Future Medicinal Chemistry

# Thio Substituted Derivatives of 4-Amino-pyrazolo[3,4-d]pyrimidine-6-thiol as antiproliferative agents



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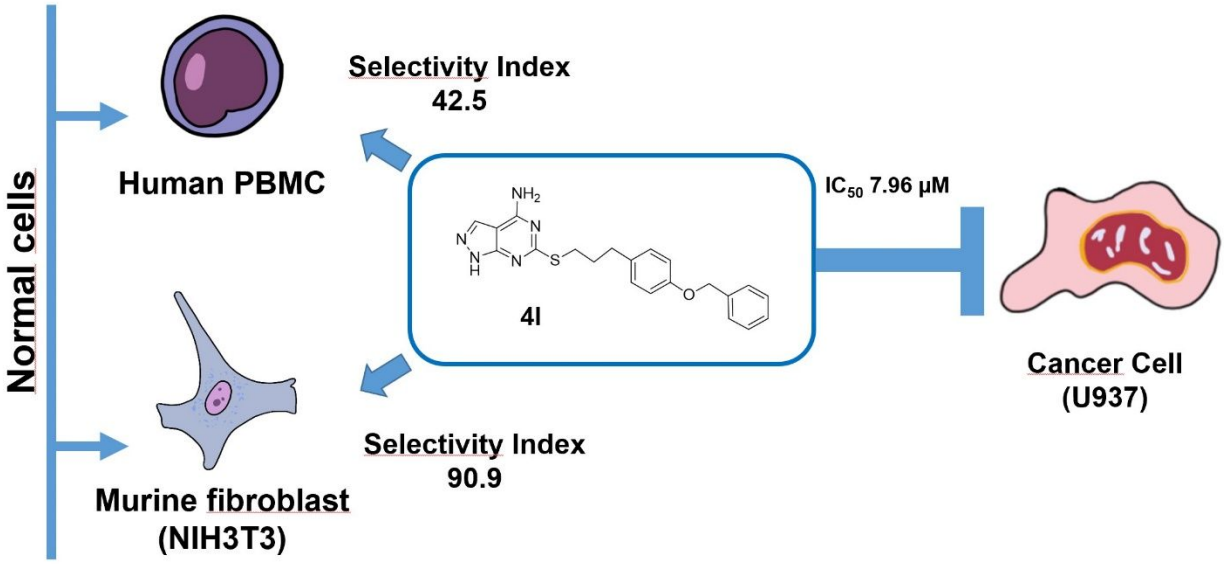
- **Abstract:**

**Aim:** Identification of new compounds as potential antiproliferative drug candidates. **Methodologies**: Synthesis of hetero aromatic bicyclic and monocyclic derivatives as purine bioisosters. Their antiproliferative activity was studied against U937 cancer cells. The most effective compounds were evaluated for their selectivity against cancer cells, the possible mechanism of cell death, and their interference with DNA replication. **Results and Conclusion:** Among the synthesized compounds, only three (4b, 4j and 4l) demonstrated a value of IC50 less than 20 µM. However, two of them (4b and 4l) were specific against cancer cells, with 4l presenting a quite high selectivity. The presence of substituted pyrazolo[3,4-d]pyrimidine core is essential for the activity, as the presence of substituents at the thiol function in 6-position.


- **Graphical abstract:** optional – a concise, visual summary of the main findings of the article, helping readers to quickly understand the findings of the paper and its relevance to them.





- **Keywords:** 4-amino-pyrazolo[3,4-d]pyrimidine-6-thiol; antiproliferative activity; U937; apoptosis; pyrazolo pyrimidine; cell cycle; cancer.

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- **Main body of text:**

## Introduction

Despite the intense efforts made during last decades to develop possible drug candidates, cancer is one of the major human health problems causing death in both developing and industrialized countries. Since the 50's, purine analogs were used as inhibitors of nucleic acid metabolism and the research in this field has led to interesting results [1]. For these reasons, and because several cancers still represent an unmet clinical need due to drug resistance, many groups are still working on the synthesis of possible modified purine derivatives. Among this class of molecules, pyrazolo-pyrimidine nucleus, which can be considered as a purine bioisostere, is one of the most important nitrogen heterocyclic derivative bearing a wide variety of biological activities. In particular, pyrazolo[3,4-d]pyrimidines are reported to encompass pharmacological potential as antiviral, antimicrobial, cardiovascular, anti-inflammatory and antitumor agents [2]. Of note, the anticancer properties of pyrazolo[3,4-d]pyrimidines can be attributed mainly to the inhibitory activity demonstrated against a large number of protein kinases, which usually demonstrate an impaired activity in a large variety of cancers [3]. In line with this, the inhibition of several kinases has proven to be an effective strategy in controlling cancer cell replication and apoptosis [3]. For instance, the substitution of the pyrazolo[3,4-d]pyrimidine scaffold with various groups confers an important anticancer activity mediated by the specific inhibition of kinases such as Src [4], CDK2 [5] and EGFR tyrosine kinases [6] (Figure 1). These represent just few examples of the multiple targets of these multifaceted compounds.

**Figure 1 to be inserted here**

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In addition to the above mentioned actions, functionalized pyrazolo[3,4-d]pyrimidine scaffold, or the simplified functionalized pyrimidine ring, are also useful tools for the synthesis of many fused heterocycles of biological interest, such as P1 purine receptors ligands [7]. Over the years we accumulated a laboratory library of structurally related compounds bearing a pyrazolo[3,4-d]pyrimidine scaffold or simplified rings. Therefore, based on the aforementioned premises we decided to test some of these structurally related compounds as possible antiproliferative agents against U937, a neoplastic histiocytic cell line [8].

Among our compounds, a series of 4-amino-1H-pyrazolo[3,4-d]pyrimidine-6-thiol derivatives, bearing different substituents on the thiol moiety (**4 a-n**), were chosen in order to evaluate the influence of: a) the chemico-physical properties; and b) the steric hindrance, on the possible biological activity. Few structurally related derivatives were also tested, such as simplified purine bioisosteres like ethyl 3-(4-amino-6-mercapto-1H-pyrazolo[3,4-d]pyrimidin-1-yl)propanoate (**4m**), ethyl 3-(4-amino-6-mercapto-2H-pyrazolo[3,4-d]pyrimidin-2-yl)propanoate (**4n**) and ethyl 3-((6-amino-9H-purin-2-yl)thio)propanoate (**6**), this last being a purine analog. Finally, we investigated the importance of the bicyclic scaffold for a possible biological activity by testing a series of variously substituted 4-aminopyrimidine-2-thiol analogs (**5a-j**), bearing some substituents of the previous series of 4-amino-1H-pyrazolo[3,4-d]pyrimidine-6-thiol derivatives (Figure 2).

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# Materials & methods

## Chemistry

Melting points (mp) (uncorrected) were obtained on a Buchi−Tottoli instrument. A Varian VXR 200 spectrometer was used to obtain $^1$H NMR data. Peak positions are provided in ppm (δ) downfield and J values in hertz. Mass spectra were obtained on a Waters ZQ 2000 electrospray ionization (ESI) single quadrupole mass spectrometer, with values given as $[M + 1]^+$. Purity (≥95%) was verified by combustion elemental analyses performed at the Microanalytical Laboratory of the Department of Chemistry and Pharmaceutical Sciences of the University of Ferrara using a Yanagimoto MT-5 CHN recorder elemental analyzer. Thin-layer chromatography was performed on glass plates from Merck coated with silica gel 60 F254, with compounds visualized by UV detection or with aqueous $KMnO_4$. Flash column chromatography was performed with 230−400-mesh silica gel and solvents as indicated. Organic solutions were dried over anhydrous $Na_2SO_4$. Commercial solvents and reagents were from Aldrich (Sigma-Aldrich) or Alfa Aesar (Johnson Matthey Company) and were used as supplied.

## General synthetic procedure of 4 a-l

A solution of 4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine-6-thiol (**7a**, 100 mg, 0.59 mmol) or 4-amino-1-methyl-pyrazolo[3,4-*d*]pyrimidine-6-thiol (**7b**, 0.59 mmol) and NaOH (23.2 mg, 1 eq) in EtOH/$H_2O$ 50% (4 mL) was added of the opportune alkyl halide (1.1 eq), and the mixture was stirred for 3 h at 90°C. Then, the solvent was removed under vacuum and the residue was purified by flash chromatography (EtOAc/ hexane 50% to EtOAc 100% to EtOAc/ MeOH 80%)

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**Characterization of compounds 4 a-l**

*Ethyl 2-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetate (4a)*

Off-white solid, 68% yield, m.p. 188-190°C. $^1$H NMR ($d_6$-DMSO): 1.18 (t, 3H, J=7); 3.96 (s, 2H); 4.12 (q, 2H, J=7); 7.85 (bs, 2H); 7.98 (s, 1H); 13.18 (bs, 1H). $[M+H]^+$ 254.15. Anal. calc for $C_9H_{11}N_5O_2S$: C, 42.68; H, 4.38; N, 27.65. Found: C, 42.66; H, 4.39; N, 27.64.

*Ethyl 3-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)propanoate (4b)*

White solid, 78% yield, m.p. 179-181°C. $^1$H NMR ($d_6$-DMSO): 1.18 (t, 3H, J=8); 2.75 (t, 2H, J=8); 3.24 (t, 2H, J=8); 4.08 (q, 2H, J=8); 7.84 (bs, 2H); 7.97 (s, 1H); 13.21 (bs, 1H). $[M+H]^+$ 267.89. Anal. calc for $C_{10}H_{13}N_5O_2S$: C, 44.93; H, 4.90; N, 26.20. Found: C, 44.95; H, 4.91; N, 26.18.

*2-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)ethanol (4c)*

Off-white solid, 67% yield, m.p. 230-232 °C with dec. $^1$H NMR ($d_6$-DMSO): 3.23 (t, 2H, J=6); 3.61, (t, 2H, J=6); 4.94 (bs, 1H); 7.63 (bs, 2H); 7.94 (s, 1H); 13.21 (bs, 1H). $[M+H]^+$ 212.11. Anal. calc for $C_7H_9N_5OS$: C, 39.80; H, 4.29; N, 33.15. Found: C, 39.81; H, 4.29; N, 33.16.

*Tert-butyl (2-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)ethyl)carbamate (4d)*

White solid, 71% yield, m.p. 214-216°C. $^1$H NMR ($d_6$-DMSO): 1.36 (s, 9H); 3.11 (t, 2H, J=6); 3.19 (t, 2H, J=6); 6.99, (bs, 1H); 7.84 (bs, 2H); 7.97 (s, 1H); 13.19 (bs, 1H). [M+H] 311.33. Anal. calc for $C_{12}H_{18}N_6O_2S$: C, 46.44; H, 5.85; N, 27.08. Found: C, 46.43; H, 5.87; N, 27.05.

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*6-((2-aminoethyl)thio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4e)*

Off-white solid, 64% yield, m.p. 204-206 °C with dec. $^1$H NMR ($d_6$ -DMSO): 3.16 (t, 2H, J=6); 3.37 (t, 2H, J=6); 3.88 (s, 3H); 8.09 (s, 1H); 8.3 (bs, 4H). $[M+H]^+$ 224.8. Anal. calc for $C_8H_{12}N_6S$: C, 42.84; H, 5.39; N, 37.47. Found: C, 42.85; H, 5.37; N, 37.44.

*6-(benzylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4f)*

White solid, 72% yield, m.p.>260°C; $^1$H NMR ($d_6$ -DMSO): 4.36 (s, 2H); 7.22-7.31 (m, 3H); 7.43-7.45 (m, 2H); 7.86 (bs, 2H); 7.98 (s,1H); 13.22 (bs, 1H). $[M+H]^+$ 258.32. Anal. calc for $C_{12}H_{11}N_5S$: C, 56.01; H, 4.31; N, 27.22. Found: C, 55.99; H, 4.30; N, 27.24.

*6-((4-methoxybenzyl)thio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4g)*

Light brown solid, 73% yield, m.p. > 260°C; $^1$H NMR ($d_6$ -DMSO): 3.71 (s, 3H); 4.3 (s, 2H); 6.85 (d, 2H, J=8); 7.35 (d, 2H, J=8); 7.86 (bs, 2H); 7.99 (s, 1H); 13.21 (bs, 1H). $[M+H]^+$ 288.23. Anal. calc for $C_{13}H_{13}N_5OS$: C, 54.34; H, 4.56; N, 24.37. Found: C, 54.35; H, 4.53; N, 24.38.

*6-(phenethylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4h)*

White solid, 71% yield, m.p. 209-211°C; $^1$H NMR ($d_6$ -DMSO): 2.98 (t, 2H, J=6); 3.24 (t, 2H, J=6); 7.09-7.18 (m, 5H); 7.85 (bs, 2H); 7.99 (s, 1H); 13.19 (bs, 1H). $[M+H]^+$ 272.23. Anal. calc for $C_{13}H_{13}N_5S$: C, 57.54; H, 4.83; N, 25.81. Found: C, 57.56; H, 4.83; N, 25.82.

*6-((3-phenylpropyl)thio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4i)*

Off-white solid, 81% yield, m.p. 195-197°C; $^1$H NMR ($d_6$ -DMSO): 1.94-1.98 (m, 2H); 2.71 (t, 2H, J=7); 3.07 (t, 2H, J=7); 7.17-7.28 (m, 5H); 7.81 (bs, 2H); 7.97 (s, 1H); 13.17 (bs, 1H). [M+H] 286.21. Anal. calc for $C_{14}H_{15}N_5S$: C, 58.92; H, 5.30; N, 24.54. Found: C, 58.91; H, 5.33; N, 24.55.

*4-(2-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)ethyl)-N-benzyl benzene sulphonamide (4j)*

White solid, 68% yield, 247-249 °C with dec. $^1$H NMR ($d_6$ -DMSO): 3.05 (t, 2H, J=6); 3.33 (t, 2H, J=6); 3.95 (d, 2H, J=6.4); 7.24-7.27 (m, 5H); 7.51 (d, 2H, J=8); 7.56 (bs, 2H); 7.75 (d, 2H, J=8); 7.98 (s, 1H); 8.1 (t, 1H, J=6.4); 13.19 (bs, 1H). $[M+H]^+$ 441.13. Anal. calc for $C_{20}H_{20}N_6O_2S_2$: C, 54.53; H, 4.58; N, 19.08. Found: C, 54.55; H, 4.57; N, 19.10.

*N-(2-((4-amino-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)ethyl)-4-methylbenzene sulphonamide (4k)*

Off-white solid, 65% yield, m.p. 242-244°C; $^1$H NMR ($d_6$ -DMSO): 2.33 (s, 3H); 3.18-3.56 (m, 5H); 7.24 (d, 2H, J=8); 7.59 (bs, 2H); 7.65 (d, 2H, J=8); 7.98 (s, 1H); 13.02 (bs, 1H). [M+H] 365.1. Anal. calc for $C_{14}H_{16}N_6O_2S_2$: C, 46.14; H, 4.43; N, 23.06. Found: C, 46.14; H, 4.45; N, 23.05.

*6-((3-(4-(benzyloxy)phenyl)propyl)thio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (4l)*

White solid, 67% yield, m.p. 194-196 °C. $^1$H NMR ($d_6$ -DMSO): 1.92-1.98 (m, 2H); 2.63-2.67 (m, 2H); 3.03-3.06 (m, 2H); 5.05 (s, 2H); 6.92 (d, 2H, J=8); 7.12 (d, 2H, J=8); 7.33-7.41 (m, 5H); 7.84 (bs, 2H); 7.97 (s, 1H); 13.18 (bs, 1H). $[M+H]^+$ 392.15. Anal. calc for $C_{21}H_{21}N_5OS$: C, 64.43; H, 5.41; N, 17.89. Found: C, 64.41; H, 5.42; N, 17.91.

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**General synthetic procedure of 4 m,n**

A solution of 5-amino-1*H*-pyrazole-4-carbonitrile (2g, 18.5 mmol) and dry $K_2CO_3$ (3.07 g, 1.2 eq) in dry DMF (15 mL) was added of ethyl 3-bromo-propanoate (2.83 mL, 1.2 eq) and the mixture was stirred at 110°C for 2h. Then, the solvent was removed under vacuum and the residue was suspended in water (15 mL) and extracted with EtOAc (10 mLx3). The organic layers were evaporated after drying (dry $Na_2SO_4$) and the dark oil was purified by flash chromatography (EtOAc/light petroleum 20% to 50%) to furnish the two isomers **9 a,b**.

To a solution of **9a or 9b** (200 mg, 0.9 mmol) in dry dioxane (10 mL), benzoylisothiocyanate (0.156 mL, 1.1 eq) was added and the mixture was stirred at 130°C for 5h in a sealed bomb. Then, the solvent was removed under vacuum and the residue was suspended in 1M NaOH (5 mL) and EtOH (5 mL) and refluxed for 35 min. At the end, the solvent was removed and the residue was suspended in EtOH and few drops of concentrated $H_2SO_4$ and refluxed for 8 hours. After removing solvent, water was added (5 mL), the pH was adjusted around 6-7 with 5% $NaHCO_3$, and the formed white precipitate was collected by filtration.

**Characterization of compounds 9 a,b and 4 m,n**

*Ethyl 3-(5-amino-4-cyano-1H-pyrazol-1-yl)propanoate (9a)*

White foam, yield 45%. $^1$H NMR ($CDCl_3$) 1.25 (t, 3H, J=8); 2.88 (t, 2H, J=6); 4.13-4.19 (m, 4H); 4.99 (bs, 2H); 7.49 (s, 1H). $[M+H]^+$ 209.33.

*Ethyl 3-(3-amino-4-cyano-1H-pyrazol-1-yl)propanoate (9b)*

Thick oil, yield 51%. $^1$H NMR ($CDCl_3$) 1.22 (t, 3H, J=8 Hz); 2.80 (t, 2H, J=6 Hz); 3.80 (bs, 2H); 4.10-4.21 (m, 4H); 7.57 (s, 1H). $[M+H]^+$ 209.14

*Ethyl 3-(4-amino-6-mercapto-1H-pyrazolo[3,4-d]pyrimidin-1-yl)propanoate (4m)*

White solid, 38% yield, m.p. 230 with dec; $^1$H NMR ($d_6$-DMSO) 1.18 (t, 3H, J=8); 2.80 (t, 2H, J=6); 4.01 (q, 2H, J=8); 4.38 (t, 2H, J=6); 7.37 (bs, 1H); 8.00 (s, 1H); 9.04 (bs, 1H); 11.85 (bs, 1H). $[M+H]^+$ 268.46. Anal. calc for $C_{10}H_{13}N_5O_2S$: C, 44.93; H, 4.90; N, 26.20. Found: C, 44.91; H, 4.90; N, 26.21.

*2-(4-amino-6-mercapto-2H-pyrazolo[3,4-d]pyrimidin-2-yl)ethyl propionate (4n)*

Off-white solid, 41% yield, m.p. 246°C with dec; $^1$H NMR ($d_6$-DMSO) 1.11 (t, 3H, J=8); 2.86 (t, 2H, J=6); 4.01 (q, 2H, J=8); 4.40 (t, 2H, J=6); 8.10 (bs, 2H); 8.17 8s, 1H); 12.39 (bs, 1H). $[M+H]^+$ 268.4. Anal. calc for $C_{10}H_{13}N_5O_2S$: C, 44.93; H, 4.90; N, 26.20. Found: C, 44.94; H, 4.89; N, 26.19.

**General synthetic procedure of 5 a-j**

A solution of 4-amino-2-thiopyrimidine-5-carbonitrile **10**, ethyl-4-amino-2-thiopyrimidine-5-carboxylate **11**, 4,6-diaminopyrimidine-2-thiol **12**, 6-amino-2-mercaptopyrimidin-4-ol **13** (0.65 mmol) and NaOH (1 eq) in EtOH/$H_2O$ 50% (4 mL) was added of the opportune alkyl halide (1.1 eq) after complete dissolution. The mixture was stirred for 3 h at 90°C, then the solvent was removed under vacuum and the residue was suspended in water (5 mL) and the pH was adjusted

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around 5 with glacial $CH_3COOH$. The collected precipitate was purified by crystallization (EtOAc) or flash chromatography (EtOAc/ hexane 50% to EtOAc 100% to EtOAc/ MeOH 80%)

**Characterization of compounds 5 a-j**

*Ethyl 3-((4-amino-5-cyanopyrimidin-2-yl)thio)propanoate (5a)*

Light yellow solid, 65% yield, m.p. 125-126 °C. $^1$H NMR ($d_6$-DMSO) 1.18 (t, 3H, J=8); 2.73 (t, 2H, J=6); 3.23 (t, 2H, J=6); 4.07 (q, 2H, J=6); 8.01 (bs, 2H); 8.45 (s, 1H). $[M+H]^+$ 253.16. Anal. calc for $C_{10}H_{12}N_4O_2S$: C, 47.61; H, 4.79; N, 22.21. Found: C, 47.62; H, 4.81; N, 22.21.

*4-(2-((4-amino-5-cyanopyrimidin-2-yl)thio)ethyl)-N-benzylbenzenesulfonamide (5b)*

Off-white solid, 58% yield, m.p. 121-123°C; $^1$H NMR ($d_6$-DMSO) 3.17 (t, 2H, J=6); 3.21 (t, 2H, J=6); 3.96 (d, 2H, J=6); 7.21-7.27 (m, 3H); 7.51-7.54 (m, 2H); 7.71-7.90 (m, 6H); 8.12 (t, 1H, J=6); 8.46 (s, 1H). $[M+H]^+$ 426.16. Anal. calc for $C_{20}H_{19}N_5O_2S_2$: C, 56.45; H, 4.50; N, 16.46. Found: C, 56.42; H, 4.50; N, 16.47.

*4-amino-2-((3-(4-(benzyloxy)phenyl)propyl)thio)pyrimidine-5-carbonitrile (5c)*

Off-white solid, 64% yield, m.p. 168-170°C; $^1$H NMR ($CDCl_3$) 1.96-2.02 (m, 2H); 2.69 (t, 2H, J=8); 3.07 (t, 2H, J=8); 5.05 (s, 2H); 5.40 (bs, 2H); 6.89 (d, 2H, J=8); 7.11 (d, 2H, J=8); 7.34-7.42 (m, 5H); 8.28 (s, 1H). $[M+H]^+$ 377.12. Anal. calc for $C_{21}H_{20}N_4OS$: C, 67.00; H, 5.35; N, 14.88. Found: C, 66.98; H, 5.34; N, 14.89.

*Ethyl 4-amino-2-((3-ethoxy-3-oxopropyl)thio)pyrimidine-5-carboxylate (5d)*

Pale yellow solid, 71% yield, m.p. 89-91°C; $^1$H NMR ($CDCl_3$) 1.39 (t, 3H, J=8); 2.79 (t, 2H, J=8); 3.38, 2H, J=8); 4.36 (q, 2H, J=8); 5.98 (bs 1H); 8.09 (bs, 1H); 8.72 (s,1H). $[M+H]^+$ 300.39. Anal. calc for $C_{12}H_{17}N_3O_4S$: C, 48.15; H, 5.72; N, 14.04. Found: C, 48.16; H, 5.74; N, 14.05.

*Ethyl 4-amino-2-((4-(N-benzylsulfamoyl)phenethyl)thio)pyrimidine-5-carboxylate (5e)*

Off-white solid, 53% yield, m.p. 158-160 °C. $^1$H NMR ($CDCl_3$) 1.37 (t, 3H, J=8); 3.06-3.09 (m, 2H); 3.31-3.36 (m, 2H); 4.12 (d, 2H, J=6); 4.33 (q, 2H, J=8); 5.78 (bs, 2H); 7.20-7.24 (m, 4H); 7.27-7.35 (m, 2H); 7.69-7.89 (m, 4H); 8.70 (s, 1H). $[M+H]^+$ 470.34. Anal. calc for $C_{22}H_{24}N_4O_4S_2$: C, 55.91; H, 5.12; N, 11.86. Found: C, 55.89; H, 5.14; N, 11.85.

*Ethyl 4-amino-2-((3-(4-(benzyloxy)phenyl)propyl)thio)pyrimidine-5-carboxylate (5f)*

Off-white solid, 65% yield, m.p. 118-120°C; $^1$H NMR ($CDCl_3$) 1.37 (t, 3H, J=8); 1.99-2.06 (m, 2H); 2.70 (t, 2H, J=8); 3.1 (t, 2H, J=8); 5.04 (s, 2H); 5.61 (bs, 1H); 6.90 (d, 2H, J=8); 7.11 (d, 2H, J=8); 7.34- 7.42 (m, 5H); 7.83 (bs, 1H); 8.68 (s, 1H). $[M+H]^+$ 424.38. Anal. calc for $C_{23}H_{25}N_3O_3S$: C, 65.23; H, 5.95; N, 9.92. Found: C, 65.25; H, 5.94; N, 9.93.

*Ethyl 3-((4,6-diaminopyrimidin-2-yl)thio)propanoate (5g)*

Off-white solid, 58% yield, m.p. 108°C; $^1$H NMR ($d_6$-DMSO) 1.17 (t, 3H, J=8); 2.69 (t, 2H, J=6); 3.11 (t, 2H, J=6); 4.07 (q, 2H, J=8); 5.13 (s, 1H); 6.09 (bs, 4H). $[M+H]^+$ 243.16.(commercially available)

*N-benzyl-4-(2-((4,6-diaminopyrimidin-2-yl)thio)ethyl)benzenesulfonamide (5h)*

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White solid, 62% yield, m.p. 146-148°C; $^1$H NMR ($d_6$-DMSO): 2.95-3.01 (m, 2H); 3.13-3.19 (m, 2H); 3.95 (d, 2H, J=6); 5.16 (s,1H); 6.17 (bs, 4H); 7.23-7.25 (m, 3H); 7.50 (d, 2H, J=8); 7.72 (d, 2H, J=8); 7.87-7.91 (m, 2H); 8.10 (t, 1H, J=6). $[M+H]^+$ 416.35. Anal. calc for $C_{19}H_{21}N_5O_2S$: C, 54.92; H, 5.09; N, 16.85. Found: C, 54.91; H, 5.11; N, 16.87.

*2-((3-(4-(benzyloxy)phenyl)propyl)thio)pyrimidine-4,6-diamine (5i)*

White solid, 54% yield, m.p. 128-130°C; $^1$H NMR ($CDCl_3$): 1.95-2.02 (m,2H); 2.69 (t, 2H, J=8); 3.04 (t, 2H, J=8); 4.51 (bs, 4H); 5.04 (s, 2H); 5.21 (s, 1H); 6.89 (d, 2H, J=8); 7.12 (d, 2H, J=8); 7.34-7.42 (m, 5H). $[M+H]^+$ 366.83. Anal. calc for $C_{20}H_{22}N_4OS$: C, 65.55; H, 6.05; N, 15.29. Found: C, 65.53; H, 6.06; N, 15.31.

*Ethyl 3-((4-amino-6-hydroxypyrimidin-2-yl)thio)propanoate (5j)*

White solid. 85% yield: $^1$H NMR ($d_6$-DMSO): 1.18 (t, 3H, J=8); 2.73 (t, 2H, J=8); 3.21 (t, 2H, J=8); 4.08 (q, 2H, J=8); 4.93 (s, 1H); 6.46 (bs, 2H); 11.41 (bs, 1H). $[M+H]^+$ 244.11 (commercially available).

**Preparation of 6**

Ethyl 3-bromopropanoate (83 µL, 1.1 eq) was added to a solution of 6-amino-9*H*-purine-2-thiol (**8**, 100 mg, 0.59 mmol) in dry DMF (1 mL) and 0.1M ethanolic NaOH (5.9 mL, 1 eq), and the mixture stirred at 100°C for 18 h. Then the pH was adjusted around 5 with glacial $CH_3COOH$ and the residue was purified by flash chromatography (EtOAc/ hexane 50% to EtOAc/MeOH 80%) after removing the solvent under reduced pressure.

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## Characterization of 6

*Ethyl 3-((6-amino-9H-purin-2-yl)thio)propanoate (6)*

Light yellow solid, 57% yield, m.p. 212-214°C with dec; $^1$H NMR ($d_6$-DMSO) 1.18 (t, 3H, J=8); 2.69 (t, 2H, J=6); 3.12 (t, 2H, J=6); 4.07 (q, 2H, J=8); 6.05 (bs, 2H); 8.05 (s,1H); 8.66 (bs, 1H). $[M+H]^+$ 268.12. Anal. calc for $C_{10}H_{13}N_5O_2S$: C, 44.93; H, 4.90; N, 26.20. Found: C, 44.94; H, 4.88; N, 26.21.

## Biology

### *Preparation of compounds*

All the compounds were prepared at a starting concentration of 5 mM in acetone with the exception of compounds **4m** and **4n**, which were dissolved in 0.1M NaOH. For the determination of IC50, the starting solutions were serially diluted, with a factor of 2, in the same solvent giving 8 further solutions with a range of starting concentrations 5-0.019 mM.

### *Cell lines and culture conditions*

The U937 histiocytic human cell line (ATCC® CRL-1593.2) was routinely cultured in complete medium composed by RPMI 1640 supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS, Microgem) at 37°C in a humidified atmosphere with 5% $CO_2$. For their use in viability assays, the cells were collected, centrifuged 5 minutes at 400g and suspended in complete medium at a cell density of 300,000 cells/ml.

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Peripheral blood mononuclear cells (PBMCs) were purified from leukocytes enriched buffy coats collected from the Blood Bank of S. Anna Hospital, Ferrara. All the data were analyzed anonymously, and the authors did not have any sensitive information about the participants. Since the buffy coats were considered as discard products of the erythrocyte isolation procedure, no ethical committee approval was needed. The cells were isolated by gradient centrifugation on Ficoll-Paque Plus (GE Healthcare, Cat. No. 17144002) as previously described with some modifications [9]. Briefly, buffy coats (around 35 ml) were diluted two times with sterile-filtered PBS (phosphate buffered saline, 150 mM NaCl, 10 mM $NaH_2PO_4$, pH 7.4), gently stratified on a Ficoll-Paque Plus cushion and subjected to centrifugation at room temperature for 15 minutes and 2200 rpm. At the end of the centrifugation, the white ring at the interface between plasma and Ficoll was collected, centrifuged at room temperature for 10 minutes at 1000 rpm and contaminating erythrocytes were removed by hypotonic lysis. The cells were then suspended in freezing medium consisting in FBS and 10% DMSO and kept under liquid nitrogen until used for the assay.

Before their use for viability assays, PBMCs were thawed and suspended in complete medium ($1.4*10^6$ cells/ml) for 24 hours. In order to promote their replication, the cells were treated with 1µg/ml phytoemoagglutinin-L (PHA-L, Sigma-Aldrich, Cat. No. L2769) for 3 days at 37°C, 5% $CO_2$. At the end of the incubation, the cells were harvested, centrifuged 5 minutes at 400g, washed 2 times with 5 ml PBS and suspended in complete medium at a cell density of $1*10^6$ cells/ml and used in viability assays.

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NIH-3T3 embryonic murine fibroblasts were routinely cultivated in DMEM high glucose (4.5 g/L) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FBS at 37°C in a humidified atmosphere with 5% $CO_2$. They were seeded at a cell density of 5000 cells/cm$^2$ until 80% confluence was reached. The cells were then detached from plastic by trypsin treatment, suspended in complete medium and used for viability assays at a cell density of $1*10^6$ cells/ml.

***Viability assays***

The general protocol used to test the effect of compounds on cell viability was as follows, with minor changes to the number of cells seeded per well depending on the cell line tested.

Briefly, 30,000 cells/well (100,000 cells/well for PBMCs, 20,000 cells/well for NIH-3T3) were seeded in sterile 96-well flat-bottomed plates (NuncIon Sterile Microplates, Thermo Fisher, Cat. No. 167008) and treated with different concentrations of compounds within the range 240-0.9 µM for 48 hours, or 72 hours for compounds **4l**, **5c, 5f**, **5i**. Cells treated with vehicle (acetone) at the same final concentration reached in treatment (4.7%) were used as negative control and treated for the exact same time. At the end of the incubation, 10 µl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, Cat. No. M5655) dissolved in water were dispensed in all wells and the plates were incubated for 3 hours (4 hours in the case of PMBCs) at 37°C and 5% $CO_2$. The reaction was stopped by adding 100 µl of stop solution (10% SDS in 0.01M HCl) each well and incubating overnight at 37°C. The absorbance of the purple colour was read at 570 nm (reference 690 nm) with a microplate reader (Tecan Infinte M200, Tecan, Switzerland). All tests were performed in triplicate, and the data obtained from at least 3 independent

experiments were used to calculate IC50 values with 95% confidence intervals (CI) by 4 parameters non-linear curve fitting using Graphpad Prism v7 software. In this case, the IC50 corresponded to the concentration of compound that inhibited of 50% the growth of cells.

The experiments on normal cell lines (PBMCs and NIH-3T3) were performed only on the most active compounds, selected based on a value of IC50 < 20 µM. Accordingly, the values of IC50 were used to calculate the Selectivity Index (SI) as the ratio: IC50 non-tumor cell line/IC50 tumor cell line [10].

***LDH assay for cytotoxicity***

The cytotoxicity of the compounds was evaluated by measuring the release of the enzyme lactate dehydrogenase (LDH) from cells as previously published [11]. Briefly, 30,000 cells (U937)/well suspended in complete medium were seeded in sterile 96-well flat-bottomed plates and incubated with the compounds at their IC50 for 48 or 72 hours at 37°C and 5% $CO_2$. Cells treated with acetone were used as control. At the end of the incubation, the plates were centrifuged for 10 minutes at 2,000 rpm and 50 µl of conditioned medium were transferred into a new 96-well flat bottomed plate (Greiner Bio-One, Cat. No. 655101). Fifty microliters of complete medium were used as reaction blank. Then, 150 µl of reaction mixture consisting in 67 mM Tris-HCl, pH 8, 17 mM Litium Lactate, 0.87 mM INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride, Sigma-Aldrich, Cat. No. I8377), 0.39 PMS (N-methylphenazonium methyl sulfate, Sigma-Aldrich, Cat. No. P9625), 1.67 mM $NAD^+$ (Sigma-Aldrich, Cat. No. N0632) were dispensed in all wells and the absorbance was read with a microplate reader at 490 nm every 30 seconds for 5 minutes at 37°C. The activity of LDH was calculated as the slope of absorbance in

function of time and the results were normalized by untreated control, which represented the 0% of release after correcting for blank reaction.

***Apoptosis evaluation through flow cytometry***

The pro-apoptotic effect of the most active compounds, selected based on a value of IC50 < 20 µM, was evaluated by flow cytometry on a BD FACS Canto II (BD Bioscience) using the Annexin V/Propidium Iodide (PI) kit from Miltenyi Biotec (Cat. No. 130-092-052) by following the manufacturer instructions. Briefly, the cells (600,000) were incubated for 48 or 72 hours with the compounds at a concentration near the theoretical value of IC25 (8 µM for compound **4b**, 6 µM for compound **4j** and 4 µM for compound **4l**) or with vehicle (acetone) as negative control. As a positive control the cells were exposed for 24 hours with 1 µM etoposide (Sigma-Aldrich, Cat. No. E2600000). At the end of the incubations, the cells were centrifuged 10 minutes at 300g, and washed with the Binding Buffer included in the kit. Then, they were suspended in the same buffer and incubated for 15 minutes in the dark at room temperature with Annexin V-FITC, centrifuged 10 minutes at 300g and washed once with Binding Buffer. Finally, they were suspended in Binding Buffer and, shortly before analysis, PI (final concentration 0.1 mg/ml) was added to all cell suspensions. A total of 20,000 events were acquired by the instrument per sample and the data were elaborated with the software FlowJo VX (BD Biosciences).

***Cell-cycle analysis through flow cytometry***

Basic information about cell-cycle were obtained through the PI staining of cells and analysis with the BD FACS Canto II. Briefly, $2*10^6$ U937cells (density 300,000 cells/ml) were suspended in complete medium and treated for 48 or 72 hours with the most active compounds at their respective

IC50 concentration. Vehicle (acetone) was used as negative control. At the end of incubation, the cells were centrifuged for 5 minutes at 400g, washed once with cold PBS and centrifuged again for 5 minutes at 400g and 4°C. The pellets were suspended in 1 ml of cold PBS and fixed with 9 ml of 70% cold ethanol and incubated at least for 24 hours at 4°C. After the fixation step, the cells were centrifuged 10 minutes at 200g and 4°C, washed with cold PBS and suspended in staining solution (20 µg/ml PI, 0.1% Triton X-100, 200 µg/ml RNAase A DNAase free in PBS). The suspensions were incubated for 15 minutes at 37°C in the dark and then kept at 4°C in the dark until analysis with flow cytometer. For each sample at least 50,000 events were recorded. FACS data were analysed with the software FlowJo VX (BD Biosciences) in order to determine the percentage of cells in each phase (G1, S and G2/M).



## Results and Discussion

### Chemistry

The synthesis of the tested compounds is reported in Scheme 1 and 2.

**Scheme 1 and Scheme 2 to be inserted here**

The compounds **4a-l** were easily synthesized starting from the commercially available 4-amino-1H-pyrazolo[3,4-d]pyrimidine-6-thiol (**7a**) or 4-amino-1-methyl-pyrazolo[3,4-d]pyrimidine-6-thiol (**7b** for the synthesis of compound **4e**) through alkylation under basic conditions for sodium hydroxide in a mixture of 50% EtOH /water, at 90°C for 3 hours, with the opportune alkyl or arylalkyl halide, usually commercially available or known in the literature. The yields are variable but generally high around 60-80%.

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Starting from the commercially available 6-amino-9H-purine-2-thiol **8**, through alkylation with ethyl 3-bromopropanoate under the same reaction conditions, compound **6** was obtained in a good yield (around 60%).

Compounds **4m,n** were obtained through reaction with benzoylisothiocyanate and subsequent cyclization in the presence of aqueous 10% NaOH at reflux of ethyl 3-(5-amino-4-cyano-1H-pyrazol-1-yl)propanoate (**9a**) and ethyl 3-(3-amino-4-cyano-1H-pyrazol-1-yl)propanoate (**9b**), respectively. The substituted amino nitrile pyrazoles **9a,b** were obtained through alkylation of 5-amino-1H-pyrazole-4-carbonitrile with ethyl 3-bromo-propanoate in the presence of anhydrous potassium carbonate in DMF. The correct structure of the two isomers was attributed with NOE experiments as reported in our previous work for similar pyrazole derivatives [12].

The same synthetic strategy was applied to obtain the derivatives **5a-j** starting from the opportune commercially available substituted pyrimidines analogs **10-13**. Each compound was alkylated in the presence of NaOH in EtOH/$H_2O$ 50%, at 95°C for 3 hours, and opportune halide, to furnish respectively **5a-c**, **5d-f**, **5g-i**, **5j** in a good yield (roughly 50-80%) [13].

**Structure-biological activity relationship**

As summarized in Table 1 and Table 2, some of the synthesized compounds showed a quite good anti-proliferative activity on U937 in low micromolar range (around 8-25 μM), whereas others were almost inactive.

**Table 1 and Table 2 to be inserted here**

From our data, we can hypothesize that the presence of 4-amino-1H-pyrazolo[3,4-d]pyrimidine core has great importance for the anti-proliferative activity of compounds (Table 1). Indeed,

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simplified pyrimidine (series **5a-j**) containing the same substituents on the thiol function of the most active compounds of the bicyclic series (**4b**, **4j** and **4l**), lose their anti-proliferative activity. The only exception is represented by the compounds **5b**, **5g** and **5h**, which retained a rather low activity (usually above 50 μM), almost 3 to 6 times lower than the most active compounds (Table 2).

Surprisingly, compound **6**, which is structurally related with **4b**, completely lose its activity despite the purine core, historically employed in antiproliferative agents.

In addition to the presence of the bicyclic core, with pyrazole and not imidazole fused with the pyrimidine cycle, the position of the substituent on the pyrazolo[3,4-d]pyrimidine core is also of paramount importance. Indeed, compounds like **4m** and **4n** which bear the same substituent of **4b** on the N1 or N2 pyrazole nitrogen, respectively, did not display any activity. Finally, we tested the compound **7** to confirm that the possible activity was not simply due to the presence of the thiol group in the precursor. As reported in Table 1, compound **7a** did not show any significant activity confirming the need of a substituent on the thiol moiety to demonstrate some biological activity.

Although our preliminary results seem encouraging and can be considered instrumental to the further optimization of novel compounds with anti-proliferative activity, definite considerations on the relationships between structure and biological effects are complicated. Indeed, the chemico-physical characteristics of substituents in the most active derivatives **4b**, **4h**, **4j** and **4l** are very different. Nonetheless, it is undoubted that the presence of a functionalized alkyl chain bearing hydrophilic moieties such as amino or hydroxy groups, is detrimental for the activity (**4c, 4e**). On

the contrary, long alkylaryl chains, substituted with hydrophobic groups at least after two carbon units seem well tolerated (**4h, 4i, 4j, 4l**). A simple benzyl group, even substituted, on the thiol function generates non-active compounds (**4f, 4g**). These results are summarized in Figure 3.

**Figure 3 to be inserted here**

**Biological mechanism of cytotoxicity**

The most active compounds, **4b**, **4j** and **4l**, which demonstrated an IC50 lower than 20 μM, were further tested to better characterize their possible mechanism of action. In particular, we evaluated: 1) the selectivity of the compounds towards cancer cells; 2) the possible mechanism of cell death triggered by the compound; 3) possible interference with DNA replication.

As summarized in Table 3, only the compounds **4j** demonstrated a quite non-specific effect against non-tumor cells since its IC50 was almost similar between the three cell types, and the SI was almost near 1 or below it (0.2 and 2.8 for PBMC and NIH3T3, respectively). On the contrary, both compounds **4b** and **4l** showed a good selectivity for cancer cells as demonstrated by a value of SI near, equal or greater than 10 (Table 3), with compound **4l** presenting a very high selectivity (SI above 40).

**Table 3 to be inserted here**

Then, the compounds were tested for the possible triggering of apoptosis (representative data are presented in Supplementary Figure 1). According to our results, all the compounds triggered apoptosis with significant fractions of cells undergoing both early and late apoptosis (Figure 4-A). These data are in agreement with the observation that LDH is not significantly increased in the medium of cells treated with **4b**, **4j** and **4l** at their IC50 concentration (Figure 4-B).

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**Figure 4 to be inserted here**

Since pyrazolo[3,4-d]pyrimidines derivatives demonstrated an inhibitor activity on CDKs, proteins involved in the control of cell cycle progression [14], we tested the possible influence of **4b**, **4j** and **4l** on the cell cycle. In the Supplementary Figure 2 are reported representative results. As outlined in Figure 5, we did not find any influence of the compounds on cell cycle. In fact, the percentage of cells in each phase was not significantly impaired when compared with control (acetone). Of note, Etoposide, a known topoisomerase II inhibitor, caused an accumulation of cells in the G2-M phase as documented in literature [15,16] thus confirming the goodness of our assay.

**Figure 5 to be inserted here**

Although we cannot exclude that our novel compounds may be inhibitors of other kinases such as Src or EGFR tyrosine kinases as documented in literature [4,5], we cannot either rule out that other proteins or pathways may be targeted. Indeed, there is a great variety of biological activities reported in the literature, attributed to pyrazolo[3,4-d]pyrimidine derivatives [17,18]. Therefore, we cannot preclude that other pathways connected with cell replication, such as the mTOR pathway [19] may be inhibited by our compounds. Thus, further studies are needed to verify the exact molecular target.

## Conclusion and future perspectives

Various series of novel 4-amino-1H-pyrazolo[3,4-d]pyrimidine-6-thiol derivatives with different substituents were synthesized. By a molecular simplification approach and by varying the substituents on the bicyclic core we determined that an intact pyrazolo[3,4-d]pyrimidine core is

essential for the anti-proliferative activity. Moreover, alkyl and alkylaryl chains specifically attached to the 6-thio position seem to be preferred for a sustained biological activity. Among the presented compounds, only three (**4b**, **4j** and **4l**) demonstrated a value of IC50 less than 20 μM. However, only two (**4b** and **4l**) were specific against cancer cells, with **4l** presenting a highly significant SI.

Considering our results, we can conclude that the pyrazolo[3,4-d]pyrimidine **4b** and **4l**, given their specificity, could be good starting point scaffolds for the design and discovery of novel agents with anti-proliferative activity and better solubility to employ in cancer research.



**Financial & competing interests disclosure**

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- **Future Perspective:** (a speculative viewpoint on how the field will evolve in 5–10 years' time)

Considering the increasing rate of cancers in the human population due to both environmental and genetic factors, there will be the need of novel drugs and strategies to overcome the replicative advantage of cancer cells. Purine nucleoside analogs, despite their first use dates back to 1980s, are still the first line of defense for aggressive cancers. Nonetheless, there is still the need of chemical compounds demonstrating the ability to specifically kill cancer cells. In this view, we can speculate that in next decades novel compounds with different mechanisms of action on cancer cells, increased specificity towards them and less side effects, might be discovered and used as adjuvants or first line of treatment for cancers.

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- **Summary Points (Research articles & Company profiles only):** 8–10 bullet point sentences highlighting the key points of the article.

  - Thio Substituted Derivatives of 4-Amino-pyrazolo[3,4-d]pyrimidine-6-thiol demonstrate an antiproliferative action.
  - They trigger apoptosis without interfering with the cell cycle.
  - Some compounds show a high specificity towards cancer cells compared to normal human or murine cells, with a mid to low micromolar activity.
  - The pyrazole ring is essential for a low micromolar antiproliferative activity.
  - The 6-thiol position in the compound needs to be substituted to show some effect on cancer cells.
  - The 6-thiol position is the main spot for substitution.
  - The preferred substituents are long hydrophobic chains, with an aryl ring, with at least two carbon units before a hydrophobic group.
  - If alkyl chains are used, they should not bear hydrophilic moieties such as amino or hydroxy groups.

- **Figure/Table legends**

## Figure Legends

**Figure 1**. Few examples of known biologically active pyrazolo[3,4-d]pyrimidine derivatives.

**Figure 2**. General structures of new synthesized compounds **4a-n**, **5a-j**, **6**

**Figure 3.** Summary of the main structural requirements of 4-Amino-pyrazolo[3,4-d]pyrimidine-6-thiol derivatives important for their biological activity.

**Figure 4**. Effects of the tested compounds on apoptosis (panel A) and LDH release (panel B).

**Figure 5.** Effects of the tested compounds on cell cycle evaluated with propidium iodide staining and flow cytometry.

**Scheme 1**. Synthesis of bicyclic derivatives **4 a-l** and **6**.

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**Scheme 2**. Synthesis of monocyclic derivatives **5a-j**.

## Table Legends

**Table 1.** *In vitro* antiproliferative activity of final compounds **4 a-n**, **6**, **7** against U937 cancer cells.

**Table 2.** *In vitro* antiproliferative activity of monocyclic final compounds **5a-j** on U937 cancer cells.

**Table 3.** Values of IC50 for **4b**, **4j** and **4l** compounds determined in U937 (cancer cells), PBMC (human normal cells) or NIH-3T3 (murine fibroblasts).



- **References:** (visit our for authors page for formatting requirements and to download our citation style files)

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- **Reference annotations**: authors should highlight 6–8 references that are of particular significance to the subject under discussion as **"* of interest"** or **"** of considerable interest"**, and provide a brief (1–2 line) synopsis.

1. Savić D, Stanković T, Lavrnja I, *et al.* Purine nucleoside analogs in the therapy of cancer and neuroinflammation. *Mol. Inhib. Target. Ther.* 1(1), 3–14 (2015).*

Interesting review showing the use of purine nucleoside analogs as antiproliferative and antinflammatory agents.

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Article showing a possible mechanistic action of Pyrazolo[3,4-d]pyrimidine derivatives.

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Interesting report showing the action of Pyrazolopyrimidines as EGFR/ErbB2 inhibitors.

17. Schenone S, Radi M, Musumeci F, Brullo C, Botta M. Biologically driven synthesis of pyrazolo[3,4-d]pyrimidines as protein kinase inhibitors: an old scaffold as a new tool for medicinal chemistry and chemical biology studies. *Chem. Rev.* 114(14), 7189–238 (2014). *

This article shows the effect of some pyrazole analogs on cell cycle, compounds that share similaryties with some of derivatives we used.

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Imporant article showing strategies to synthesize pyrazolo[3,4-d]pyrimidine derivatives and some biological actions of such derivatives.

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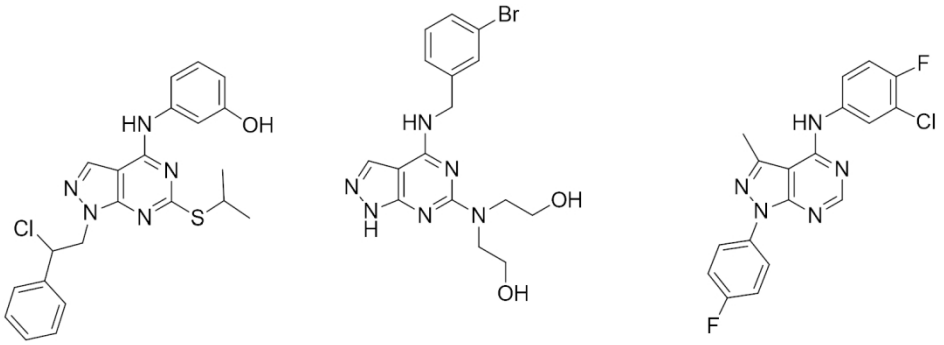
This work shows a novel pathway, PI3K/mTOR, that can be targeted Pyrazolopyrimidine derivatives.




**1**
Src kinase inhibitor
Ki 0.01μM

**2**
CDK2 inhibitor
$IC_{50}$ 3.8 μM

**3**
EGFR tyrosine kinases inhibitor
$IC_{50}$ 0.186 μM

Figure 1. Few examples of known biologically active pyrazolo[3,4-d]pyrimidine derivatives.

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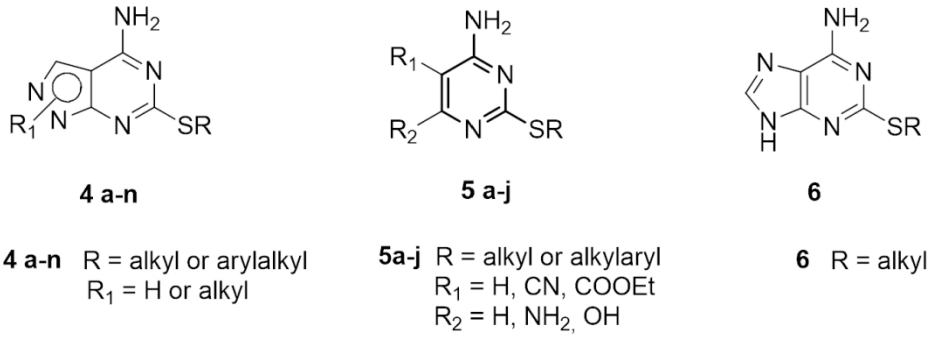



Figure 2. General structures of new synthesized compounds 4a-n, 5a-j, 6

122x46mm (300 x 300 DPI)

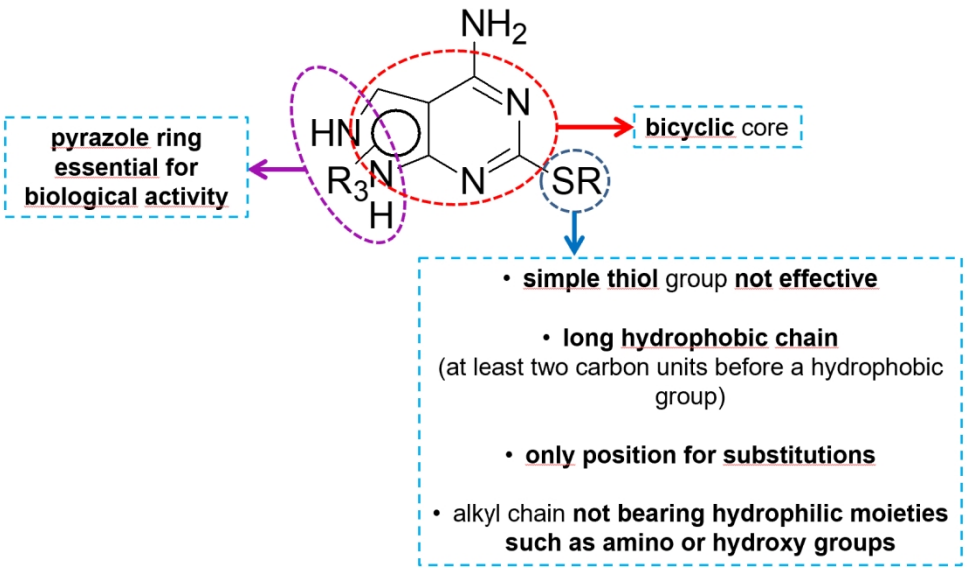



Figure 3. Summary of the main structural requirements of 4-Amino-pyrazolo[3,4-d]pyrimidine-6-thiol derivatives important for their biological activity.

148x90mm (300 x 300 DPI)

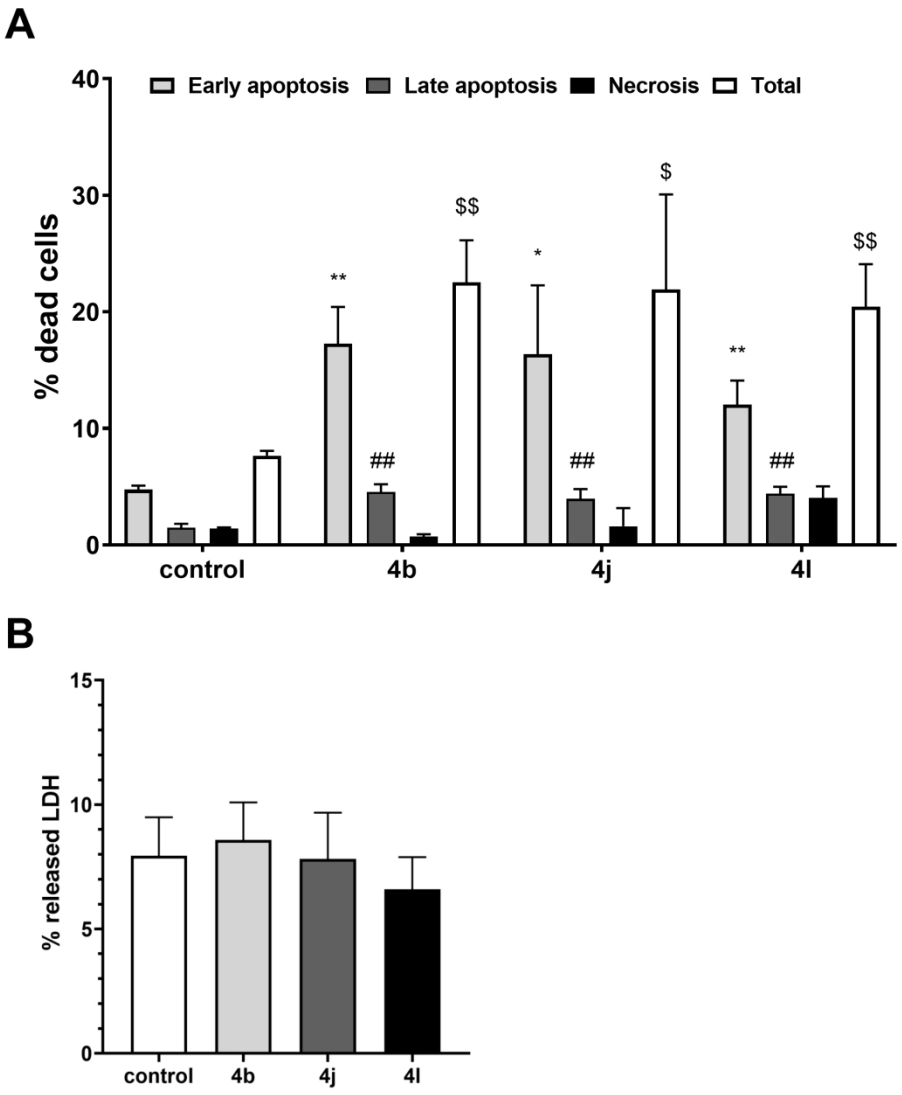



Figure 4. Effects of the tested compounds on apoptosis (panel A) and LDH release (panel B).

201x230mm (300 x 300 DPI)

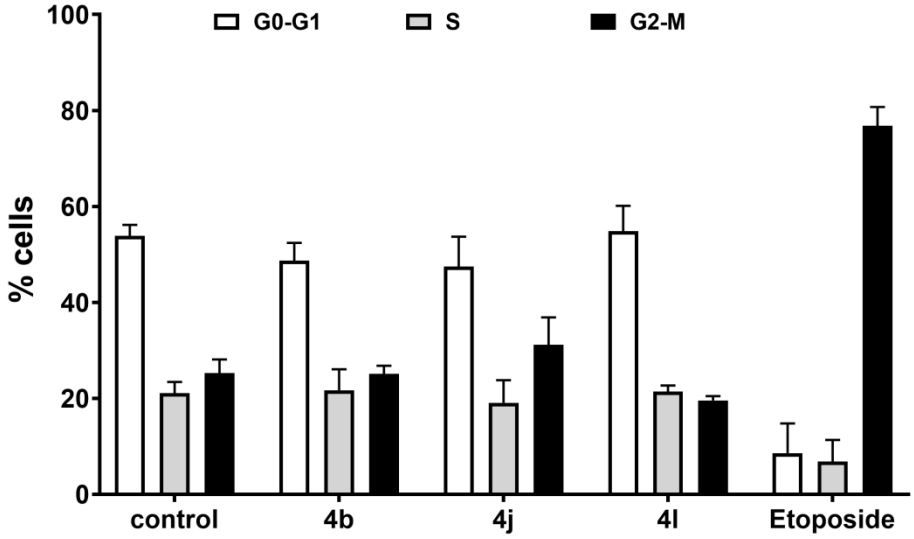
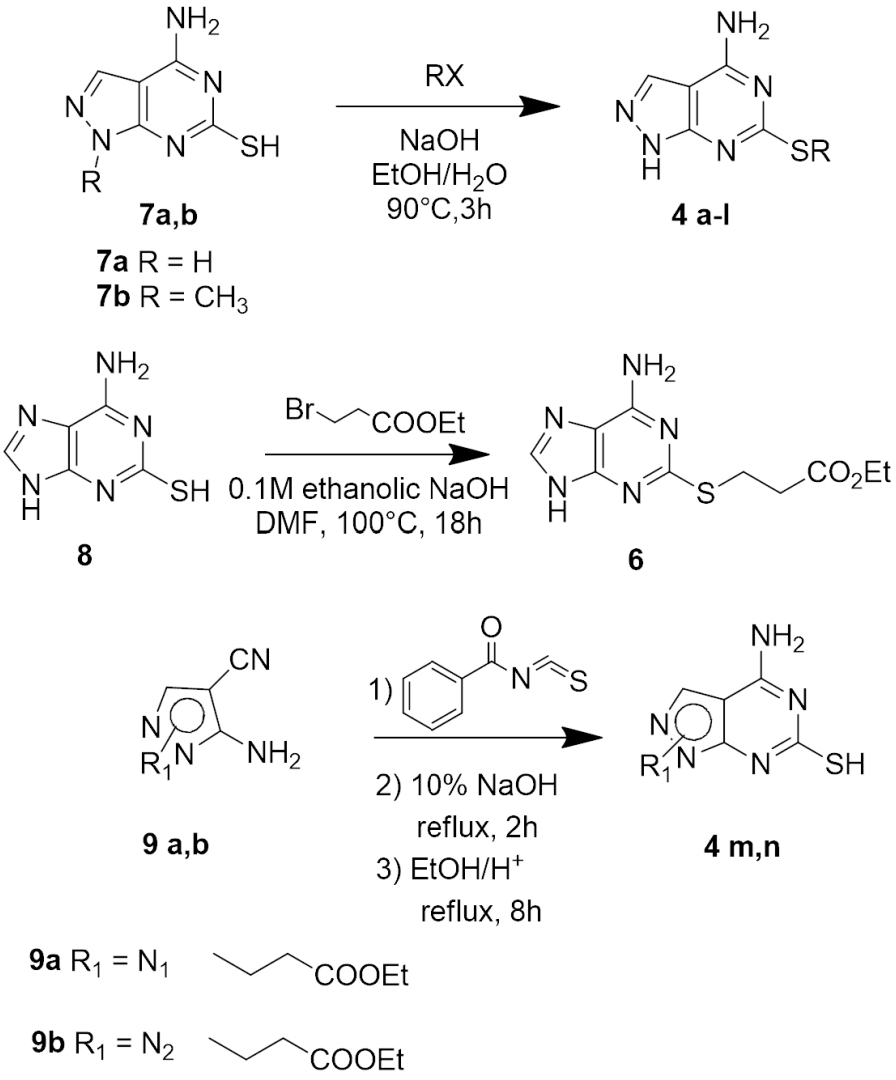


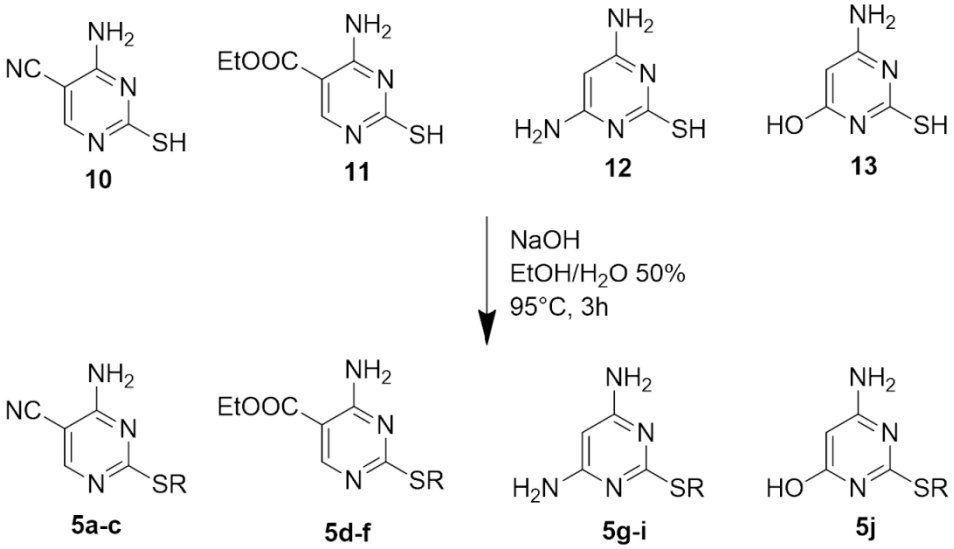

Figure 5. Effects of the tested compounds on cell cycle evaluated with propidium iodide staining and flow cytometry.

215x126mm (300 x 300 DPI)



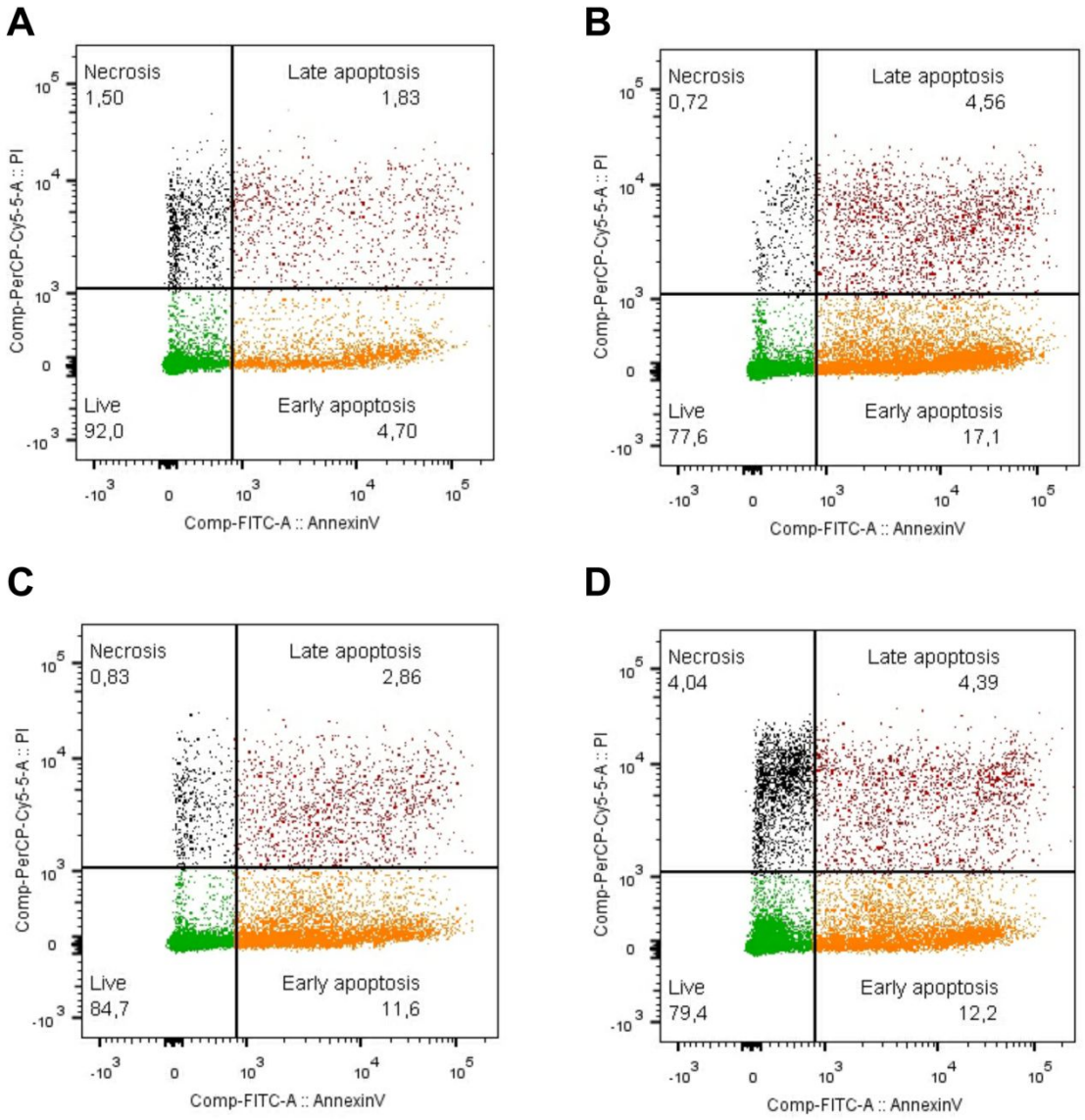

Scheme 1. Synthesis of bicyclic derivatives 4 a-l and 6.

104x117mm (300 x 300 DPI)



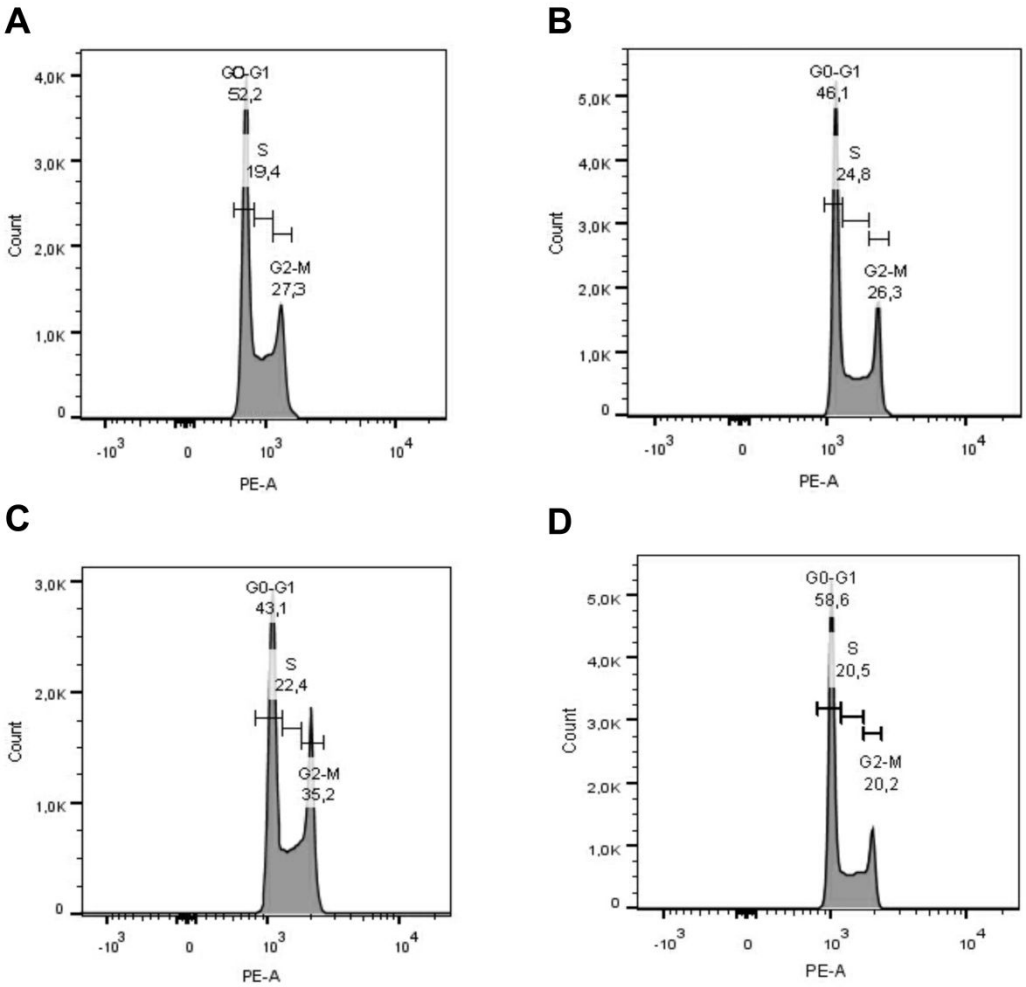

Scheme 2. Synthesis of monocyclic derivatives 5a-j.

121x69mm (300 x 300 DPI)



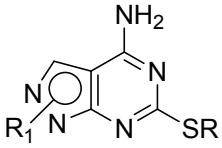

**Supplementary Figure 1**. Representative data of apoptosis analysis with Annexin V and flow cytometry.

A: negative control (acetone); B: compound **4b** at IC25 (8 µM) concentration; C: compound **4j** at IC25 (6 µM) concentration; D: compound **4l** at IC25 (4 µM) concentration. Cells were treated for 48 hours in the case of compounds **4b** and **4j**, and 72 hours for compound **4l**.

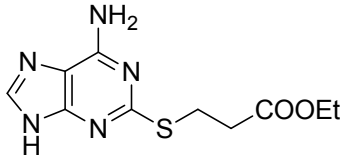



**Supplementary Figure 2**. Representative results of cell cycle analysis with propidium iodide labeling and flow cytometry. A: negative control (acetone); B: compound **4b** at IC25 (8 µM) concentration; C: compound **4j** at IC25 (6 µM) concentration; D: compound **4l** at IC25 (4 µM) concentration. Cells were treated for 48 hours in the case of compounds **4b** and **4j**, and 72 hours for compound **4l**.

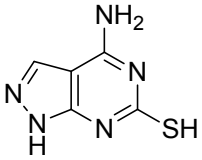
**Table 1.** *In vitro* antiproliferative activity of final compounds **4 a-n**, **6**, **7** against U937 cancer cells.



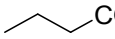
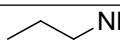
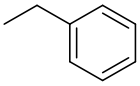
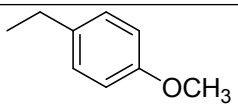
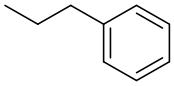
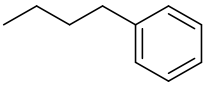
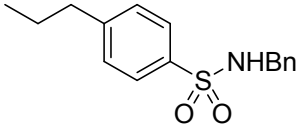

**4 a-n**

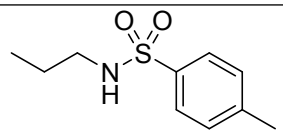
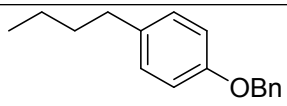
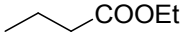
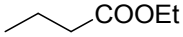



**6**



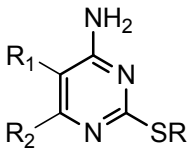

**7a**

| Compound | R | $R_1$ | IC50 (μM) | 95% CI (μM) |
|---|---|---|---|---|
| **4a** |  | H | 53.3 | 50.46-56.1 |
| **4b** |  | H | 16.64 | 13.34-20.4 |
| **4c** |  | H | n.a. | |
| **4d** |  | H | 42.5 | 30.4-58.3 |
| **4e** |  | (N1) $CH_3$ | 134.2 | 117.0-151.2 |
| **4f** |  | H | n.a. | |
| **4g** |  | H | 134.5 | 116.8-152.5 |
| **4h** |  | H | 25.7 | 24.1-27.3 |
| **4i** |  | H | 38.5 | 35.2-42.0 |
| **4j** |  | H | 12.56 | 8.55-18.27 |

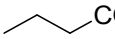
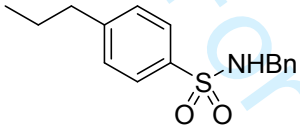
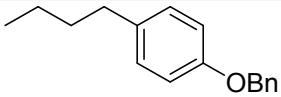
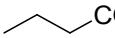
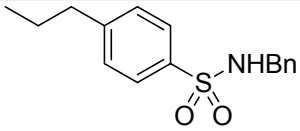
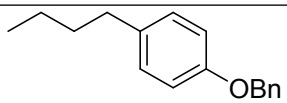
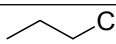
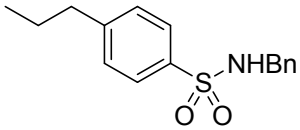
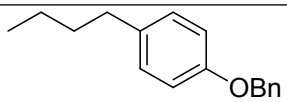
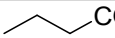
| | | | | |
|---|---|---|---|---|
| **4k** |  | H | 172.2 | 146.6-210.1 |
| **4l** |  | H | 7.96 | 5.87-10.82 |
| **4m** | H | (N1)  | n.a. | |
| **4n** | H | (N2)  | n.a. | |
| **6** | | | n.a. | |
| **7a** | | | n.a. | |

For the determination of IC50, the starting solutions were serially diluted with a range of starting concentrations 5-0.019 mM. The U937 histiocytic human cell line (ATCC® CRL-1593.2) was routinely cultured in complete medium composed by RPMI 1640 supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS, Microgem) at 37°C in a humidified atmosphere with 5% $CO_2$. CI: confidence interval; n.a.: not active.

**Table 2.** *In vitro* antiproliferative activity of monocyclic final compounds **5a-j** on U937 cancer cells.




**5 a-j**

| Compound | R | $R_1$ | $R_2$ | IC50 (μM) | 95% CI (μM) |
|---|---|---|---|---|---|
| **5a** |  | CN | H | 141.2 | 129.3-154.6 |
| **5b** |  | CN | H | 47.65 | 41.66-54.74 |
| **5c** |  | CN | H | n.a. | |
| **5d** |  | COOEt | H | n.a. | |
| **5e** |  | COOEt | H | n.a. | |
| **5f** |  | COOEt | H | n.a. | |
| **5g** |  | H | $NH_2$ | 54.38 | 51.37-57.37 |
| **5h** |  | H | $NH_2$ | 56.6 | 50.48-63.74 |
| **5i** |  | H | $NH_2$ | n.a. | |
| **5j** |  | H | OH | n.a. | |

For the determination of IC50, the starting solutions were serially diluted with a range of starting concentrations 5-0.019 mM. The U937 histiocytic human cell line (ATCC® CRL-1593.2) was routinely cultured in complete medium composed by RPMI 1640 supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS, Microgem) at 37°C in a humidified atmosphere with 5% $CO_2$. CI: confidence interval; n.a.: not active.

**Table 3.** Values of IC50 for **4b**, **4j** and **4l** compounds determined in U937 (cancer cells), PBMC (human normal cells) or NIH-3T3 (murine fibroblasts).

| Compound | IC50 (95%CI) in U937 | IC50 (95%CI) in PBMCs | IC50 (95%CI) in NIH-3T3 | Selectivity Index (SI) | |
|---|---|---|---|---|---|
| | | | | vs. PBMC | vs. NIH3T3 |
| **4b** | 16.6 (13.34-20.4) | 166.4 (114.4-373.2) | 249.5 (199.8-322.7) | 10.0 | 15.0 |
| **4j** | 12.6 (8.55-18.27) | 2.9 (1.7-4.9) | 35.4 (26.9-47.2) | 0.2 | 2.8 |
| **4l** | 7.9 (5.87-10.82) | 335.6 (142.8-1111.0) | 718.6 (318.3-2569) | 42.5 | 90.9 |