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RHAMNAZIN INHIBITS PROLIFERATION AND INDUCES APOPTOSIS OF HUMAN JURKAT LEUKEMIA CELLS *IN VITRO*

A. A. PHILCHENKOV, M. P. ZAVELEVYCH

R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, Kyiv;
e-mail: apoclub@i.ua

Antiproliferative and apoptogenic effects of rhamnazin, a dimethoxylated derivative of quercetin, were studied in human acute lymphoblastic leukemia Jurkat cells. The cytotoxicity and apoptogenic activity of rhamnazin in vitro are inferior to that of quercetin. The apoptogenic activity of rhamnazin is realized via mitochondrial pathway and associated with activation of caspase-9 and -3. The additive apoptogenic effect of rhamnazin and suboptimal doses of etoposide, a DNA topoisomerase II inhibitor, is demonstrated. Therefore, methylation of quercetin modifies its biological effects considerably.

K e y w o r d s: acute lymphoblastic leukemia, flavonoids, cell cycle, apoptosis, caspases, flow cytometry.

Flavonoids constitute the largest class (over 6,500 compounds) of biologically active plant polyphenols [1]. Most of them by far are derivatives of 2-phenylbenzopyran (flavan) or 2-phenylbenzopyran-4-one (flavone). Flavonoids display antioxidant properties and may also induce apoptotic cell death, depending on concentration [2]. It has been demonstrated that the proapoptotic and cytostatic activity of flavonoids could not be explained by their antioxidant or prooxidant properties only. Specific target molecules for flavonoids in tumor cells are currently under spotlight. The data available allows us to perceive certain flavonoids as signal transducer analogs, which makes them especially attractive as candidates for specific antitumor pharmaceuticals. Importantly, flavonoids are also known to potentiate or modify the effects of cytotoxic and antitumor agents and are thus viewed as suitable for adjuvant therapies.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the most studied of flavonoids. Its well-documented pleiotropic biological activity includes, beyond potent antioxidant properties, an antinflammatory, antiangiogenic, cytostatic, and proapoptotic effects (as cited in [3]). Cytotoxic effects of micromolar concentrations of quercetin and its derivatives towards human leukemia cells have been demonstrated [4-7]. Methylated quercetin derivatives with variations in the number and positions of methyl groups are common natural substances. Monomethylated derivatives have been shown to inhibit proliferation and induce apoptosis in cancer cells [8], or to sensitize malignant cells to other cytotoxic agents [9]. Yet there is only a limited amount of data concerning efficiency of rhamnazin (3′,7-dimethylquercetin), a quercetin derivative differing only by two methyl groups (Fig. 1), against infection agents [10]. Antiproliferative activity of rhamnazin has also been demonstrated recently *in vitro* in human cancer cell lines [11]. To the best of our knowledge, its effects upon leukemia cells have not been studied. Rhamnazin interaction with cytotoxic antitumor drugs is also unknown.

Physical-and-chemical as well as biological properties of methylated derivatives of quercetin may vary considerably, depending on the number and positions of methyl groups. There are few data relating certain properties of quercetin derivatives, namely their anticancer activity, to structural characteristics of the particular molecules [12]. The aim of the present work was to investigate *in vitro* biological effects of rhamnazin in human acute lymphoblastic leukemia Jurkat cells, in particular its ability to induce apoptosis and involvement of caspases in the induction of apoptosis in these cells. The effects of rhamnazin were compared to those of quercetin, which is important for attributing biological effects of flavonoids, e. g. quercetin and its dimethylated de-

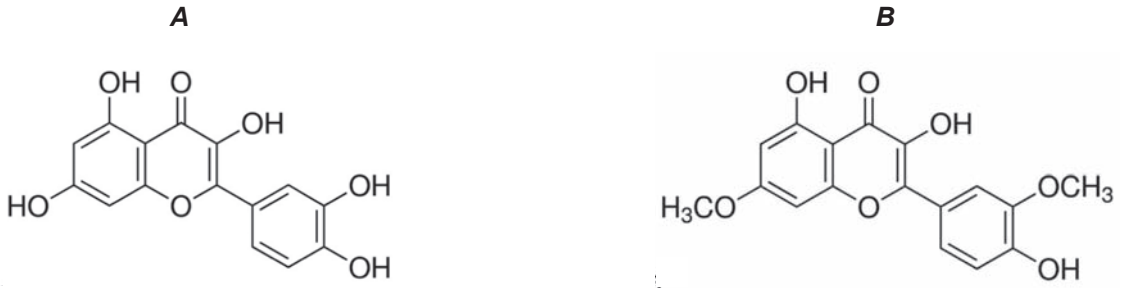

Fig. 1. Structure of quercetin (A) and rhamnazin (B)

rivative, to their structure, and for further targeted selection and synthesis of compounds with particular properties.

Materials and Methods

Human T-cell acute lymphoblastic leukemia cell line Jurkat was obtained from the National Collection of Cell Lines of the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). The cells were grown in RPMI-1640 medium with addition of 10% fetal bovine serum (Sigma, USA) and 2 mM of glutamine at 37 °C. The cells were subcultured at density of 0.8×10^6 to 0.9×10^6 per ml.

Rhamnazin (99% pure, Sigma, USA) was dissolved in dimethyl sulfoxide. Quercetin (Merck, USA) was dissolved in ethanol. The stock solutions were stored at -20 °C. Etoposide was purchased from Brystol-Myers Squibb SpA (Italy) as commercially available solution at a concentration of 20 mg/ml.

The investigated compounds were added into culture medium in the beginning of the logarithmic cell growth to analyze growth kinetics and cytotoxicity. Final concentrations of ethanol or dimethyl sulfoxide in culture media did not exceed 0.5%. Cell viability was determined by trypan blue exclusion.

Cells distribution by phases of cell cycle and percentage of hypodiploid (apoptotic) cells [13] were estimated after incubating them with propidium iodide (50 μg/ml) solution in 0.1% sodium citrate and 0.1% Triton X-100. Cell fluorescence was measured using FACScan flow cytometer (Becton Dickinson, USA). The percentage of cells containing active form of caspase-3 was assessed by flow cytometry using Caspase-3, Active Form, mAb Apoptosis Kit: FITC (BD Biosciences Pharmingen, USA) according to the procedure recommended by the manufacturer. The flow cytometry data were analyzed with ModFit LT2.0 software (Verity Software House, USA) and CELLQuest (BD Biosciences Pharmingen, USA).

For Western blot, cells were washed in phosphate buffer solution and lysed on ice in 20 mM Tris-HCl (pH 7.4), 1% Triton X-100 and 150 mM NaCl with addition of protease inhibitor cocktail (Roche Diagnostics, Germany). The lysate was centrifuged at 12,000 g, the supernatant was mixed with Laemmli sample buffer and boiled on water bath. Proteins were separated by electrophoresis in 12% PAAG with SDS and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, USA). The membranes were blocked for 12 h in 5% nonfat dry milk in phosphate buffered saline with 0.05% Tween 20 at 4 °C, then incubated with anti-caspase-9 (5B4 clone recognizing both proform and active form of caspase-9) or anti-caspase-8 (5F7 clone, recognizing only procaspase-8) monoclonal antibodies (Immunotech, France). To confirm equal protein loading, each membrane was probed with anti-β-actin MoAb (clone AC-15, Sigma, USA). Anti-mouse IgG conjugated with horseradish peroxidase (Promega, USA) was used as secondary antibody. The affinity binding bands were identified with enhanced chemiluminescence Amersham ECL Western Blotting Detection kit (GE Healthcare Life Sciences, USA).

The statistical analysis was performed using Student's *t*-test, the results were considered significant if $P < 0.05$.

Results and Discussion

Jurkat cells were incubated with 15 μM or 150 μM rhamnazin to evaluate its antiproliferative activity. The cells were counted after 24 or 48 h of incubation. According to the results, rhamnazin does not affect Jurkat cell growth kinetics at 15 μM, but inhibits cell growth noticeably if the concentration is increased tenfold (Fig. 2).

As antiproliferative activity is generally realized via cell cycle arrest, the next series of experiments involved investigation of rhamnazin effect on

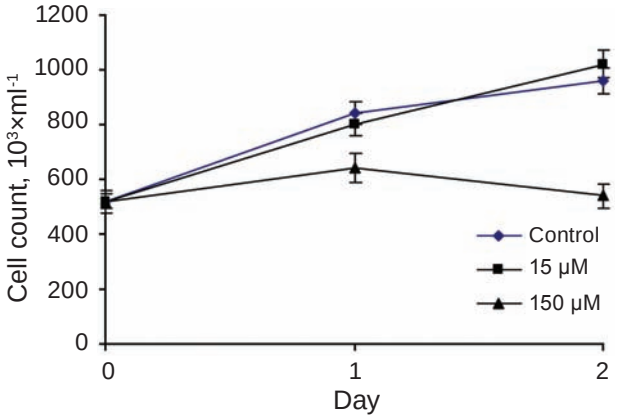

Fig. 2. Jurkat cell growth in culture incubated with rhamnazin

the mitotic cycle of Jurkat cells. The results indicate increased percentage of Jurkat cells in G_2/M phase if rhamnazin concentration is raised from 75 μM to 150 μM (Fig. 3), While the percentage of cells in G_2/M induced by 150 μM rhamnazin is significantly less as compared to that under effect of 40 μM quercetin, the total fraction of cells in phases S and G_2/M is approximately the same under both 150 μM rhamnazin and 40 μM quercetin due to the partial arrest in S phase of the cells incubated with rhamnazin.

Rhamnazin exhibited low toxicity in Jurkat cells even at the relatively high concentrations (150 μM) with only a minor death fraction as compared to that induced by quercetin (Fig. 4). These results corroborate the data of N. Li et al. [14], who showed that methylation of flavonols (including quercetin) in 3′ and 4′ position leads to significant decrease or total loss of cytotoxic properties.

In order to evaluate the contribution of apoptosis in overall cell death induced by high rhamnazin concentrations we analyzed in parallel hypodiploid cell fraction and the content of the active form of effector caspase-3. As demonstrated in Fig. 5, rhamnazin causes apoptotic cell death (Fig. 5), and apoptosis induction by rhamnazin is accompanied with increased number of cells with the active form of caspase-3. The maximum percentage of apoptotic cells and cells containing the active form of caspase-3 was detected after 72 h of incubation with rhamnazin. Delayed cell death and, probably, protracted inhibition of cell growth may in part explain cell cycle effects of rhamnazin in comparison to those of quercetin (see Fig. 3).

As at least two main pathways have been identified in cells for activation of caspase-3, namely receptor-mediated (via caspase-8) and mitochondrial (via caspase-9) [15], it was important to determine activation of these initiator caspases in rhamnazin-treated cells. To this end, we used immunoblot assay with monoclonal antibodies specific to procaspase-8 or active form of caspase-9. The active form of caspase-9 was detectable in Jurkat cells incubated with rhamnazin or quercetin (Fig. 6, *A*). On the other hand, the induction of apoptosis in Jurkat cells was not associated with changes in procaspase-8 content (Fig. 6, *C*). There is a good amount of evidence that caspase-8 activation is not restricted to receptor-

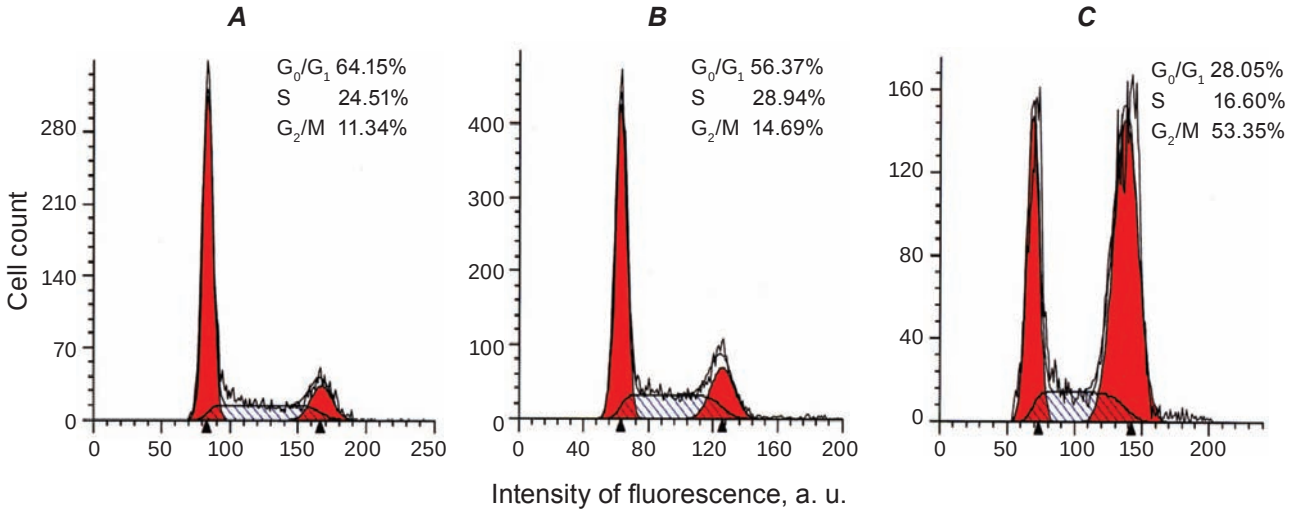

Fig. 3. Distribution of Jurkat cell population incubated for 48 h with rhamnazin or quercetin by cell cycle phases. A – 75 μM rhamnazin; B –150 μM rhamnazin; C – 40 μM quercetin

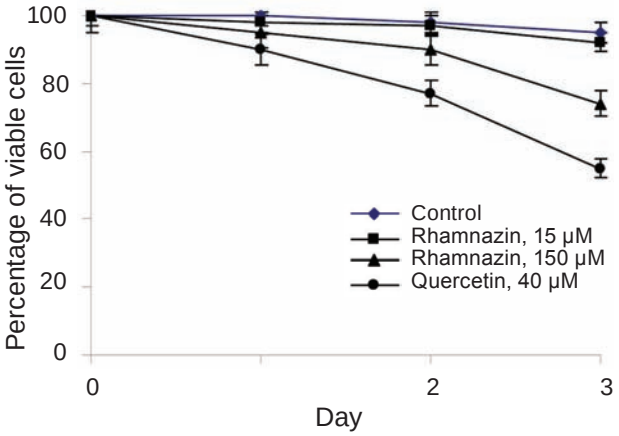

Fig. 4. Cell death of Jurkat cells incubated with rhamnazin

mediated apoptosis. Caspase-8 may be involved in apoptosis induced by various cytotoxic agents, including etoposide [16], although the significance of this phenomenon for activation of apoptotic effector pathways has not been yet clarified. Our data distinctly indicate caspase-8 involvement in apoptosis induction by etoposide in Jurkat cells. On the contrary, neither quercetin nor rhamnazin caused significant changes in procaspase-8 content in Jurkat cells, which is in accordance with the results of our previous studies [7]. It is worth noting that the pattern of involvement for various initiator caspases in response to an apoptotic inducer depends on cell type and experimental conditions. For instance, some authors failed to detect caspase-8 activation in apoptosis induction by quercetin, which corroborates

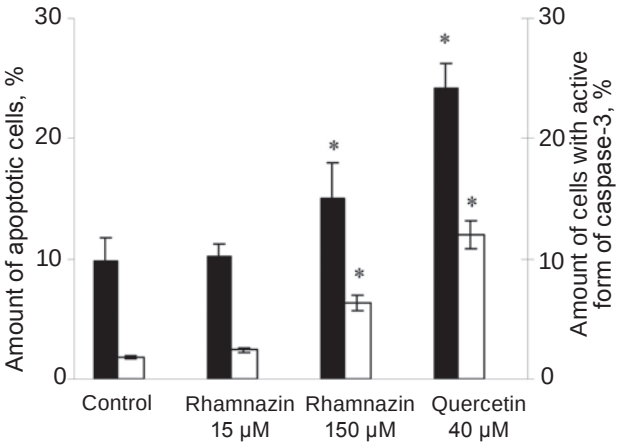

*Fig. 5. Induction of apoptosis (black bars) and caspase-3 activation (white bars) in Jurkat cells incubated for 72 h with rhamnazin or quercetin; * $P < 0.05$ compared to control*

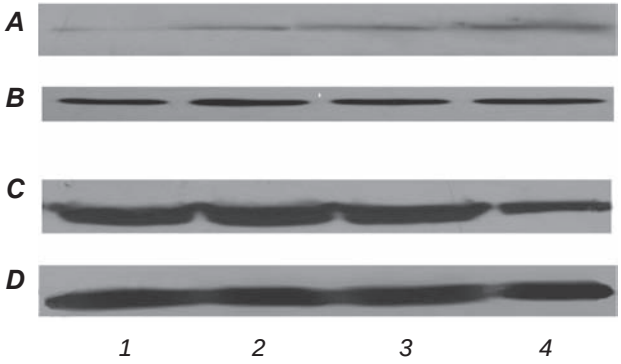

Fig. 6. Western blot assay of lysates of Jurkat cells incubated with rhamnazin or quercetin. Affinity binding of monoclonal antibodies against active form of caspase-9 (A) or procaspase-8 (C); anti-β-actin antibodies (B and D, correspondingly) were used to monitor the uniformity of loading per well: 1 – control; 2 – 150 μM rhamnazin, 48 h; 3 – 40 μM quercetin, 48 h; 4 – 2 μM etoposide, 18 h

our results [17]. On the other hand, caspase-8 activation by quercetin has been proven in other studies, i.e. in HL-60 cells [18].

Our results indicate the additive total effect by percentage of hypodiploid cells for induction of apoptosis by suboptimal dose of etoposide in cells that had been preincubated with rhamnazin for 72 h (Fig. 7). It is worth to note that the additive effect varied considerably with different modes of exposure to rhamnazin (data not shown), which requires further study in order to select the optimal conditions for combinations of the investigated compounds. It is well known that flavonoids, quercetin in particular, are not just capable of inducing apoptosis as single agents at higher concentrations, but may also interact with known apoptosis inducers – various classes of cytotoxic chemotherapeutic agents – and potentiate their cytotoxic and proapoptotic effects. For example, quercetin may potentiate cytotoxic effect of doxorubicin in human hepatocarcinoma SMMC7721 cells and cisplatin in human ovary adenocarcinoma cells [19, 20].

Therefore, in experiments with rhamnazin, a quercetin dimethoxylated derivative, we established that methylation of hydroxyl groups in 3′ and 7 positions significantly alters the biological activity of quercetin, in particular diminishing its cytotoxic potential and ability to induce apoptosis in Jurkat cells. However, at higher concentrations (150 μM) rhamnazin is capable of inhibiting cell growth, inducing

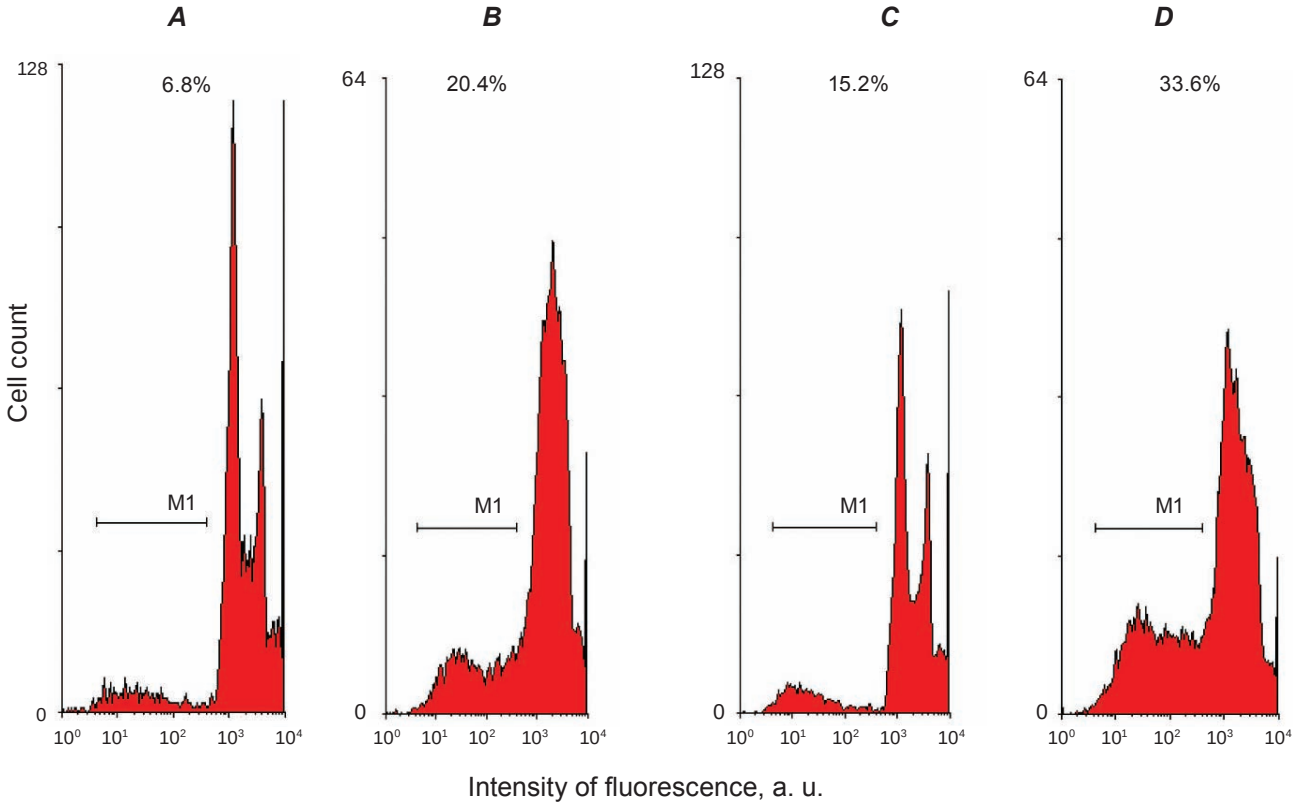

Fig. 7. Etoposide-induced apoptosis in Jurkat cells that had been incubated with rhamnazin. A – control; B – 0.5 μM etoposide, 18 h; C – 150 μM rhamnazin, 72 h; D – 0.5 μM etoposide, 18 h after incubation with 150 μM rhamnazin for 72 h. M1 bar represents cells with hypodiploid DNA content

apoptosis in a minor fraction of cell population without massive cell death. Rhamnazin arrests cells in G_2/M phase less efficiently than quercetin, although total increase in the number of cells in G_2/M and S phases is quite noticeable. The peculiar properties of phase-specific rhamnazin effects in comparison to those of quercetin may be explained in part by delayed cell death with protracted growth inhibition in cell population. Proapoptotic effect of rhamnazin is realized via mitochondrial pathway and involves activation of caspase-9 and caspase-3. We observed an additive effect on the percentage of hypodiploid cells in experiments with induction of apoptosis by suboptimal dose of etoposide, a DNA topoisomerase II inhibitor, in Jurkat cells pretreated with rhamnazin. Our results demonstrate a high investigative potential of studies on effects of structurally related flavonoid compounds, which is important for understanding of structure-function relations and for further search of substances with particular properties.

ПРИГНІЧЕННЯ ПРОЛІФЕРАЦІЇ ТА ІНДУКЦІЯ АПОПТОЗУ ЛЕЙКЕМІЧНИХ КЛІТИН ЛЮДИНИ ЗА ДІЇ РАМНАЗИНУ *IN VITRO*

О. О. Фільченков, М. П. Завелевич

Інститут експериментальної
патології, онкології та радіобіології
ім. Р. Є. Кавецького НАН України, Київ;
e-mail: apoclub@i.ua

На клітинах лінії Jurkat гострої лімфобластної лейкемії людини проаналізовано пригнічення проліферації та апоптогенні ефекти диметоксильованого похідного кверцетину – рамназину. Показано, що цитотоксична та проапоптотична активність рамназину *in vitro* зменшена в порівнянні з такою кверцетину. Проапоптотична дія рамназину реалізується за мітохондріальним шляхом і пов'язана з активацією каспази-9 і каспази-3. За індукції

апоптозу субоптимальними дозами інгібітора ДНК-топоізомерази II вепезиду в клітинах Jurkat, культивованих із рамназином, досягається адитивний ефект за відсотком гіподиплоїдних клітин. Таким чином, метилування кверцетину суттєво модифікує його біологічні ефекти.

Ключові слова: гостра лімфобластна лейкемія, флавоноїди, клітинний цикл, апоптоз, каспази, проточна цитометрія.

УГНЕТЕНИЕ ПРОЛИФЕРАЦИИ И ИНДУКЦИЯ АПОПТОЗА ЛЕЙКОЗНЫХ КЛЕТОК ЧЕЛОВЕКА ПРИ ДЕЙСТВИИ РАМНАЗИНА *IN VITRO*

А. А. Фильченков, М. П. Завелевич

Институт экспериментальной патологии, онкологии и радиобиологии им. Р. Е. Кавецкого НАН Украины, Киев;
e-mail: apoclub@i.ua

На клетках линии Jurkat острого лимфобластного лейкоза человека проанализировали угнетение пролиферации и апоптогенные эффекты диметоксилированного производного кверцетина – рамназина. Показано, что цитотоксическая и проапоптотическая активность рамназина *in vitro* снижена по сравнению с таковой кверцетина. Проапоптотическое действие рамназина реализуется митохондриальным путем и связано с активацией каспазы-9 и каспазы-3. При индукции апоптоза субоптимальными дозами ингибитора ДНК-топоизомеразы II вепезида в клетках Jurkat, культивированных с рамназином, достигается аддитивный эффект по процентному содержанию апоптотических клеток. Таким образом, метилирование кверцетина существенно модифицирует его биологические эффекты.

Ключевые слова: острый лимфобластный лейкоз, флавоноиды, клеточный цикл, апоптоз, каспазы, проточная цитометрия.

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