

**APOPTOSIS AND CONTENT OF MOBILE LIPID DOMAINS IN HUMAN LEUKEMIA K-562 CELLS INDUCED TO DIFFERENTIATE BY QUERCETIN OR DIMETHYL SULFOXIDE**

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*The apoptotic index, cell cycle progression and caspase-3 activation in K-562 cells induced to differentiate by DMSO or quercetin have been studied. Quercetin treatment of K-562 cells was accompanied by cell cycle arrest in  $G_2/M$  and apoptosis with caspase-3 activation. In contrast, DMSO-induced differentiation was accompanied by the complete cell cycle arrest in  $G_1/G_0$  with negligible caspase-3 activation. In spite of the appearance of benzidine-positive cells and the decreased CD71 level in K-562 cells after exposure to quercetin, the analysis of  $^1H$  NMR spectra revealed the overall balance in favor of apoptosis, namely the increase in the content of NMR-visible mobile lipid domains and the decreased intensity of choline-containing metabolites.*

*Key words: apoptosis, caspase-3, cell cycle, chronic myelogenous leukemia, DMSO, flavonoids, mobile lipid domains, quercetin.*

The induction of leukemic cell differentiation may provide an alternative approach to the conventional cytotoxic chemotherapy of leukemia [1]. Nevertheless, the application of differentiation therapy in leukemia patients is currently limited only to several forms of leukemias. Meanwhile, the precise mechanisms of pharmacologically induced differentiation of leukemic cells and their relations to apoptotic cell pathways are not well understood.

Quercetin (3,3',4',5,7-pentahydroxy flavone), which is one of the most widely distributed flavonoids in nature, has been reported to possess antiproliferative and proapoptotic activities in leukemic cells [2–4]. These activities do not seem to be related only to the conventional antioxidant properties of this substance. Several mechanisms of the apoptogenic effects of quercetin have been suggested, with death receptor-5, anti-apoptotic Bcl-2 and Bcl-x<sub>L</sub> proteins, caspases, Akt/PKB kinase, Cu-Zn superoxide dismutase, fatty acid synthase and heat shock proteins being among the cellular targets [5–7]. Few reports have shown that quercetin may also induce differentiation in human chronic myelogenous leukemia [8, 9]. Quercetin was also shown to induce apoptosis in K562 and several other human leukemic cell lines [10]. Nevertheless, the relations between apoptosis and differentiations in quercetin-treated cells as well as the mechanisms of quercetin-triggered differentiation have not been studied in detail.

Abundant experimental evidence has demonstrated that besides their role in the initiation and execution of apoptosis, caspases have been also involved in such physiological processes as cell survival, proliferation, differentiation, and inflammation [11, 12]. Several data suggest the roles of caspases in erythroid differentiation in the bone marrow [13, 14].

The dual effect of quercetin as well as some other flavonoids inducing both apoptosis and cell differentiation makes it rather difficult to analyze these effects separately in cell populations. The use of the approaches allowing one to assess the overall effects may be advantageous for characterizing the balance of these two processes in cell populations. While  $^1H$  NMR has been shown to detect the apoptotic changes in cell membranes by the shifts in the content of NMR-visible mobile lipid domains and choline-containing metabolite intensity [15], the changes in  $^1H$  NMR detectable parameters in the systems of cell differentiation have not yet been followed.

Therefore, the major objective of the study was to compare the apoptosis induction, cell cycle progression and caspase-3 activation in K-562 cells induced to differentiate by dimethyl sulfoxide (DMSO) or quercetin. We also have attempted to analyze the patterns of  $^1H$  NMR spectra, namely the content of NMR-visible mobile lipid domains and choline-containing metabolite intensity in cells undergoing apoptosis or differentiation with

the aim of assessing the overall balance of apoptosis and differentiation in K-562 cells treated with quercetin.

### Materials and Methods

**Chemicals.** Quercetin purchased from Sigma Chem. Co. (USA) was dissolved in 96% ethanol and further diluted in culture medium. Vesipide was purchased from Bristol-Myers Squibb SpA (Italy).

**Cell culture and treatment.** Human K-562 cell line was obtained from the National Collection of Cell Lines of the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). Quercetin or DMSO was added to the cells at the beginning of the exponential growth phase. Cell growth and viability were assessed by direct counting of trypan blue dye-excluding cells. The percentage of hemoglobin-producing cells was determined by a benzidine staining method as previously described [16].

**Proton NMR-spectroscopy.** The high-resolution  $^1\text{H}$  NMR spectra were acquired using a 300 MHz Varian Mercury 300BB NMR spectrometer (Varian, USA). The details of the technique were described [17]. The areas of signals at 0.9 and 1.3 ppm arising from  $-\text{CH}_3$  and  $(-\text{CH}_2-)_n$  groups of fatty acyl chains of lipids as well as signals at 3.2 ppm arising from choline-containing metabolites (Cho), which include choline and phosphorylcholine, were integrated using VNMR software (Varian, USA) and expressed in relative units.

**Flow cytometric analysis.** A) *Cell cycle distribution and apoptosis.* The cells were resuspended in hypotonic buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 5  $\mu\text{g}/\text{ml}$  propidium iodide. 250  $\mu\text{g}/\text{ml}$  of RNase A was added to each sample, and the cells were stained for 15 min at 37 °C. Flow cytometry was performed on a FACScan automated system (Becton Dickinson, USA), and data were analyzed using CellQuest software package and ModFit LT 2.0 program.

B) *Immunostaining.* The percentage of cells with active form of caspase-3 was assessed using FITC-conjugated MAb active caspase-3 kit (BD Biosciences, USA) according to the manufacturer's recommendations. Expression of CD71 on the surface of K-562 cells was evaluated by flow cytometry as described previously [18].

**Statistical analysis.** Each experiment was replicated three times. The results of the experimental and control groups were tested for statistical significance by a one-tailed Student's *t* test.

### Results and Discussion

First, the effects of quercetin and DMSO on cell growth, cell cycle and differentiation were compared. K-562 cells were treated with quercetin at concentrations up to 40  $\mu\text{M}$  or 1.9% DMSO. Quercetin slightly inhibited cell proliferation, while DMSO suppressed completely cell growth without increasing cell death. In cells treated with quercetin, the increase in hypodiploid cell fraction up to 15% was evident. Meanwhile, the cells grown in the presence of DMSO have shown no increase in apoptotic cell fraction (Fig. 1, A). As shown in Fig. 1, B, quercetin treatment for 48 h resulted in relatively moderate increase (about 25%) in the percentage of cells with the active form of caspase-3. In contrast, DMSO treatment has not induced caspase-3 activation relative to baseline level. In addition, the opposite effects of quercetin and DMSO on cell cycle traverse have been shown. Namely, DMSO induced complete arrest in  $G_0/G_1$  phase, while quercetin suppressed the growth of K-562 cells by blocking the cell cycle predominantly in  $G_2/M$  phase (Fig. 2).

In contrast to DMSO-induced differentiation of K-562 cells involving practically the whole cell population, morphological and cytochemical analysis of quercetin-treated cells reveal only partial features of erythroid differentiation in 15–20% of cells. Nevertheless, the differentiation effect of quercetin was confirmed by the increasing percentage of hemoglobin-containing (benzidine-positive) cells ( $38 \pm 4\%$  over the baseline level  $16 \pm 2\%$ ).

We next investigated the expression of cell surface transferrin receptor (CD71) as a non-lineage specific marker of early erythroid progenitor cells in the course of DMSO- or quercetin-induced differentiation of K-562 cells. CD71 is known to be lost as these cells differentiate to mature erythrocytes. Flow cytometric analysis demonstrated strong decrease in the percentage of CD71-positive cells upon DMSO treatment: in 72 h transferrin receptor was practically undetectable (Fig. 3). In quercetin-treated cells, slight reduction of the percentage of CD71-positive cells was observed (75% at 48 h as compared with the basal level). The differences between DMSO and quercetin treatments in CD71 are quite in line with morphological changes and assessment of benzidine-positive cells – therefore indicating only partial differentiation within cell population and in a different cell cycle setting.

Since the findings above suggest that the differentiation of K562 cells induced by quercetin but not DMSO is accompanied by apoptosis,

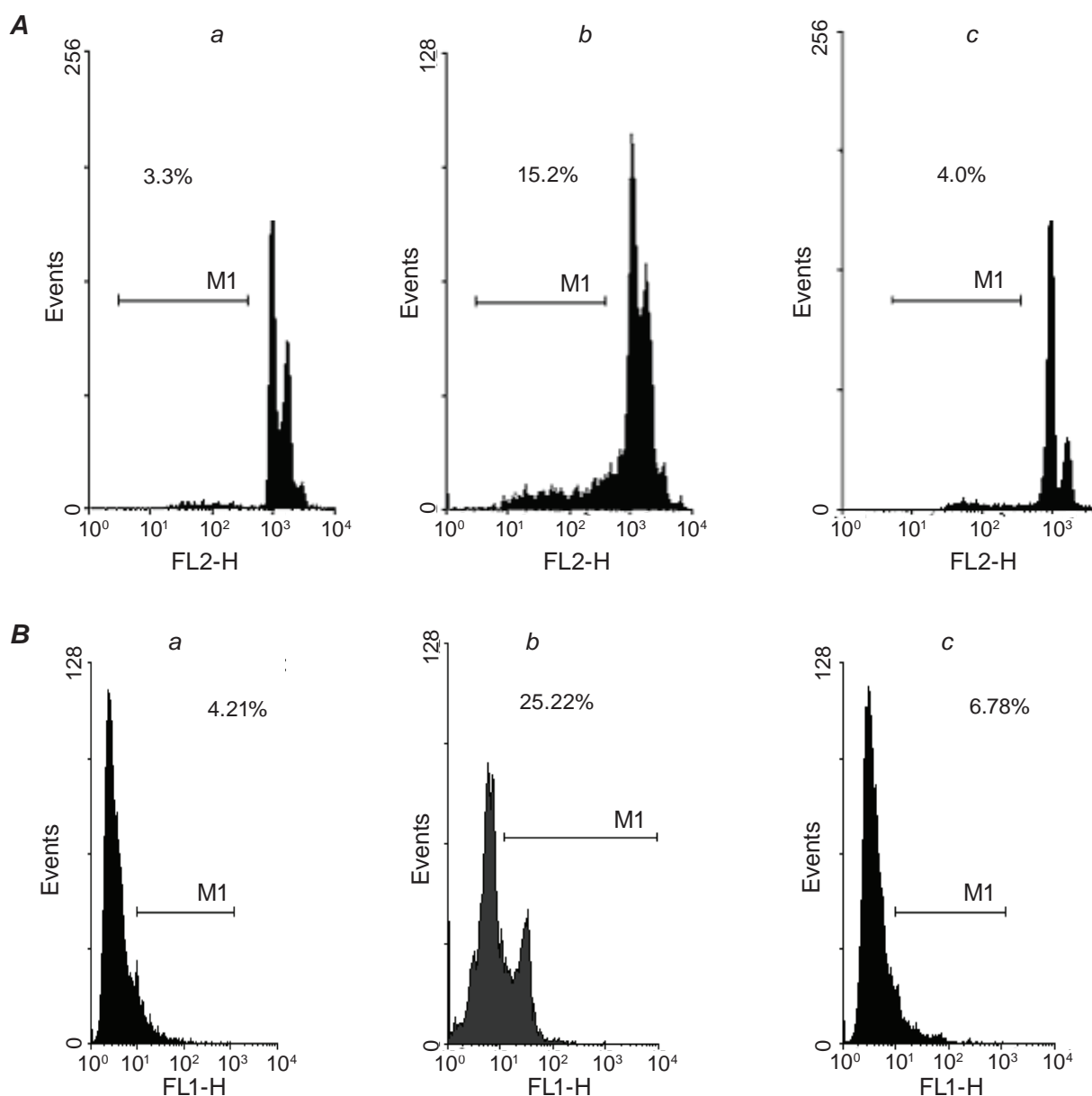
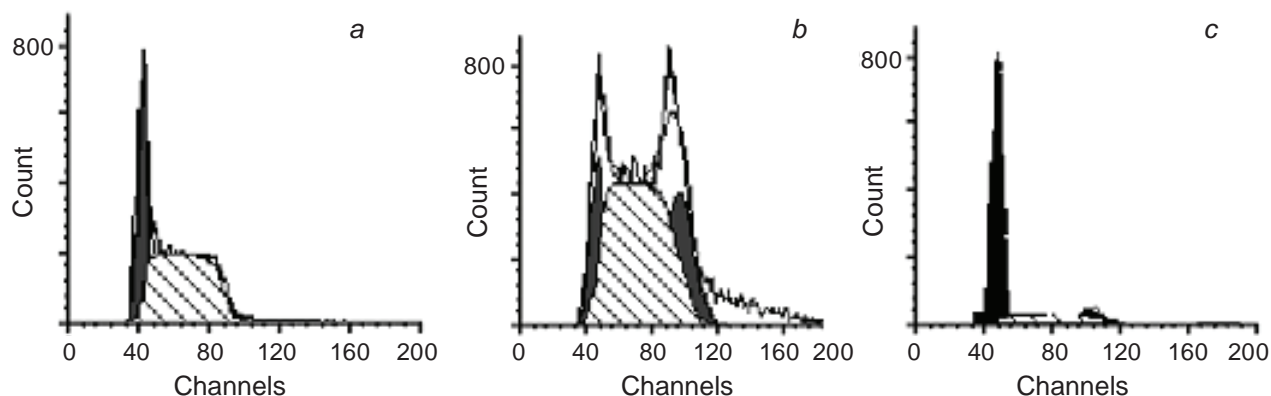


Fig. 1. Induction of apoptosis (A) and activation of caspase-3 (B) in K-562 cells treated with quercetin or DMSO. a – control; b – 40 μM quercetin, 48 h; c – 1.9% DMSO, 48 h. The figures show a representative staining profile for 10,000 cells per experiment. M1 is the cell population defined as apoptotic (A) or caspase-3-positive (B)

it would be of interest to assess the overall balance of differentiation and apoptosis in this system by <sup>1</sup>H NMR analysis. Some representative spectra given in Fig. 4 showed 1.6-fold decrease of the CH<sub>2</sub>/CH<sub>3</sub> signal intensity ratio corresponding to MLD content and 2.4-fold increase of the intensity of Cho resonance in K-562 cells treated with DMSO. In contrast, the CH<sub>2</sub>/CH<sub>3</sub> signal intensity ratio in the same cells treated with vesipide as a typical apoptosis inducer increased about two-fold, while the intensity of Cho resonance was thrice as

low as that in untreated cells. The analysis of <sup>1</sup>H NMR spectra in K-562 cells exposed to quercetin revealed the overall balance of changes in favor of apoptosis, namely the 1.6-fold increase in MLD content and 1.8-fold decrease in Cho resonance intensity as compared to the control values.

The human K562 cell line has been used widely as a valuable model for erythroid differentiation *in vitro* which is induced by various substances. The intrinsic commitment of at least part of K562 cell population to differentiate has been also



| Treatment       | Cell cycle phase distribution in % |              |                   |
|-----------------|------------------------------------|--------------|-------------------|
|                 | G <sub>0</sub> /G <sub>1</sub>     | S            | G <sub>2</sub> /M |
| Control         | 37.92 ± 0.87                       | 57.78 ± 0.41 | 4.29 ± 0.29       |
| Quercetin 40 μM | 15.48 ± 0.73                       | 60.30 ± 0.54 | 24.22 ± 0.25      |
| DMSO 1.9%       | 77.24 ± 0.26                       | 15.04 ± 0.39 | 7.73 ± 0.95       |

Fig. 2. Cell cycle analysis of K-562 cells after 48 h treatment with quercetin or DMSO: a – control; b – 40 μM quercetin; c – 1.9% DMSO. Cells were stained with PI for flow cytometry analysis and DNA histograms were plotted with ModFit software

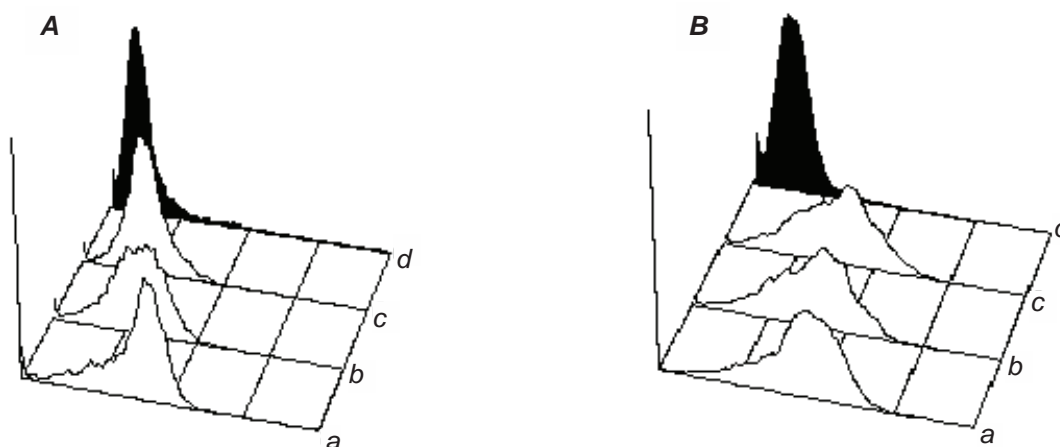


Fig. 3. Cell surface transferrin receptor expression in K-562 cells treated with 1.9% DMSO (A) or 40 μM quercetin (B): a – control; b – 48 h; c – 72 h. Black peaks (d) show negative control (anti-transferrin receptor antibody is omitted). The figures show a representative staining profile for 10,000 cells per experiment. The log fluorescence intensity is shown on the horizontal axis. On the vertical axis the cell number is plotted

demonstrated [19]. Molecular mechanisms of such differentiation induced by unrelated agents and their relevance to the normal process of erythroid differentiation have been still far from being elucidated. The existence of various regulatory pathways involved in the final differentiation effects has not been excluded. Among the agents capable of inducing the differentiation of K562 cells are cytotoxic chemotherapeutics drugs as well, which

induce differentiation at the subtoxic doses [20]. Therefore, the relation between the differentiation and the involvement of the apoptotic pathway mechanisms is still to be investigated. Nevertheless, several authors believe that apoptosis and erythroid differentiation of chronic myelogenous leukemia cells represent the different processes, which may be easily dissociated [21]. Although some investigators suggested that quercetin can trigger dif-

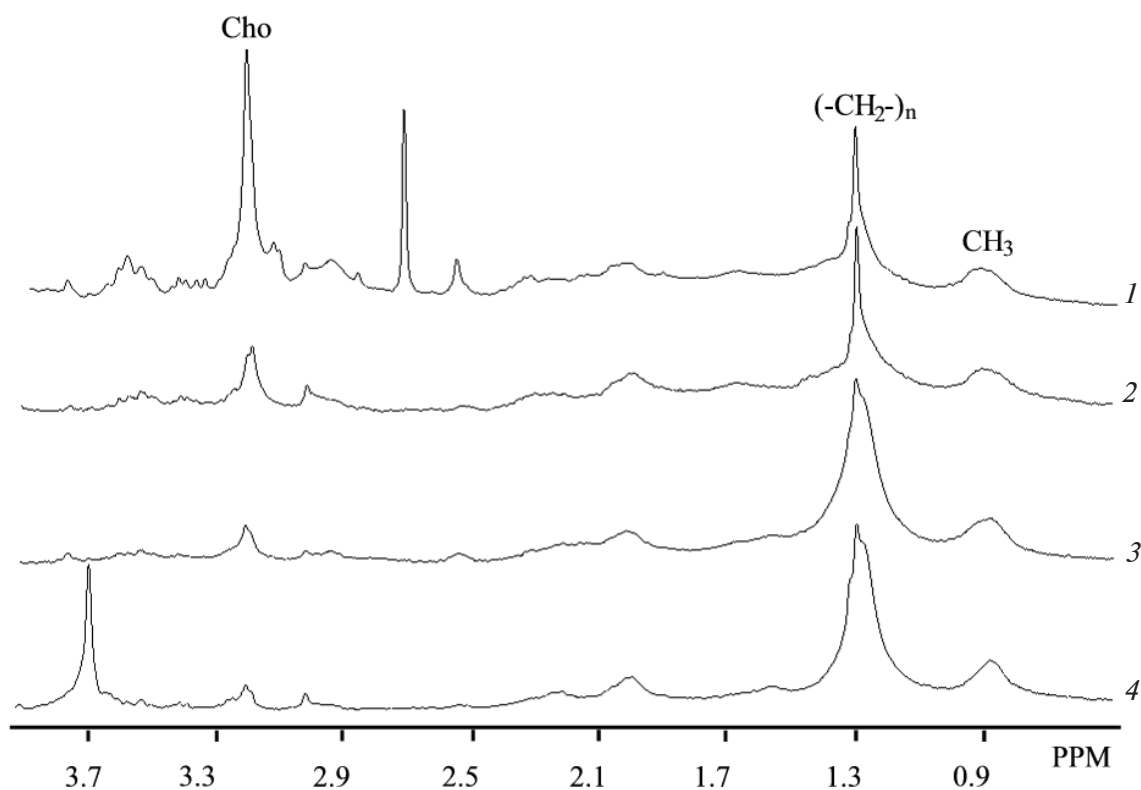


Fig. 4. Representative high-resolution 300 MHz spectra of K-562 cells. 1 – cells treated with DMSO (1.9%, 18 h); 2 – control; 3 – cells treated with quercetin (40  $\mu$ M, 48 h); 4 – cells treated with vepeside (20  $\mu$ M, 18 h)

ferentiation in K-562 cells [8, 9], this could not be confirmed by others [22]. Furthermore, in some cases quercetin was shown to inhibit the environmental stress-induced differentiation of K-562 cells [23].

Our studies demonstrated some features of erythroid differentiation of K562 cells induced by quercetin. In particular, the exposure to quercetin resulted in the increase of benzidine-positive cell count in K-562 culture over the baseline level. Treatment of those cells with either DMSO or quercetin downregulated cell surface transferrin receptors, which may be important in causing cessation of transferrin-mediated iron transport required for cell growth.

Nevertheless, the features of differentiation of K562 cells induced by quercetin are distinct from DMSO-induced differentiation. The most important is that quercetin-induced differentiation is accompanied by caspase-3 activation and apoptosis in a fraction of cell population. This result is in agreement with the data obtained in human acute myelogenous or promyelocytic leukemia cells treated with flavonoids [4, 24]. In contrast, DMSO-induced differentiation was not accompanied by

caspase-3 activation suggesting that at least in this system caspase-3 is not involved in differentiation pathways. While quercetin-treated K-562 cells are arrested in G<sub>2</sub>/M transition phase, DMSO blocked cell cycle progression in G<sub>0</sub>/G<sub>1</sub> phase. However, it is not possible to demonstrate whether caspase-3 activation in quercetin-treated cells is associated with the fraction of cells undergoing differentiation or apoptosis.

It is known that flavonoids may induce both apoptosis and differentiation in leukemic cells [25]. Therefore, we have attempted to assess the overall balance of apoptosis and differentiation in cell population with the aid of <sup>1</sup>H NMR-spectroscopy. An increased <sup>1</sup>H NMR-visible MLD formation has been reported as a peculiar feature of cells undergoing apoptosis or proliferating cells [15]. Earlier, we have observed MLD accumulation and Cho decrease in apoptotic malignant lymphoid cells upon quercetin treatment [17]. In the present study, the opposite spectral changes in cells undergoing erythroid differentiation were identified. Namely, DMSO-induced differentiation in K-562 cells was accompanied by the decrease of the CH<sub>2</sub>/CH<sub>3</sub> signal intensity ratio and the marked increase of Cho



resonance intensity being evident as early as 18 h after the onset of differentiation. These data are in line with those of Agris and Campbell [26] who found a dramatic increase of a signal at 3.2 ppm in Friend leukemia cells induced to differentiate by DMSO. Furthermore, it was proposed that an increased phosphorylcholine level might be a marker of differentiation in diverse cell systems (see in [27]). According to our data, the decrease in  $^1\text{H}$  NMR-visible MLD formation may be regarded as one of the features characteristic of the increased differentiation status of the cells, at least in the system of DMSO-induced differentiation in K-562 cells. As to quercetin treatment of K-562 cells, the data on MLD content suggest the overall shift towards apoptosis.

To sum up, our results demonstrated that quercetin in contrast to DMSO induced both apoptosis and erythroid differentiation in K-562 cells with the apoptotic trend in the overall effects. The potential role of the apoptotic effectors in the control of cell differentiation merits further investigation using other differentiating agents.

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### **АПОПТОЗ ТА ВМІСТ МОБІЛЬНИХ ЛІПІДНИХ ДОМЕНІВ В КЛІТИНАХ К-562 ЛЕЙКЕМІЇ ЛЮДИНИ ПРИ ЇХНЬОМУ ДИФЕРЕНЦІЮВАННІ, ІНДУКОВАНОМУ КВЕРЦЕТИНОМ АБО ДИМЕТИЛСУЛЬФОКСИДОМ**

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Визначали апоптотичний індекс, проходження клітинного циклу та активацію каспази-3 в клітинах К-562 в разі диференціювання, індукованого кверцетином або диметилсульфоксидом (ДМСО). Обробка клітин кверцетином супроводжувалась зупинкою клітинного циклу в фазі  $G_2/M$  та призводила до апоптозу і активації каспази-3. Навпаки, в разі диференціювання, індукованого ДМСО, спостерігали повну зупинку клітинного циклу у фазі  $G_1/G_0$

без активації каспази-3. Незважаючи на появу бензидин-позитивних клітин та зниження вмісту CD71 після інкубації з кверцетином, аналіз спектрів  $^1\text{H}$  ЯМР виявив баланс змін у бік апоптозу, а саме збільшення вмісту ЯМР-візуалізованих мобільних ліпідних доменів та зниження інтенсивності сигналу холін-вмісних метаболітів.

**Ключові слова:** апоптоз, каспаза-3, клітинний цикл, хронічна мієлоїдна лейкемія, ДМСО, флавоноїди, мобільні ліпідні домени, кверцетин.

### **АПОПТОЗ И СОДЕРЖАНИЕ МОБИЛЬНЫХ ЛИПИДНЫХ ДОМЕНОВ В КЛЕТКАХ К-562 ЛЕЙКОЗА ЧЕЛОВЕКА ПРИ ИХ ДИФФЕРЕНЦИРОВКЕ, ИНДУЦИРОВАННОЙ КВЕРЦЕТИНОМ ИЛИ ДИМЕТИЛСУЛЬФОКСИДОМ**

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Определяли апоптотический индекс, проходжение клеточного цикла и активацию каспазы-3 в клетках К-562 при индукции дифференцировки кверцетином или диметилсульфоксидом (ДМСО). Обработка клеток кверцетином сопровождалась остановкой клеточного цикла в фазе  $G_2/M$  и активацией каспазы-3. В противоположность этому при дифференцировке, индуцированной ДМСО, отмечали полную остановку клеточного цикла в фазе  $G_1/G_0$  без активации каспазы-3. Несмотря на появление бензидин-положительных клеток и снижение содержания CD71 после инкубации с кверцетином, анализ спектров  $^1\text{H}$  ЯМР выявил баланс изменений в сторону апоптоза, а именно, увеличение содержания ЯМР-визуализируемых мобильных липидных доменов и снижение интенсивности сигнала холин-содержащих метаболитов.

**Ключевые слова:** апоптоз, каспаза-3, клеточный цикл, хронический миелолейкоз, ДМСО, флавоноиды, мобильные липидные домены, кверцетин.

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