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1 # C(5) modified uracil derivatives showing antiproliferative
2 and erythroid differentiation inducing activities on human
3 chronic myelogenous leukemia K562 cells

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16 ## Supplementary Data

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18 ### 1. Supplementary methods

19 *1.1. Benzidine assay*
20 In order to determine effects on erythroid differentiation, benzidine-positive cells was
21 identified using a solution containing 0.2% benzidine in 0.5 M glacial acetic acid
22 (10% H_2O_2) as previously described (Bianchi et al., 2001). Benzidine positivity
23 indicates the presence of intracellular hemoglobin.
24

25 *1.2. High Performance Liquid Chromatography*
26 K562 cells were harvested, washed once with phosphate buffered saline (PBS), and
27 the pellets were lysed in water. After incubation on ice for 15 min and spinning for 5

28 min at 10,000 rcf in a microcentrifuge, the supernatant was collected and
29 hemoglobin proteins were separated by cation-exchange HPLC, using a Beckman
30 Coulter instrument System Gold 126 Solvent Module-166 Detector and a 3.5 x 0.46
31 cm column packed with porous (100-nm pore size) 5-mm microparticulate
32 polyaspartic acid-silica, Poly CAT A (Poly LC, Columbia MD, USA) (Ching et al.,
33 1993). Samples were eluted in a solvent gradient using aqueous sodium chloride-
34 BisTris-KCN buffers, and detection was performed at 415 nm. The gradient was
35 made up of mobile phase A (20 mM Bis Tris, 2 mM KCN, pH 6.98) and mobile
36 phase B (20 mM Bis Tris, 2 mM KCN, 200 mM NaCl, pH 6.57), with a flow rate of
37 1.7 ml/min. Elution of hemoglobins was performed by increasing buffer B from 11%
38 to 40% and to 100% at 8 and 12 min, respectively, and then decreasing to 11%
39 buffer B for the least 8 min before application of next sample. The peak areas were
40 used for quantitation of individual hemoglobin peaks, utilizing standard controls of
41 HbA and HbF (Analytical Control Systems, Fishers IN, USA) (Fibach et al., 2003).
42

43 *1.3. Flow cytometry*

44 Cell cycle analysis was performed by flow cytometry of propidium iodide-treated
45 cells, using the FACS Calibur Becton Dickinson Immunocytometry System (Becton
46 Dickinson, San Jose, CA, USA). The cells were collected, washed with PBS and
47 centrifuged at 400 rcf for 5 minutes. The supernatant was eliminated and the cells
48 were stained in the dark for 30 minutes with propidium iodide (50μg/ml), NP40 and
49 RNase (10 μg/μl). After incubation, cells were washed twice with PBS, centrifuged
50 at 400 rcf for 5 minutes and analyzed (Chui et al., 2010).

51

52 *1.4. Measurement of apoptosis*

53 Apoptotic cells were detected by the DeadEnd^{TM} Colorimetric TUNEL System
54 (Promega Italia, Milan, Italy) according to the manufacturer's instructions. K562 cells
55 were treated with the increasing concentrations of compounds; after 5 days, (when
56 differentiated) the cells were rinsed twice with PBS solution and fixed for 25 min in
57 4% paraformaldehyde at room temperature. Measurement of apoptosis was

calculated as a percentage of apoptotic nuclei (dark brown nuclei) versus total nuclei, evaluated in three independent experiments. A dark brown DAB signal indicates positive staining, while shades of blue-green to greenish tan indicate a non-reactive cell. The positive-control was the treatment with DNA-se 1 (Lampronti et al., 2009). Apoptosis was also detected with Annexin V/PI release assay. Cells were treated with the compounds and, after 72 hours, cells were collected, centrifuged for 5 min at 400 rcf. Cells were rinsed with Binding Buffer and then annexin V was added and the samples were incubated at room temperature for 15 min in the dark, according to the manufacturer’s instructions. For the analysis we have used FACS Calibur Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA, USA) (Viola et al., 2008).

2. Supplementary References

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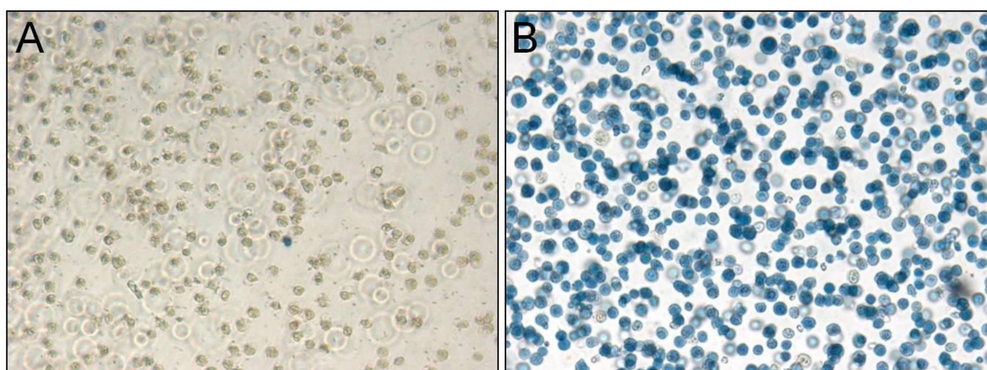
95 **3. Supplementary Figures**

96



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98 **Supplementary Fig. 1.** Effects on erythroid differentiation of K562 cells. The
99 images of benzidine staining assay were taken after 6 days of K562 cell culture in
100 the absence (A) or in presence (B) of 30 μM compound **9**.

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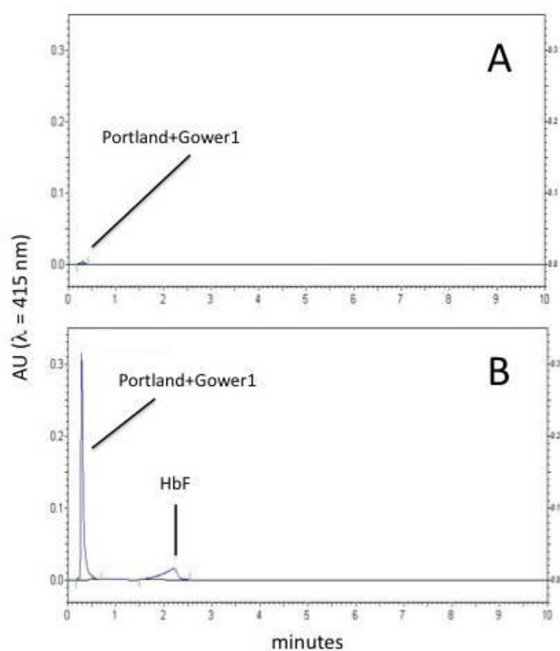

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103 **Supplementary Fig. 2.** HPLC analysis of K562 cellular lysates: chromatogram A
104 shows the hemoglobin profile (Hb Portland and Hb Gower1) in untreated cells,
105 chromatogram B presents the increased production of Hb Portland/Gower1 and HbF
106 in K562 cells treated with compound **9**.

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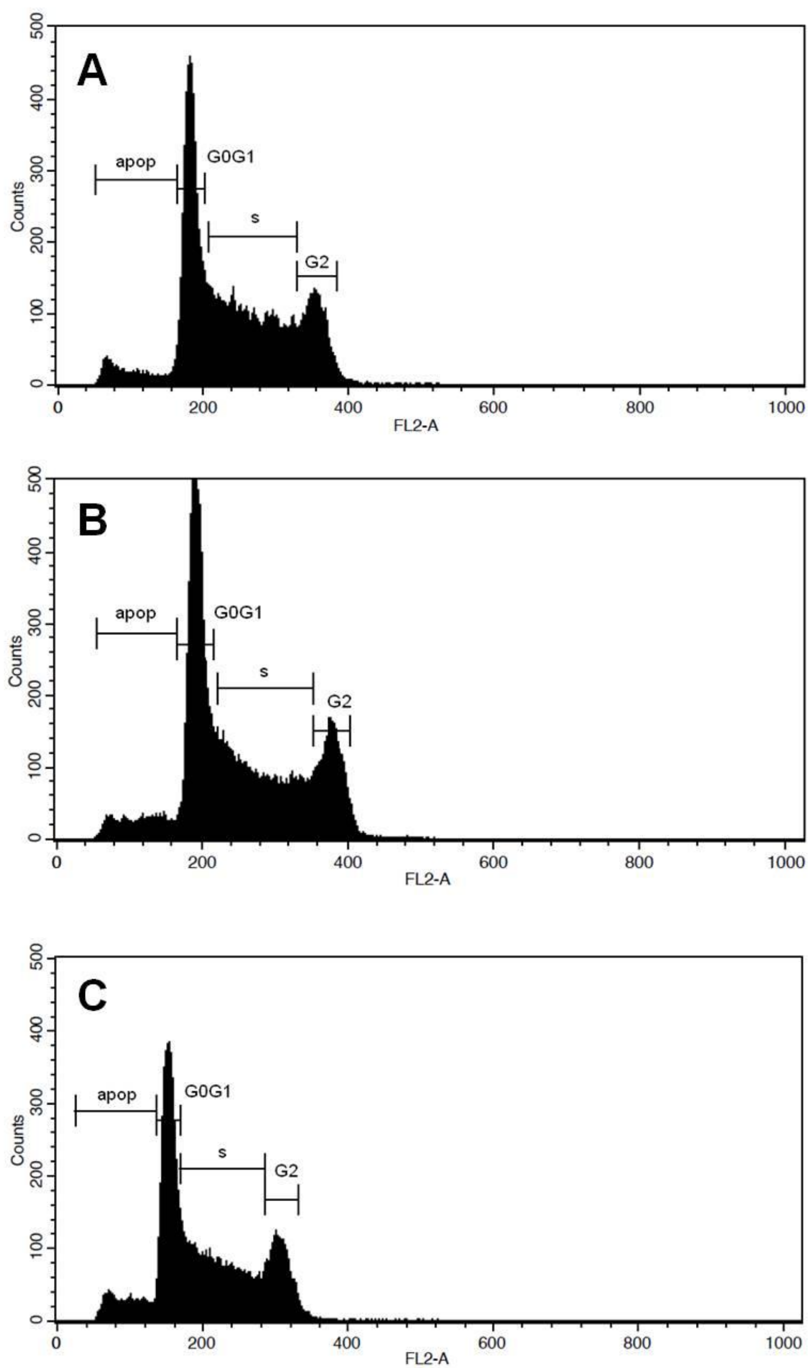

108 **Supplementary Fig. 3.** Analysis of cell cycle using a fluorescence-activated cell
109 sorter (FACS). A: untreated K562 cells; B,C: K562 cells treated with 30 μM (B) and
110 50 μM (C) compound **9**. Similar data were reproducibly obtained in three
111 independent experiments.

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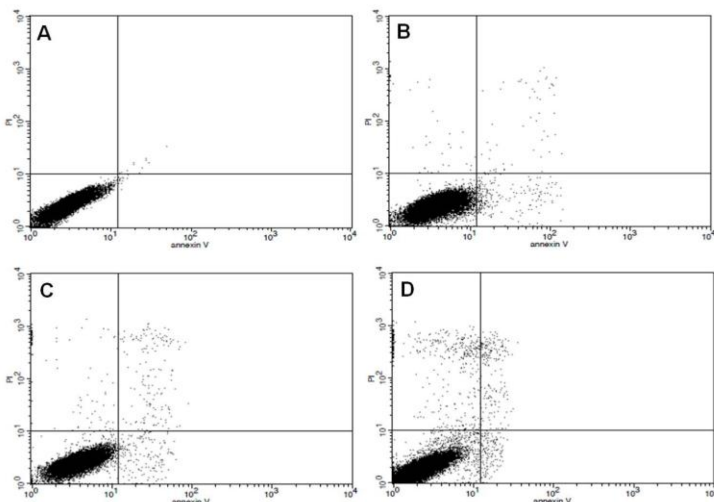
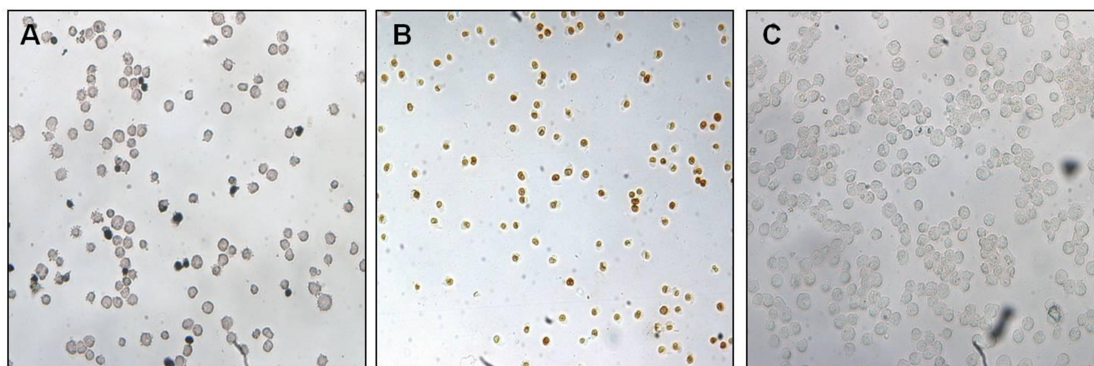


Supplementary Fig. 4. Apoptosis of K562 cells were detected by flow cytometry after annexin V–FITC staining. For this analysis the cells were treated with annexin V and PI (B), 30 μM (C) and 50 μM (D) compound 9. Panel A represents untreated K562 cells.



Supplementary Fig. 5. Detection of apoptosis by DeadEnd TUNEL assay in K562 cell line (A), treated with DNAse I (1U/ml) (B), 30 μM compound **9** (C). Brown color reaction, visible only in the treatment with the DNAse I, indicates apoptotic cells. Cells were photographed at a magnification of 20X.