HL60 and multidrug resistant (MDR) HL60 cell lines. Compound **15** showed a pro-apoptotic activity against HL60 and K562 resistant cell lines markedly higher than etoposide and busulfan, respectively. Flow cytometry studies carried out on K562 cells allowed to establish that **15** induces G0–G1 phase arrest followed by apoptosis.

Keywords: 1,2,3,5-Tetrazepinones; Pyrazolo[3,4-*f*][1,2,3,5]tetrazepinones; Drug resistance; G0–G1 arrest; Apoptosis; Antiproliferative activity

1. Introduction

Drug resistance in cancer treatment calls for the availability of new chemotherapeutic agents able to overcome this phenomenon. In the literature is reported the synthesis of some benzo-1,2,3,5-tetrazepin-4(3*H*)-ones of type **1** (see Fig. 1) which, like temozolomide **2** [1,2], contain the N=N–N(CH_3)CO–N atomic sequence [3–5]. Benzotetrazepinones suffer from instability unless they bear in the benzene moiety an electron-withdrawing group. In fact, the above group destabilizes the diazonium ion which is afforded during the heterolytic opening of the N_2–N_3 bond of the tetrazepinone ring and, consequently, stabilizes the parent tetrazepinone [4] (see Scheme 1).

Tetrazepinones were shown to be much more active than temozolomide when tested against a variety of alkylating agent resistant cell lines such as SF-188 (human brain cancer), WiDR (human colon cancer), OVCAR-3 (epithelial ovarian cancer) and MCF-7 (human breast cancer). All the experimental results so far obtained suggest that tetrazepinones follow a mechanism of action different from that of temozolomide [6], but still it is not well established [7].

Literature provides few examples of derivatives containing the tetrazepinone ring fused to a heterocyclic nucleus [3,8] and therefore we thought that it would be of interest to synthesize new derivatives of this class, containing other heterocycles, in order to gain more insight into the structure–activity relationship of fused 1,2,3,5-tetrazepinones. Recently we have described the synthesis of the pyrazolo[3,4-*f*][1,2,3,5]tetrazepinone derivative **3**, but owing to its inherent instability at r.t. it could not be tested in vitro to evaluate its possible antiproliferative activity [9]. In order to circumvent the instability of the above pyrazolotetrazepinone we designed the derivative **15** which contains the pyrazole nucleus substituted at the 3-position with a trifluoromethyl group, able to exert a strong inductive electron-withdrawing effect on the 4-position of the

* Corresponding author. Tel.: +39 091 6236116; fax: +39 091 6236119.
E-mail address: gdaidone@unipa.it (G. Daidone).

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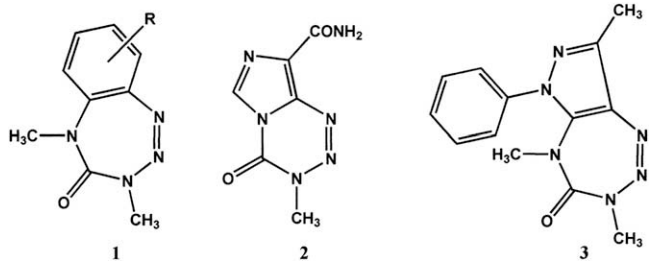


Fig. 1.

above nucleus and, as a consequence, to increase the stability of **15** than **3** (see above) [4]. Finally, due to our interest in antileukemic compounds [10] and considering that no tetrazepinone derivative has been so far tested against leukemic cell lines, we tested **15** against some sensitive and resistant ones.

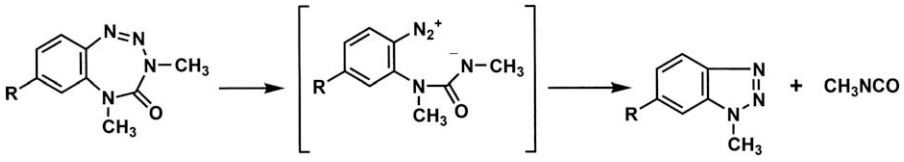
2. Chemistry

The synthesis of the novel tetrazepinone **15** was carried out as outlined in Scheme 2. The starting material **4**, prepared according to literature procedures from ethyl (1-hydroxy-2,2,2-trifluoroethyl)ether by a three-step route [11], was hydrolyzed with potassium hydroxide in order to obtain the 5-amino-pyrazole-4-carboxylic acid derivative **5** (Scheme 1). Compound **5** was decarboxylated at 215 °C affording the 5-aminopyrazole derivative **6** [12] which, in turn, was acetylated. The acetyl derivative **7** was methylated with methyl iodide in the presence of potassium hydroxide to give **8** which was transformed into the 5-methylaminopyrazole derivative **9** by hydrolysis with potassium hydroxide solution under reflux for 10 h. Nitrosation of **9** with nitrosyl hydrogen sulphate, obtained in situ by sodium nitrite and concentrated sulphuric acid, afforded the nitrous derivative **10**. The attempt to nitrosate **9** with sodium nitrite in acetic acid [13] was unsuccessful as the 4-position of the pyrazole nucleus is deactivated by the electron-withdrawing trifluoromethyl group. Compound **10** was reduced with tin(II) chloride in 36.5% aqueous hydrochloric acid to give the corresponding 4-amino derivative **11**. At this point the amino group of **11** was protected with a benzyloxycarbonyl group, by treatment with benzyl chloroformate, affording **12** which was then reacted with methyl isocyanate. The obtained ureido derivative **13** was deprotected by treatment with hydrogen and 10% Pd–C to give the derivative **14** which, in turn, was diazotized at 0 °C with nitrous acid. Finally, the diazotization solution of **14** was adjusted to pH 8 to give the desired 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-*f*][1,2,3,5]tetrazepin-4(3*H*)-one **15**. The latter transformed easily to pyrazolotriazole derivative **16** when it was heated until its melting point, probably following the same decomposition pathway of benzotetrazepinones [4]. All the above compounds were identified on the basis of satisfactory elemental and spectroscopic data. In particular, the 4-nitroso structure of the green compound **10** was based on the lack of the H-4 pyrazole signal in the ^{1}H NMR spectrum, which ruled out an *N*-nitrosamine structure, and on the absorption band in the visible spectrum at 600 nm, with low molar absorptivity value (96), indicative of a nitrous group [14]. The ^{13}C NMR spectrum of **15** accorded fully with the other spectral data. In fact, it showed, among the other signals, two singlets at 36.08 and 38.52 δ, respectively, for two *N*-methyl groups, the singlet at 155.91 δ for a carbonyl group and, finally, a quadruplet at 121.67 δ ($J_{C-F} = 269.53$ Hz) for a trifluoromethyl group.

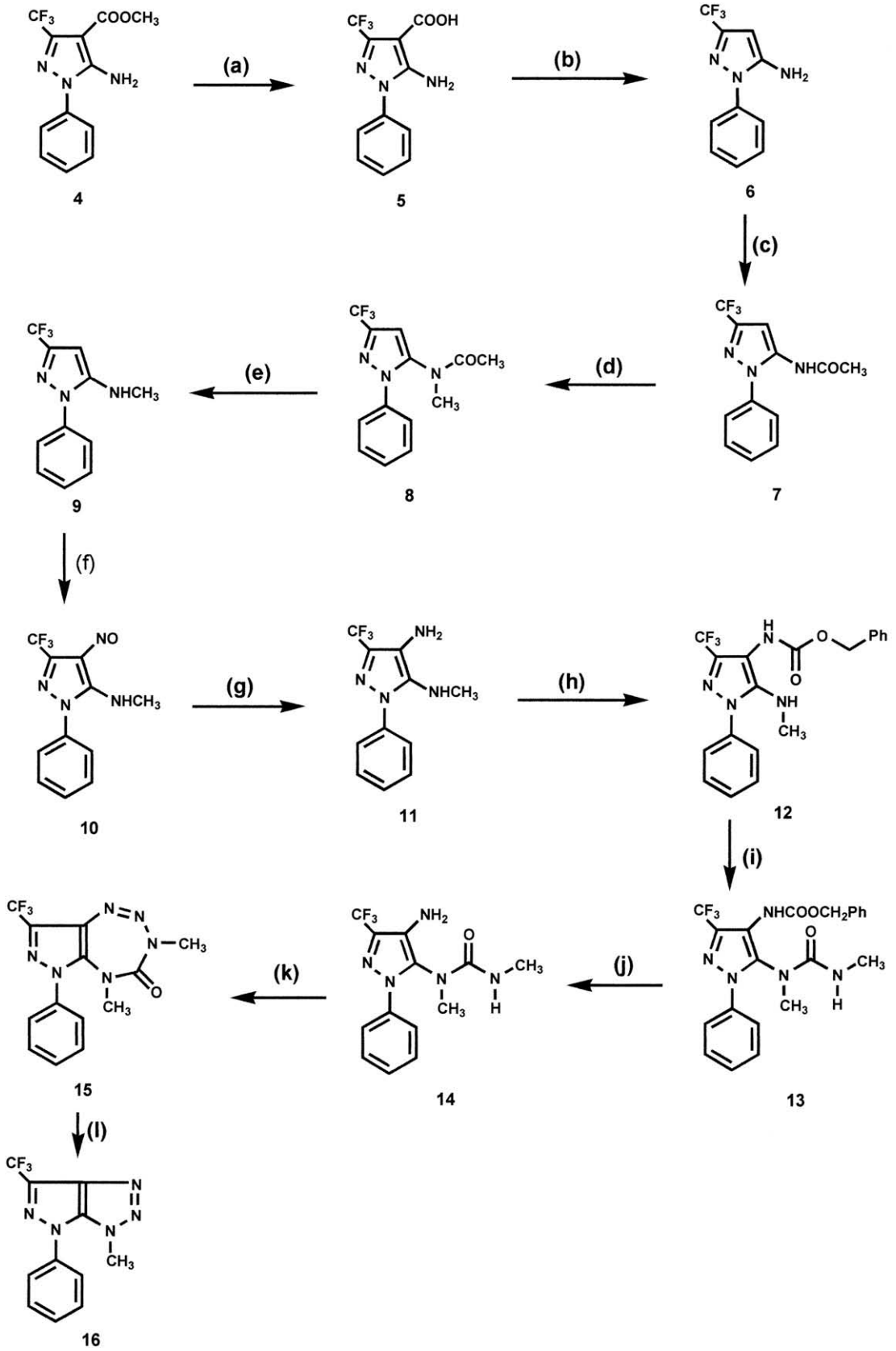
3. Pharmacological results and discussion

3.1. Cytotoxicity

Compound **15** was tested against sensitive HL60 cells (acute promyelocytic leukemia), P-glycoprotein expressing (MDR) HL60-R cells, K562 cells (a Bcr-Abl expressing leukemia) and K562-R cells that are resistant to the Bcr-Abl tyrosine-kinase inhibitor imatinib mesilate (Gleevec®). Cells were exposed to different concentrations of compound **15** and the number of living cells and the percentage of apoptotic cells were determined after 48 h as reported in Section 5. Antiproliferative and apoptosis-inducing activities were expressed as IC_{50} (concentration able to inhibit 50% of cell growth) and AC_{50} (concentration able to induce apoptosis in 50% of cells), respectively (Table 1). As shown in Fig. 2(a) and (b) and in Table 1, the antiproliferative and apoptotic activities of compound **15** were similar in sensitive HL60 and HL60-R cell lines, respectively. This indicated that the presence of P-glycoprotein in cells does not modify the activity of compound **15**. Compared to other chemotherapeutic drugs commonly used in acute leukemias, such as daunorubicin (**17**) or etoposide (**18**) (see Fig. 3), that show in HL60 cells AC_{50} values of 0.1 μM and 0.4 μM, respectively (data not shown), compound **15** was substantially less active (AC_{50} 36 μM). However, as shown in Fig. 4, in MDR cells the apoptotic inducing activity of **15** was markedly higher than etoposide (AC_{50} >400 μM) and identical to that of daunorubicin (AC_{50} 38 μM). Similar results were obtained in K562 and K562-R cells. K562 is a cell line resistant to apoptosis induced by different stimuli including chemotherapeutic agents [15] but it is sensitive toward apoptosis induced by imatinib mesilate (**19**) which is the lead drug used in all Bcr-Abl expressing leukemias. K562-R is a cell line derived from K562 by exposure to increasing concentrations of imatinib mesilate. As shown in Fig. 5(a)



Scheme 1. Suggested decomposition pathway of benzotetrazepinones [4].



Scheme 2. Synthetic pathway for the formation of tetrazepinone **15**. Reagents: (a) EtOH/KOH, reflux, 30 min; HCl; (b) heating (215 °C), 1 h; (c) $(CH_3CO)_2O$, 24 h; (d) CH_3I/KOH, reflux, 1 h; (e) KOH, H_2O/EtOH, reflux, 10 h; (f) $NaNO_2/H_2SO_4$; (g) $SnCl_2$/HCl; (h) 2 M NaOH/$PhCH_2OCOCl$; (i) CH_3NCO, reflux, 190 h; (j) H_2/10% Pd–C, 20 h; (k) $NaNO_2$/2 N HCl; $NaHCO_3$; (l) heating (105 °C), 5 min.

Table 1
IC_{50} (inhibition concentration 50%) and AC_{50} (concentration able to induce 50% apoptosis) of compound **15** in sensitive and resistant HL60 and K562 cell lines

Cells	IC50 (μM)	AC50 (μM)
HL60	21	36
HL60-R	30	38
K562	40	57
K562-R	38	62

IC_{50} and AC_{50} were calculated after 48 h drug exposure.

and (b), compound **15** was effective as antiproliferative and apoptotic agent in both K562 and K562-R cell lines. Fig. 6 shows the apoptotic effect of compound **15** compared to busulphan (**20**) (a drug commonly used in Bcr-Abl positive chronic myelogenous leukemia) and to imatinib mesilate in K562-R cells. Compound **15** (AC_{50} 56 μM) was markedly more active than busulphan (AC_{50} >800 μM) and slightly less active than imatinib mesilate (AC_{50} 62 μM).

3.2. Cell cycle

The effect of compound **15** on cell cycle distribution was analyzed in K562 cells. Cells were cultured in the presence of different concentrations of compound **15**. Flow cytometric analysis of cell cycle was carried out after 24 h and 48 h of culture as described in Section 5. As shown in Fig. 7, compound **15** caused a dose dependent arrest of cells in G0–G1 phase after 24 h of treatment. No subG0–G1 apoptotic peak was observed after 24 h of treatment (Fig. 7). After 48 h the G0–G1 peak decreased and an evident apoptotic subG0–G1 peak appeared. The percentage of cells in subG0–G1 phase at 48 h was correlated to the concentration of compound **15** used and to the percentage of cells recruited in G0–G1 after 24 h. As shown in Fig. 7, after 24 h of treatment with compound **15** (50 μM), the percentage of cells in G0–G1 phase increased from 35.8% (control) to 60.7%. After 48 h the percentage of cells in G0–G1 decreased to 28.7% with a concomitant increase of apoptotic cells (subG0–G1 peak) from 5% (24 h) to 52.2% (48 h). In contrast, the modifications in the percentage of cells in S and G2–M phases observed at 24 h and 48 h of treatment were less evident. This indicates that compound **15** induces a block of cells in G0–G1 and, afterward, it kills these cells by activating apoptosis. Most chemotherapeutic drugs used in the treatment of malignancies act in S or G2–M phase of cell cycle but not in G0–G1. Thus, a variable percentage of cells in G0–G1 could escape from the cytotoxic effects of the therapy (kinetic resistance) causing a relapse of the disease months or years after therapy [16]. Recently, several compounds capable of arresting neoplastic cells in G0–G1 have been described, nevertheless they don't kill them.

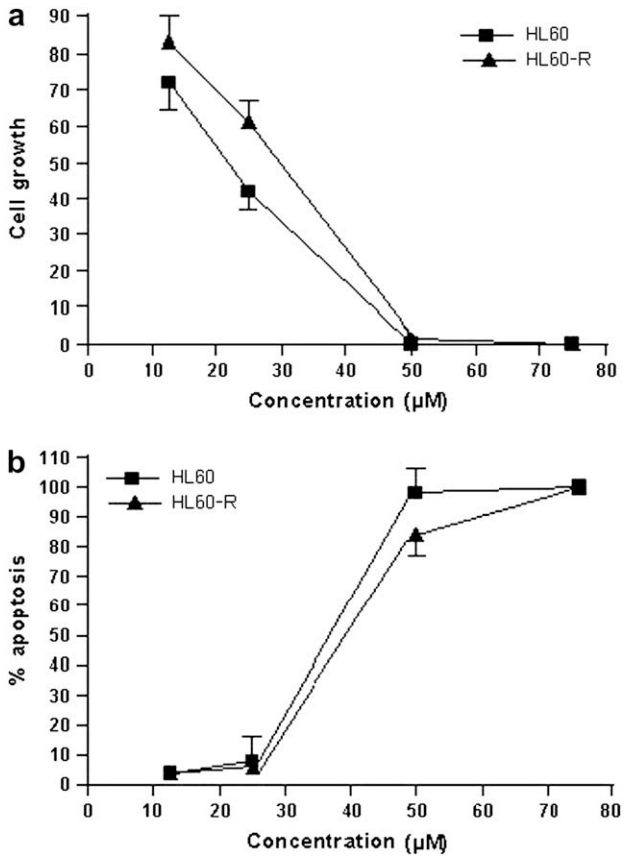


Fig. 2. Antiproliferative (a) and apoptotic (b) effects of compound **15** on HL60 and HL60-R cells. The antiproliferative activity was evaluated by counting living cells after 48 h exposure to different concentrations of compound **15**. The number of living cells was expressed as percentage of the control. The percentage of apoptotic cells was determined after 48 h exposure to different concentrations of compound **15** as reported in Section 5. Bars: ±SE.

4. Conclusion

To summarize, the tetrazepinone **15** was able to act on sensitive and resistant HL60 and K562 cell lines. This is probably due to the fact that tetrazepinones follow a new mechanism of action and therefore they are able to overcome the defences of neoplastic cells. Finally, the compound showed the advantage, than most chemotherapeutic drugs, to block cells in the G0–G1 phase and, in the same time, to kill them. The above results encourage the research of other heterocycle-fused tetrazepinones.

5. Experimental protocols

5.1. Chemistry

5.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F_{254}, 0.2 mm). Organic solutions were dried over Na_2SO_4. 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 ^{13}C 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

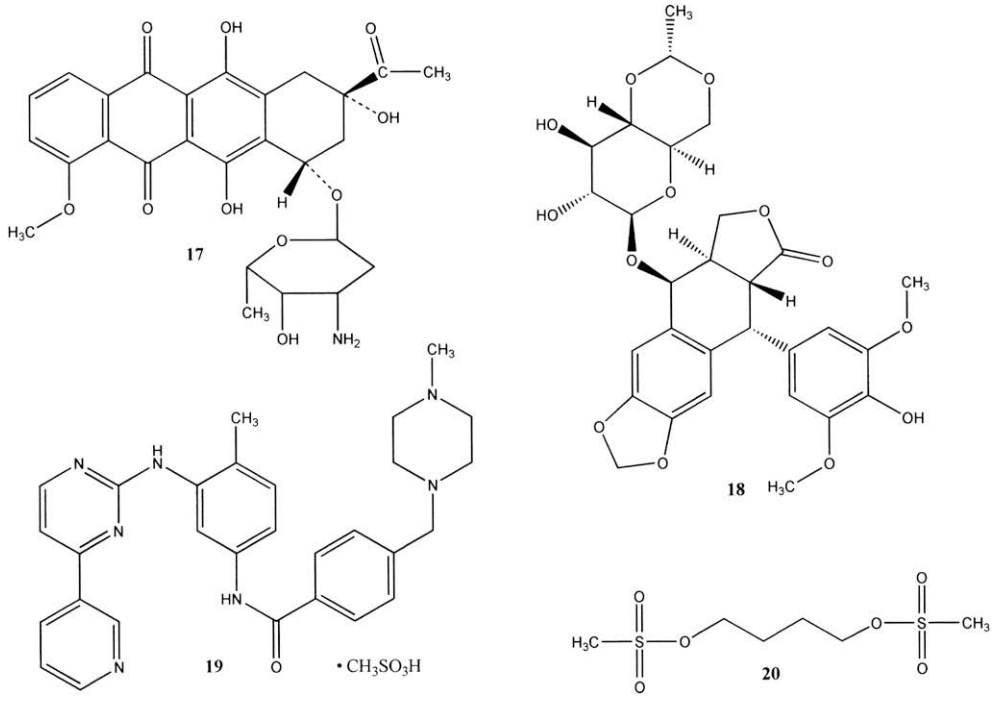


Fig. 3. Structural formulas of commercial drugs tested for comparison.

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 (Kiesegel 60/230–400 mesh) was used for flash chromatography columns. Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and are within ±0.4% of the theoretical values. Yields refer to purified products.

5.1.2. 5-Amino-1-phenyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxylic acid [5]

To 2.47 g (8.7 mmol) of methyl 5-amino-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylate (**4**) [10] dissolved in 3.4 ml of ethanol, a solution of potassium hydroxide (1.56 g of KOH in 1.12 ml of water) was added, and the reaction mixture was refluxed for 30 min. The above mixture was evaporated and the solid residue was dissolved in 3.2 ml of water adjusting its pH with aqueous 37% hydrochloric acid until acidity. The white solid which separated was filtered off and then crystallized to give compound **5**. Yield 78%.

Compound ***5***. Mp 215–216 °C (ethyl acetate/petroleum ether b.p. 40–60 °C). Anal Calcd for $C_{11}H_8F_3N_3O_2$: C, 48.72; H, 2.97; N, 15.49. Found: C, 48.51; H, 3.10; N, 15.30; MS (*m/z*) 227 ($M^+ - CO_2$); IR (KBr) (cm^{-1}) 3440, 3336 (NH_2), 3039–2660 (OH) 1664 (CO); ^1H NMR (DMSO-d_6) (δ) 6.66 (2H, s, exchangeable with D_2O, NH_2); 7.51–7.59 (5H, a set of signals, C_6H_5); 12.78 (1H, br s, exchangeable with D_2O, OH).

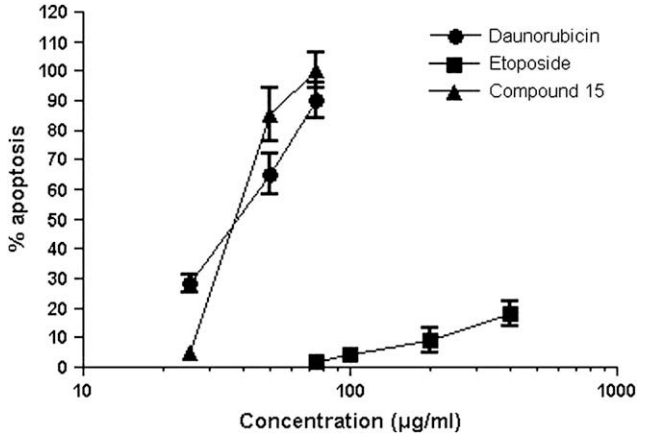


Fig. 4. Apoptotic effects of compound **15** compared to daunorubicin and etoposide in HL60-R cells. The percentage of apoptotic cells was determined after 48 h exposure to different concentrations of each compound as reported in Section 5. Bars: ±SE.

5.1.3. 1-Phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine [6]

The compound 5-amino-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid **5** (1 g, 3.7 mmol) was allowed to stand at its melting point for 1 h, then the crude product was crystallized. Yield 30%.

Compound ***6***. Mp 101–102 °C (ethanol). Anal Calcd for $C_{10}H_8F_3N_3$: C, 52.87; H, 3.55; N, 18.50. Found: C, 52.95; H, 3.70; N, 18.71; MS (*m/z*) 227 (M^+); IR (KBr) (cm^{-1}) 3452, 3324 (NH_2); ^1H NMR ($CDCl_3$) (δ) 3.96 (2H, br s, exchangeable with D_2O, NH_2); 5.81 (1H, s, pyrazole H-4); 7.40–7.54 (5H, a set of signals, C_6H_5). [Lit. [11]: 3.92 (2H, br, s), 5.87 (1H, s), 7.40–7.58 (5H, m).]

5.1.4. N-[1-Phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-acetamide [7]

A mixture of 1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-amine **6** (1 g, 4.40 mmol) and acetic anhydride (4 ml) was

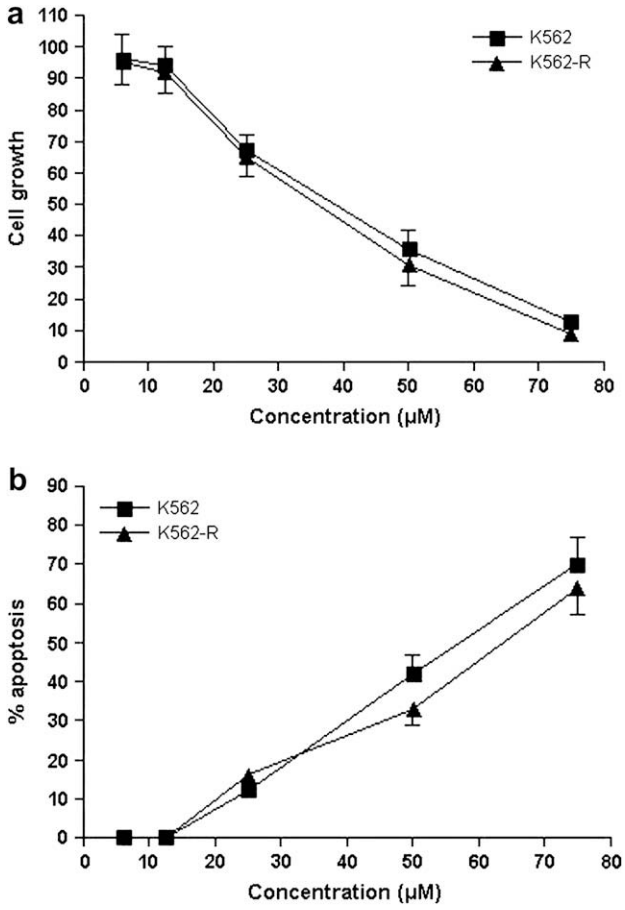


Fig. 5. Antiproliferative (a) and apoptotic (b) effects of compound **15** on K562 and K562-R cells. The antiproliferative activity was evaluated by counting living cells after 48 h exposure to different concentrations of compound **15**. The number of living cells was expressed as percentage of the control. The percentage of apoptotic cells was determined after 48 h exposure to different concentrations of compound **15** as reported in Section 5. Bars: ±SE.

stirred at room temperature for 24 h, and then cold water was added until precipitation of an oily material which easily solidified. The solid was filtered off and then crystallized to give **7**. Yield 88%.

Compound ***7***. Mp 146–148 °C (ethyl acetate). Anal Calcd for $C_{12}H_{10}F_3N_3O$: C, 53.54; H, 3.74; N, 15.61. Found: C, 53.60; H, 3.90; N, 15.40; MS (*m/z*) 269 (M^+); IR (KBr) (cm^{-1}) 3241 (NH), 1673 (CO); ^1H NMR ($CDCl_3$) (δ) 2.01 (3H, s, CH_3); 6.79 (1H, s, pyrazole H-4); 7.35–7.48 (5H, a set of signals, C_6H_5); 7.82 (1H, s, exchangeable with D_2O, NH).

5.1.5. N-Methyl-N-[1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-acetamide [8]

To 1 g (3.71 mmol) of *N*-[1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]acetamide **7** dissolved in 13.6 ml of acetone was added 1 g of powdered potassium hydroxide and then the mixture was refluxed gently for 10 min. After this time 0.35 ml (5.62 mmol) of iodomethane in 2 ml of acetone was added and reflux was continued for 1 h. The mixture was filtered off, the filtrate was evaporated affording a residue which was treated with water (25 ml) and extracted three times

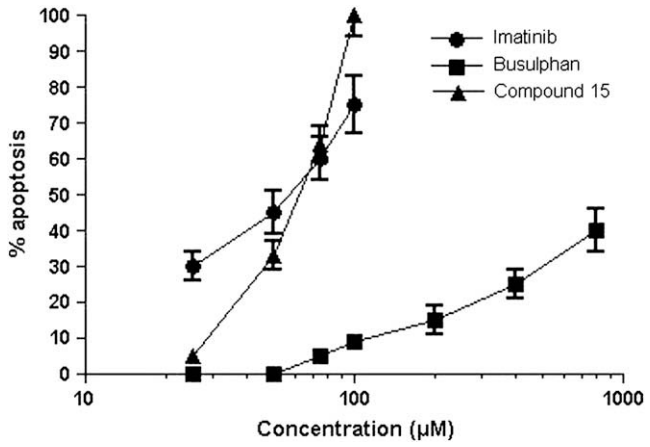


Fig. 6. Apoptotic effects of compound **15** compared to imatinib mesilate and busulphan in K562-R cells. The percentage of apoptotic cells was determined after 48 h exposure to different concentrations of each compound as reported in the Section 5. Bars: ±SE.

with diethyl ether (3 × 25 ml). Evaporation of the extracts allowed to obtain an oily residue which was crystallized to give **8**. Yield 66%.

Compound ***8***. Mp 58–60 °C (petroleum ether b.p. 40–60 °C). Anal Calcd for $C_{13}H_{12}F_3N_3O$: C, 55.12; H, 4.27; N, 14.84. Found: C, 55.30; H, 4.45; N, 14.60; MS (*m/z*) 283 (M^+); IR (KBr) (cm^{-1}) 1690 (CO); ^1H NMR ($CDCl_3$) (δ) 1.85 (3H, s, CH_3); 3.12 (3H, s, CH_3); 6.60 (1H, s, pyrazole H-4); 7.45–7.49 (5H, a set of signals, C_6H_5).

5.1.6. N-Methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine [9]

To a solution of 1 g (3.53 mmol) of *N*-methyl-*N*-[1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]acetamide **8** in 6 ml of ethanol, 3.4 ml of a 5 N aqueous potassium hydroxide solution was added, and the mixture was refluxed for 10 h. The mixture was filtered off, and the filtrate when evaporated left an oily residue which was chromatographed following the flash chromatography procedure [17]: external diameter of the column 4 cm, ethyl acetate/petroleum ether b.p. 40–60 °C (3:7 v/v) as eluent (1.5 l). Fractions 12–17 (each 50 ml) were collected and evaporated to give **6** as a pure brown oil. Yield 82%.

Compound ***9***. Anal Calcd for $C_{11}H_{10}F_3N_3$: C, 54.77; H, 4.18; N, 17.42. Found: C, 54.90; H, 4.36; N, 17.55; MS (*m/z*) 241 (M^+); IR (hexachloro-1,3-butadiene) (cm^{-1}) 3200–3420 (multiple bands, NH); ^1H NMR ($CDCl_3$) (δ) 2.81 (3H, d, CH_3, $J = 5.20$ Hz); 3.89 (1H, br s, exchangeable with D_2O, NH); 5.73 (1H, s, pyrazole H-4); 7.35–7.46 (5H, a set of signals, C_6H_5).

5.1.7. N-Methyl-4-nitroso-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-amine [10]

To a magnetically stirred cold solution (ice bath 5 °C) of 1 g (4.15 mmol) of *N*-methyl-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-amine **9** in 3.1 ml of acetic acid, a mixture of 0.31 g (4.49 mmol) of sodium nitrite in 0.82 ml of 98% sulphuric acid was added in portions. Stirring was continued

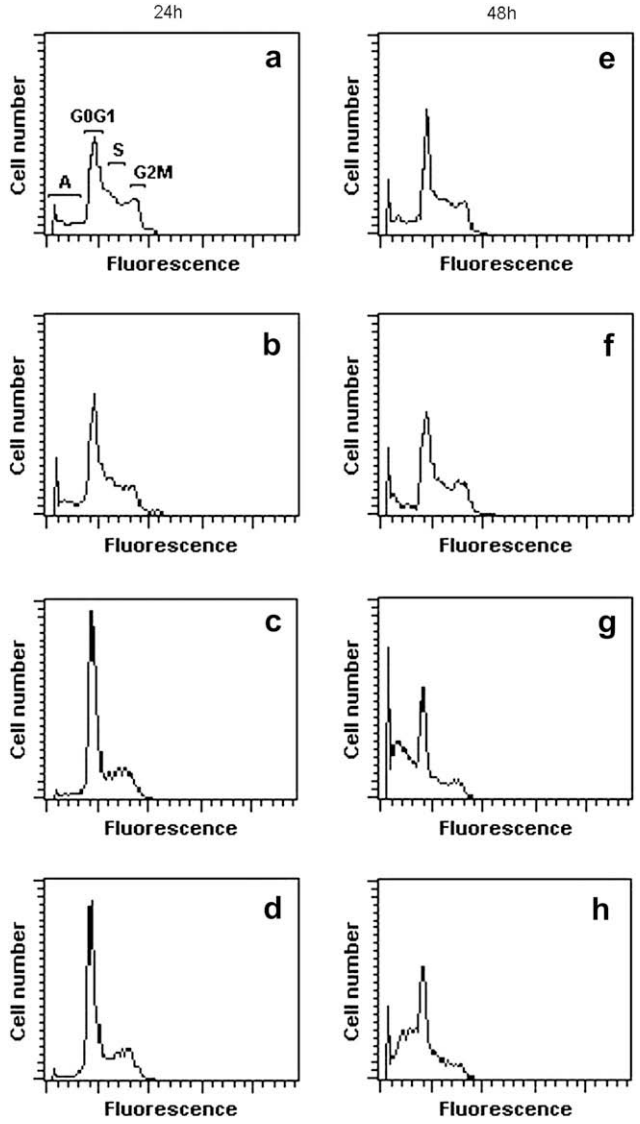


Fig. 7. Effects of compound **15** on DNA content/cell following treatment of K562 cells for 24 h and 48 h. The cells were cultured without compound (control, panels a and e) or with the compound at the following concentrations: 25 μM (panels b and f), 50 μM (panels c and g), 75 μM (panels d and h). Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in Section 5. SubG0–G1 (A), G0–G1, S, and G2–M cells are indicated in panel a.

overnight at room temperature and then the mixture was poured in crushed ice. The green solid separated was filtered off, washed with a saturated aqueous sodium hydrogen carbonate solution until pH of washing filtrate was neutral and then crystallized to afford *N*-methyl-4-nitroso-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-amine **7**. Yield 38%.

*Compound **10***. Anal Calcd for $C_{11}H_9F_3N_4O$: C, 48.89; H, 3.36; N, 20.73. Found: C, 48.80; H, 3.50; N, 20.93; mp 138–140 °C (ethyl acetate/petroleum ether, b.p. 40–60 °C); MS (*m/z*) 270 (M^+); IR (KBr) (cm^{-1}) 3231 (NH); vis (methanol) λ_{max} nm (ε) 600 (96), 360 (6859), 284 (7214); ^{1}H NMR ($CDCl_3$) (δ) 2.54 (3H, s, CH_3); 7.49–7.54 (5H, a set of signals, C_6H_5); 9.72 (1H, s, exchangeable with D_2O, NH).

5.1.8. N^5-Methyl-1-phenyl-3-(trifluoromethyl)-1H-pyrazole-4,5-diamine [11]

To a magnetically stirred cold solution ($T = 5–10$ °C) of 3.18 g of tin(II) chloride in 6.52 ml of 37% aqueous hydrochloric acid, 1.5 g (5.55 mmol) of *N*-methyl-4-nitroso-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-amine **10** was added in portions. Stirring was continued overnight at room temperature and then the mixture was diluted with 66 ml of cold water. After adjusting its pH with a 40% aqueous sodium hydroxide solution until alkalinity, the aqueous layer was extracted with diethyl ether (3 × 50 ml) and the combined extracts were dried over anhydrous sodium sulphate and then evaporated to give N^5-methyl-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4,5-diamine **11** as a pure oily residue. Yield 90%.

*Compound **11***. Anal Calcd for $C_{11}H_{11}F_3N_4$: C, 51.56; H, 4.33; N, 21.87. Found: C, 51.76; H, 4.50; N, 21.55; MS (*m/z*) 256 (M^+); IR (KBr) (cm^{-1}) 3222–3435 (multiple bands, NH and NH_2); ^{1}H NMR ($CDCl_3$) (δ) 2.71 (3H, s, CH_3); 3.11 (3H, s, exchangeable with D_2O, NH and NH_2); 7.36–7.56 (5H, a set of signals, C_6H_5).

5.1.9. Benzyl [5-(methylamino)-1-phenyl-3-(trifluoro methyl)-1H-pyrazol-4-yl]carbamate [12]

To a magnetically stirred cold solution ($T = 0–5$ °C) of 0.256 g (1 mmol) of N^5-methyl-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazole-4,5-diamine **11** in 2 ml of 2 M sodium hydroxide water/dioxane (1:1) (v/v) solution and 0.157 ml (1.1 mmol) of benzyl chloroformate were added dropwise. Stirring was continued overnight at room temperature and then the mixture was diluted with cold water and extracted with diethyl ether (3 × 3 ml). After evaporation of the extracts the obtained oily residue was crystallized to give **12**. Yield 82%.

*Compound **12***. Anal Calcd for $C_{19}H_{17}F_3N_4O_2$: C, 58.46; H, 4.39; N, 14.35. Found: C, 58.29; H, 4.37; N, 14.28; mp 95–96 °C (diethyl ether); MS (*m/z*) 390 (M^+); IR (KBr) (cm^{-1}) 3386–3198 (multiple bands, 2 × NH), 1740 (CO); ^{1}H NMR ($CDCl_3$) (δ) 2.69 (3H, s, CH_3); 3.51 (1H, s, exchangeable with D_2O, NH); 5.19 (2H, s, CH_2); 6.14 (1H, br s, exchangeable with D_2O, NH); 7.36–7.57 (10H, a set of signals, 2 × C_6H_5).

5.1.10. Benzyl (5-{methyl[(methylamino)carbonyl]-amino}-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-4-yl) carbamate [13]

To a solution of 0.46 g (1.18 mmol) of benzyl [5-(methylamino)-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-4-yl]carbamate **12** in 12 ml of anhydrous chloroform, 2.4 ml (36.9 mmol) of methyl isocyanate was added, and the mixture was refluxed for 190 h. The solution was evaporated obtaining an oily residue which was crystallized to give **13**. Yield 41%.

*Compound **13***. Anal Calcd for $C_{21}H_{20}F_3N_5O_3$: C, 56.37; H, 4.51; N, 15.65. Found: C, 56.45; H, 4.60; N, 15.78; mp 164–166 °C (diethyl ether); MS (*m/z*) 447 (M^+); IR (KBr) (cm^{-1}) 3421 and 3214 (2 × NH), 1716 and 1663 (2 × CO); ^{1}H NMR ($CDCl_3$) (δ) 2.65 (3H, br s, CH_3); 2.86 (3H, s, CH_3); 5.17 (3H, broad signal exchangeable with D_2O for 1H, CH_2 and NH);

6.21 (1H, s, exchangeable with D_2O, NH); 7.36–7.46 (10H, a set of signals, $2 \times C_6H_5$).

5.1.11. 1-[4-Amino-1-phenyl-3-(trifluoromethyl)-1H-pyrazol-5-yl]-1,3-dimethylurea [14]

To a solution of 0.5 g (1.11 mmol) of benzyl (5-{methyl [(methylamino)carbonyl]amino}-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-4-yl)carbamate **13** in 50 ml of methanol, 50 mg of 10% Pd–C as a catalyst was added. The mixture was left under hydrogenation in a Parr apparatus at 50 psi for 20 h. After this time the suspension was filtered, and the filtrate was evaporated affording an oily residue which was crystallized to give **14**. Yield 58%.

Compound ***14***. Anal Calcd for $C_{13}H_{14}F_3N_5O$: C, 49.84; H, 4.50; N, 22.36. Found: C, 49.70; H, 4.65; N, 22.50; mp 164–165 °C (benzene); MS (*m/z*) 313 (M^+); IR (KBr) (cm^{-1}) 3452–3316 (multiple bands, NH_2 and NH), 1656 (CO); ^1H NMR ($CDCl_3$) (δ) 2.82 (3H, d, N–CH_3, $J = 4.12$ Hz); 2.92 (3H, s, CH_3); 3.58 (2H, br s, exchangeable with D_2O, NH_2); 4.91 (1H, br s, NH); 7.37–7.44 (5H, a set of signals, C_6H_5).

5.1.12. 3,5-Dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-f][1,2,3,5]tetrazepin-4(3H)-one [15]

To a magnetically stirred cold solution ($T = -5$ °C) of 0.3 g (0.96 mmol) of 1-[4-amino-1-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-1,3-dimethylurea **14** in 2.8 ml of 2 N aqueous hydrochloric acid solution, 0.35 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) (3 × 5 ml). The pH of aqueous layer was adjusted to 8 with a saturated aqueous sodium hydrogen carbonate solution (0 °C), and the resulting solution was extracted again with cold dichloromethane (5 × 5 ml). The combined extracts were dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure (rotary vacuum pump) at r.t. to give **15** as a pure yellow powder. 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 the crystal product was filtered off. Yield 58%.

Compound ***15***. Anal Calcd for $C_{13}H_{11}F_3N_6O$: C, 48.15; H, 3.42; N, 25.92. Found: C, 48.35; H, 3.52; N, 25.70; mp 108–109 °C dec.; MS (*m/z*) 324 (M^+); IR (KBr) (cm^{-1}) 1656 (CO); ^1H NMR ($CDCl_3$) (δ) 2.83 (3H, s, CH_3); 3.49 (3H, s, CH_3); 7.48–7.57 (5H, a set of signals, C_6H_5). ^{13}C NMR ($CDCl_3$) (δ) 35.66 (CH_3), 38.11 (CH_3), 121.67 (q, $J_{C-F} = 269.53$, CF_3), 123.57, 124.29, 126.57, 131.15, 131.31, 138.97, 139.05, 155.91 (CO).

5.1.13. 3-Methyl-4-phenyl-6-(trifluoromethyl)-3,4-dihydropyrazolo[3,4-d][1,2,3]triazole [16]

The compound 3,5-dimethyl-6-phenyl-8-(trifluoromethyl)-5,6-dihydropyrazolo[3,4-*f*][1,2,3,5]tetrazepin-4(3*H*)-one **15** (0.05 g, 0.15 mmol) was allowed to stand at its melting point for 5 min affording quantitatively **16** as a pure oily product.

Compound ***16***. Anal Calcd for $C_{11}H_8F_3N_5$: C, 49.44; H, 3.02; N, 26.21. Found: C, 49.66; H, 3.22; N, 26.45; MS (*m/z*) 267 (M^+); ^1H NMR ($CDCl_3$) (δ) 4.16 (3H, s, CH_3); 7.49–7.59 (5H, a set of signals, C_6H_5).

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, 2×10^5 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 were determined morphologically after labeling with acridine orange and ethidium bromide. Cells (2×10^5) were centrifuged ($300 \times g$) 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 **15** on cell cycle distribution were studied on K562 cells (myeloblastic leukemia) by flow cytometric analysis after staining with propidium iodide. Cells were exposed for 24 h to compound **15**. After treatment cells were washed once in ice-cold phosphate buffered saline medium and resuspended at 10^6/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 an FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the cell cycle and the apoptotic subG0–G1 peak were analyzed with the ModFit LT3 program (Verity Software House, Inc.).

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