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Effect of maleimide derivative, protein kinases inhibitor, on the morphofunctional state of human neoplastic monoblast cell line U-937.

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ABSTRACT

Maleimide derivative (MI-1) (1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione) is the inhibitor of VEGF-R1,2,3 (h), Src (h), Syk(h), PDK1, and other protein kinases, *in vivo* decreases the number of colon tumors and monocytes in the blood of rats with colon carcinogenesis. The abovementioned protein kinases are involved in the differentiation and functioning of monocytes, the aim of this study was to investigate the effect of MI-1 on the morphofunctional state of U-937. Proliferative activity and viability of U-937 were assessed using the hemocytometer with trypan blue staining; the apoptotic, mitotic and necrotic cells – in the cytospin specimens after Pappenheim's staining; the cell cycle – by flow cytometry. Results. MI-1 inhibits the proliferation of U-937 by 50 % at 0.009 mM; causes a double increase in G_0/G_1 and the decrease in 3.5 times of S phases, reduces the number of mitotic cells by 34 % ($p = 0.050$) at 0.008 mM and by 65 % ($p = 0.003$) at 0.016 mM after 48 h of treatment, and elevates the number of apoptotic cells three- and five-fold ($p = 0.001$; $p = 0.016$). Conclusion. MI-1 inhibits the proliferative activity of U-937 cells via block cells in G_0/G_1 phase, the decrease in the mitotic activity and the induction of apoptosis.

Keywords: maleimide derivative, U-937, cell cycle, mitosis, apoptosis.

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INTRODUCTION

Maleimide derivative (MI-1) (1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione) was synthesized *in silico* by Research and Industrial Chemistry and Biology Centre of Taras Shevchenko National University of Kyiv as a competitive inhibitor of the ATP-binding site of protein kinases [1]. MI-1 *in vitro* inhibits VEGF-R1,2,3(h), PDK1 (h), FGF-R1 (h), YES (h), EGF-R(h), Src (h), ZAP70, Syk(h), and other protein kinases as well as the proliferation of cancer cell lines: HCT-116 and SW-620 (Colon Cancer), MALME-3M and UACC (Melanoma), A549/ATCC and NCI-H226 (Non-Small Cell Lung Cancer) *etc*. [1-3]. The studies *in vivo* demonstrated that MI-1 decreases the number of colon tumors and the affected area of the colon [4], and normalizes the number of monocytes and platelets in the blood of rats with 1,2-dimethylhydrazine-induced colon carcinogenesis [5]. The abovementioned protein kinases are involved in the signal transduction in response to cytokines, initiating the proliferation, survival and differentiation of various cells, including the hematopoietic ones [6–9].

The impairment of the expression and functioning of the mentioned protein kinases is associated with the development of leukemia [10]. For instance, the vascular endothelial growth factor (VEGF) family is expressed in leukemic cells, with the highest expression levels in myeloma cells, monocytic and megakaryocytic progenitor cells [11]. It also stimulates the proliferation of leukemic cells and angiogenesis in the bone marrow [12]. PDK1 kinase is overexpressed in myelo-monocytic leukemic cells for both acute and chronic leukemias [9, 13]. High expression of families of VEGF-R, EGF-R, FGF-R and PDK1 kinases in leukemic cells causes low sensitivity to the therapy and poor survival prognosis [14–17]. VEGF-R, PDK1 and Src kinase inhibitors inhibit the proliferation of leukemic cells and are compounds, selected for the complex therapy of leukemia [13, 18, 19].

Since families of VEGF-R, FGF-R, EGF-R and PDK1, Src and other protein kinases are involved in the differentiation and functioning of monocytes [7–10] and MI-1 inhibits the former [1-3] as well as the growth of colon tumors [4] and normalizes the increased number of monocytes in the blood of rats with 1,2-dimethylhydrazine-induced colon carcinogenesis [5], the purpose of this study was to investigate the effect of MI-1 on the morphofunctional state of human neoplastic monoblast cell line U-937.

MATERIALS AND METHODS

Cell culture and reagents

U-937 cells were incubated in 96 well plates under normal conditions (5 % CO2, 100% humidity, 37 ^{o}C) in the culture medium RPMI-1640 («Sigma», USA) with the introduction of 10 % FBS («Sigma»), 2 mM Glutamine and 40 µg/ml gentamicin (Biopharma, Ukraine) (cell line were kindly provided by prof. Filonenko V.V. Institute of Molecular Biology and Genetics National academy of Science of Ukraine). MI-1 in the final concentration range from 0,00025 to 0,032 mM was added to the cell cultures after 24 h of the cell adaptation under normal conditions with subsequent incubation for 24 or 48 h. The number of living and dead cells was calculated using the hemocytometer with 0.1 % trypan blue staining.

Quantitative analysis of apoptotic, mitotic and necrotic cells

The percentage of U-937 cells at the apoptotic, mitotic or necrotic stage was calculated by microscopic detection per 1,000 cells using the cytospin prepared specimens, stained according to Pappenheim's method [20] after treatment with MI-1 at the concentration of 0.008 or 0.016 mM.

Cell cycle distribution

The proportions of cells in different phases of the cell cycle after treatment with MI-1 at the concentration of 0.008 or 0.016 mM were measured by flow cytometry with argon laser (excitation = 488 µm, emission = 585/40 µm) («Becton Dickinson», USA) following propidium iodide staining. The samples were analyzed using the Mod Fit LT 3.0 (BDIS, USA) software.

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Statistical analysis

The data are normally distributed, according Shapiro-Wilk test result p>0.05. The data were statistically processed using One-Way ANOVA followed by the post-hoc Dunnett t (2-sided)-test for number of live and dead cells (equal variances assumed according Levene test result p>0.05) and DunnettT3 for apoptotic, mitotic and necrotic cells (homogeneity of variances not assumed) using SPSS 16.0 for Windows. Mean values and standard deviation are presented.

RESULTS AND DISSCUSION

MI-1 affects the proliferative activity and viability of U-937 (Fig. 1). A 50 % reduction in the proliferative activity of cells, treated with MI-1 at 0.022 mM for 24 h (Fig. 1 A) and at 0.009 mM for 48 h (Fig. 1 B) was observed.

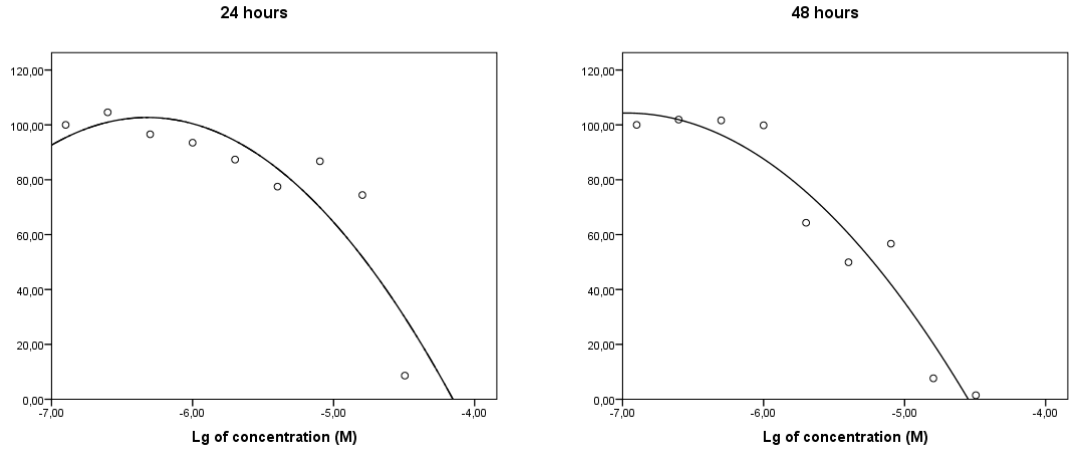

Fig.1. The viability of U-937 (live cells, percent of control) treated with MI-1 at the increasing concentration for 24 (A) and 48 (B) h; data represents the mean of 5 independent replicate experiments (n=5).

The mode of MI-1 treatment was investigated at the concentrations of 0.008 and 0.016 mM. One-Way ANOVA results revealed that under the indicated concentration MI-1 changes the number of live cells after 24 h of exposure (p = 0.049) and its effect is persisted after 48 h (p <0.001). At the same time the number of dead cells (stained with trypan blue) changes after 48 h of MI-1 action (p <0.001). According to the results of Dunnett's test, after 24 h of treatment of MI-1 at 0.008 mM slightly inhibits the proliferative activity of U-937 as evidenced by the decrease in the number of cells by 13 % ($1.10\pm0.14 \times 10^6$/ml), compared to the untreated control ($1.27\pm0.28\times10^6$/ml) (Fig. 2 A), whereas 48 h of the exposure entail the inhibition of the proliferative activity by 43 % (1.51±0.25 vs. $2.66\pm0.18\times10^6$/ml respectively, p<0.001). Double concentrations of MI-1 lead to the decrease in the proliferative activity by 25 % ($0.95\pm0.13\times10^6$/ml; p=0.032) after 24 h and by 92 % ($0.20\pm0.12\times10^6$/ml; p<0.001) after 48 h of exposure. Thus, the prolongation of U-937 incubation with MI-1 up to 48 h results in the persisted cytostatic effect of the maleimide derivative which testifies to the prolongation of its action. The reduction in the proliferative activity of the cells due to MI-1 is conditioned by the cell-cycle block in the phase of proliferative rest G_0/G_1 to a greater degree (a two-fold increase - up to 76.35 ± 3.67 % ; p<0.01) and in G_2/M – to a lesser degree (7.86 ± 0.32 % ; p<0.01), with the relevant 3.5-fold reduction of the S phase (15.70 ± 3.49 % ; p<0.001) compared to untreated U937 (38.55 ± 3.05, 4.97 ± 0.25, 56.47 ± 3.30 % respectively) (Fig. 2 B). After 48 h of action MI-1 at 0.016 mM also causes a considerable increase in the cells stained with trypan blue (dead) ($0.27\pm0.08\times10^6$/ml, p<0.001) (Fig. 2 C) vs. control ($0.11\pm0.05\times10^6$/ml). Since the cells get stained with the trypan blue at the stage of necrotic death and at the late stages of apoptosis, we performed a morphological analysis of U-937 in cytospin specimens and assessed their mitotic activity.

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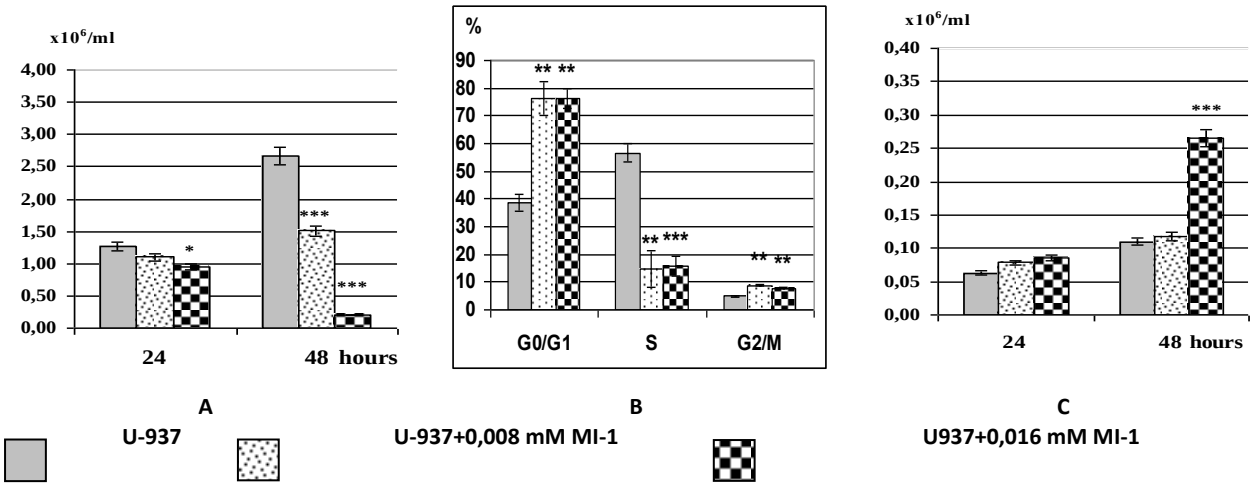

Fig. 2. The number of live cells (A) and dead cells (C) U-937 after 24 and 48 h and cell cycle distribution (B) after 48 h of MI-1 treatment at the concentrations of 0.008 and 0.016 mM; * p<0.05; ***p<0.001 compared with the untreated (control) U-937, Dunnett t (2-sided)-test result. Each data A and C represents the mean of 5 independent replicate experiments (n=5), B - n=3.

One-Way ANOVA results demonstrate MI-1 effect on the mitotic activity (p = 0.024) and the level of apoptosis (p < 0.001) for U-937 both 24 and 48 h after the exposure (p = 0.005, p < 0.001, respectively). According to the results of the DunnettT3 test, the mitotic activity of cells does not change significantly after 24 h of MI-1 action for both studied concentrations (2.40±0.36 %, p = 0.093; 2.70±0.26 %, p = 0.180, respectively) compared with the control (3.63±0.55 %) (Fig. 3 A; Fig. 4 A, B). The inhibition of the mitotic activity by 65 % after 48 h of MI-1 effect at 0.016 mM (1.10±0.26, p = 0.027) relative to the control (3.13±0.55 %) (Fig. 3 A; Fig. 4 G, H) was established. At the same time, as early as after 24 h of MI-1 effect the number of cells at the apoptotic stage increases three and ten times for both investigated concentrations, respectively, (3.27±0.95 % p = 0.093; 10.40±2.03 %, p = 0.030 compared with the control 0.90±0.20 %) (Fig. 3 B, Fig. 4 B, D) and is still increased three- and five-fold after 48 h of the influence (3.33±0.25 %, p = 0.001; 5.47±0.76 %, p = 0.016, respectively) relative to the controls (0.97±0.15%) (Fig. 3 B, Fig. 4 G, H, J, K, L). Increased number of dead cells after 48 h of treatment with MI-1 at 0.016 mM (Fig. 2 C) probably due to fragmentation of apoptotic cells at the late stage of apoptosis and their detection in the cell suspension. At the same time, the fragments may be lost in cytospin and staining process and a lower percentage of apoptotic cells in cytospin spesiments can be observed (Fig. 3 B). The number of necrotic cells (Fig. 3 C; Fig. 4 A, E, F, H, I) is not significantly different from the control values after the exposure to MI-1 under both concentrations for both exposure variants.

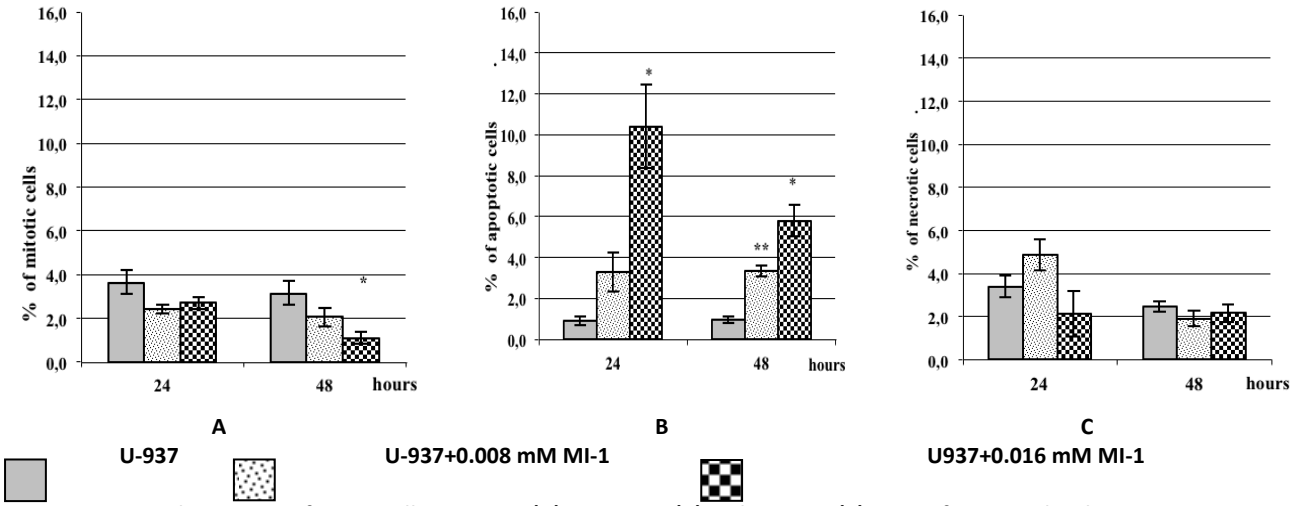

Fig. 3. The content of U-937 cells in mitotic (A), apoptotic (B) and necrotic (C) stage after 24 and 48 h treatment with MI-1 at the 0.008 and 0.016 mM;

* p <0.05; ** p <0.01 compared with the untreated (control) U-937, DunnettT3-test result (n=3).

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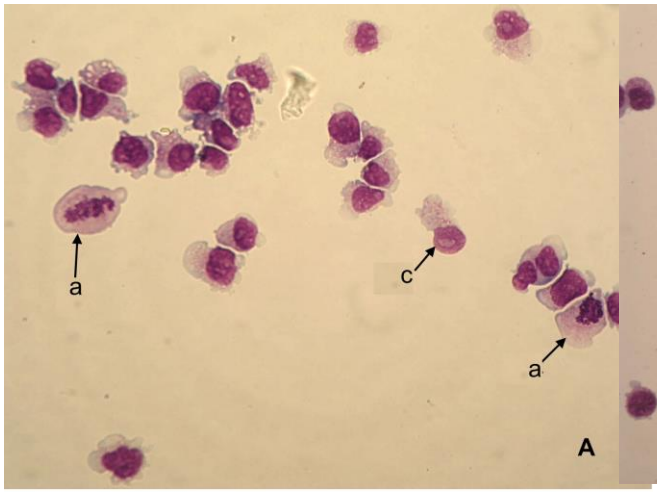
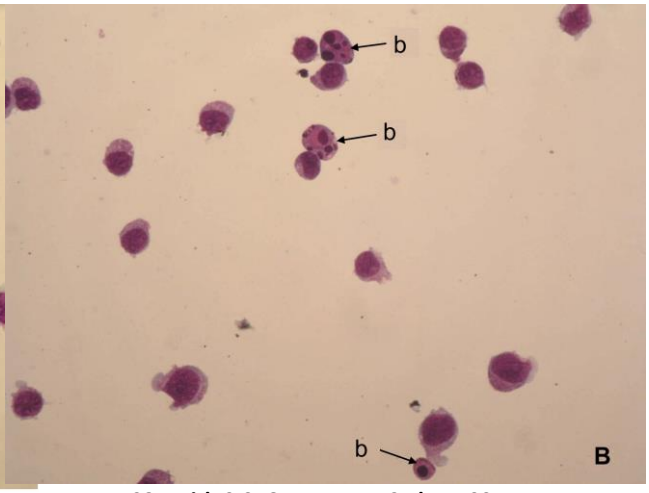
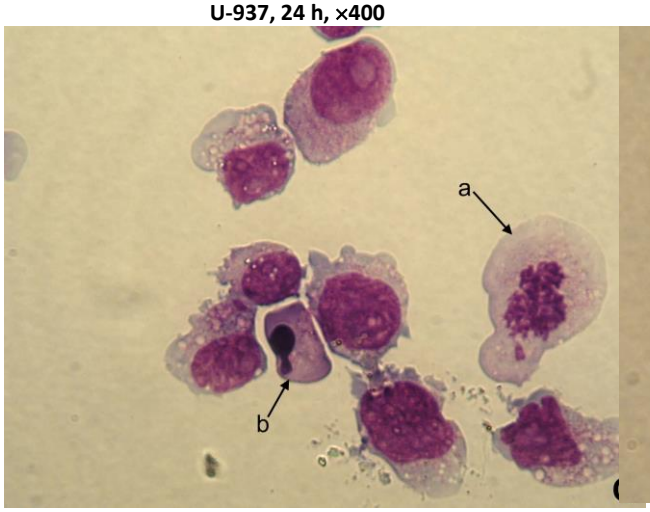


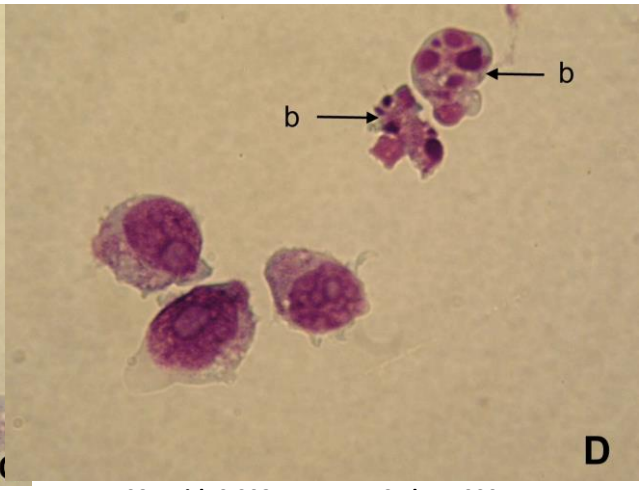
U-937, 24 h, ×400



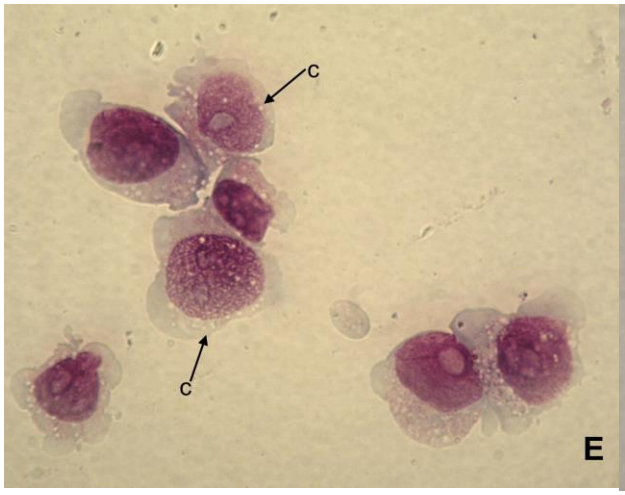
U-937 with 0.016 mM MI-1, 24 h, ×400



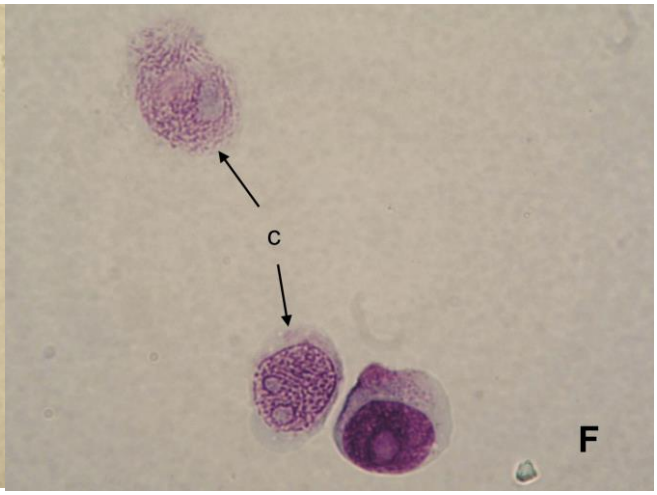
U-937, 24 h, ×1000



U-937 with 0.008 mM MI-1, 24 h, ×1000



U-937, 24 h, ×1000



U-937 with 0.008 mM MI-1, 24 h, ×1000

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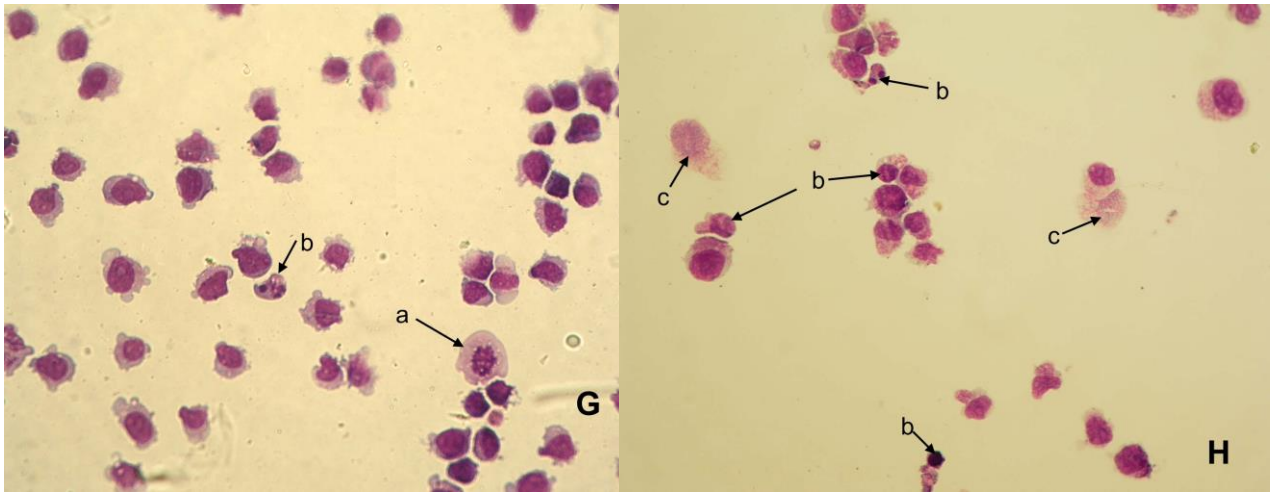
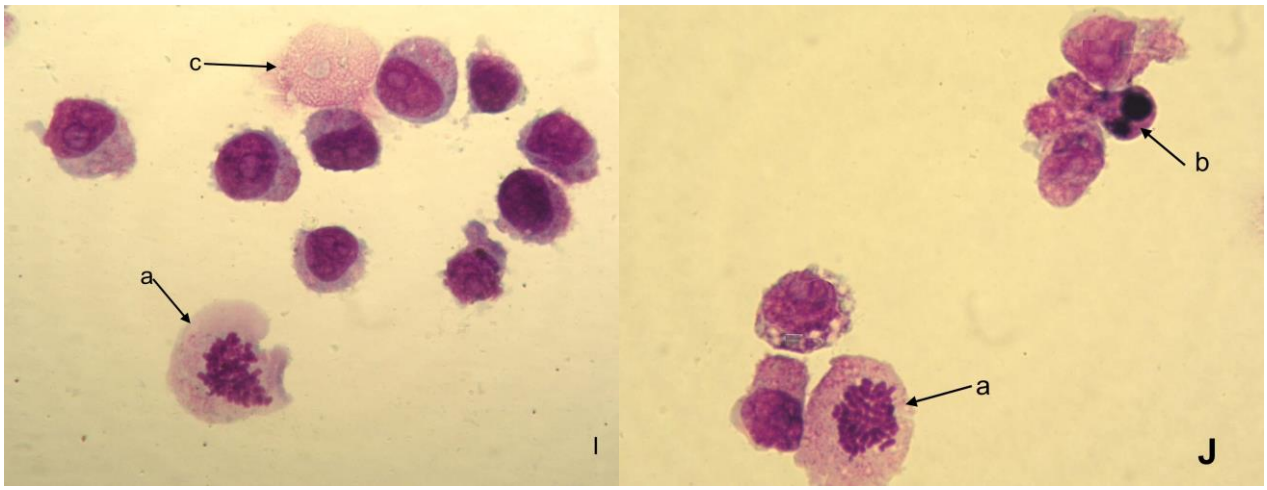
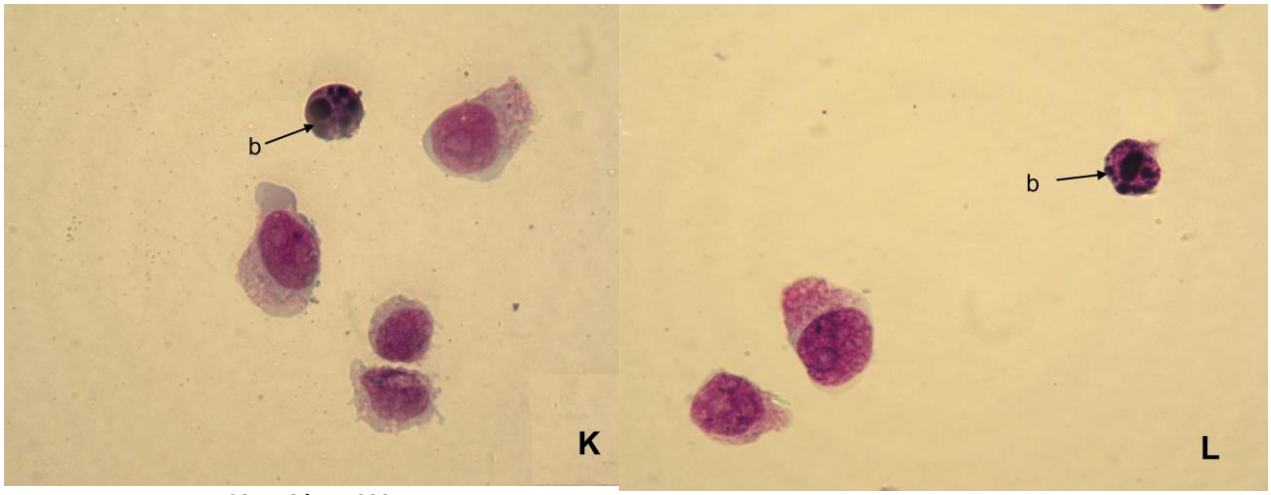


U-937, 48 h, ×400 U-937 with 0.016 mM MI-1, 48 h, ×400



U-937, 48 h, ×1000 U-937 with 0.016 mM MI-1, 48 h, ×1000



U-937, 48 h, ×1000 U-937 with 0.008 mM MI-1, 48 h, ×1000

Fig. 4. Photomicrographs of cytospin specimens of U-937 cells after 24 and 48 h of treatment with MI-1 at the 0.008 and 0.016 mM, Pappenheim stained; a - mitosis, b - apoptosis, c - necrosis

Thus, the results obtained demonstrate that as an inhibitor of VEGF-R1,2,3 (h), PDK1, FGF-R1 (h), EGF-R (h), Src (h), Syk (h), and other protein kinases [1] MI-1 inhibits the proliferative activity of human neoplastic monocytic U-937 cells via increasing the number of cells in the phase of proliferative rest G_0/G_1, the reduction in the mitotic activity and apoptosis induction.

The elaboration of an inhibitor of chimeric protein kinase BCR/ABL [21] and its successful application in the clinical treatment of chronic myeloid leukemia [22] prompted a large number of studies of signaling cascades of neoplastic cells, including leukemic ones, as targets for new generation therapeutic drugs [18, 19, 21, 22]. The advantages of this therapy are high specificity to tumor cells and low toxicity to normal hyperproliferative cells, including hematopoietic ones.

The patients with hematologic diseases (myelodysplastic syndrome, acute and chronic myeloid leukemia, etc.) have elevated concentrations of families of VEGF, FGF, EGF in serum, the latter are expressed in leukemic cells and their expression level correlates with low sensitivity to the therapy and poor survival prognosis [17, 23–27]. The high expression of indicated growth factor and their kinases in cells is associated with active proliferation of monocytic and megakaryocytic hematopoietic cells in myelomonoblast, monoblast and megakaryoblast leukemias [11, 24, 26]. The blocking of VEGF receptors by antibodies or by low molecular weight compounds of receptor-protein kinases leads to a significant decrease in their concentration in serum, but without any reduction in the number of leukemic cells. Therefore, anti-VEGF monotherapy is not effective in the treatment of leukemia, but it has therapeutic effect in the combination with the cytotoxic therapy [15].

Activation of PI3K signaling pathway by growth factor leads to activation downstrem PDK1, Src and Syk kinases that regulate cell cycle, proliferation, viability and apoptosis [28-30]. Monocytic hematopoietic cells with high expression of PDK1-kinase have high proliferative potential and viability, resistance to cytotoxic drugs, resulting in partial response to the cytostatic therapy of patients [9, 13]. Low molecular weight PDK1-kinase inhibitor reduces proliferative activity and induces apoptosis in neoplastic monocytes cells [18]. Low expression of indicated kinase reduces cell proliferation, delays G_0/G_1 to S phase and impaires G_2/M progresion due to an increases level of p27Kip1 (cyclin-dependent kinase inhibitor 1B) expression and a reduces level of cyclin D1 expression [31, 32]. Syk-kinase is expressed in hematopoietic and leukemic cells and its blockade in acute myeloid leukemia cells leads to the differentiation in the monocytic direction [33], cell-cycle arrest in G_0/G_1 phase and induction of apoptosis [34] and its combined inhibition with PI3K enhances this effect [35]. The blocking of activated Src-kinase in chronic myeloid leukemia cells (K562), resistant to imatinib, an inhibitor of BCR/ABL-protein kinase, induces G_0/G_1 arrest and apoptosis of these cells [19]. Another maleimide derivatives are inhibitors of different serine/threonine and tyrosine kinases [36]. These data evidence that the VEGF-R1,2,3 (h), PDK1, FGF-R1 (h), EGF-R (h), Src (h), Syk (h) protein kinases are hyperexpressed at the proliferation of monocytic hematopoietic cells and their inhibition induces the differentiation, cell-cycle arrest in G_0/G_1 phase and apoptosis of these cells. Similar results were obtained for U-937 cells, treated with maleimide derivative MI-1, an inhibitor of indicated protein kinases. In addition, according to the results of our previous studies, MI-1 normalizes the increased number of monocytes in the blood of rats with 1,2-dimethylhydrazine-induced colon carcinogenesis [5], does not affect the differentiation of blood cells in healthy rats [37], has low toxicity to the gastrointestinal tract, liver [4], kidneys [38], reproductive system [39] and is remarkable for its antioxidant properties [40, 41]. MI-1 inhibits the proliferation of tumor cells of epithelial origin, increasing the number of cells in G_0/G_1 phase [42] and reduces the number of colon tumors of rats with 1,2-dimethylhydrazine-induced colon carcinogenesis [4, 43]. Thus, MI-1 is a target compound with low toxicity and promising potential for complex therapy of both colon cancer and monoblast leukemia.

CONCLUSION

MI-1 inhibits the proliferative activity of human neoplastic monoblast U-937 cells, causes block cells in G_0/G_1 phase, the decrease in the mitotic activity and the induction of apoptosis. A 50 % reduction in the proliferative activity of the cells, treated with MI-1 at 0.009 mM for 48 h was observed, indicating its evident cytostatic effect. MI-1 is a promising agent with antitumor antiproliferative activity.

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