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JURKAT/A4 CELLS WITH MULTIDRUG RESISTANCE EXHIBIT REDUCED SENSITIVITY TO QUERCETIN

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Background: **While multidrug resistance of cancer cells is a well-known phenomenon, little is known on the cross resistance between cytotoxic chemotherapeutical agents and unrelated substances such as natural flavonoids.** ***Aim:*** **To compare the effects of cytotoxic drug, vepeside and natural flavonoid, quercetin in Jurkat cells and their multidrug-resistant subline Jurkat/A4, in particular to analyze the effector mechanisms of apoptosis and the profiles of several pro- and antiapoptotic proteins in these cells upon exposure to vepeside or quercetin.** ***Methods:*** **Apoptosis and poly (ADP-ribose) polymerase cleavage were assessed by flow cytometry. Expression of apoptosis-related proteins was analyzed by Western blotting.** ***Results:*** **Jurkat/A4 cells are less sensitive to antiproliferative effects of quercetin as compared with the parental Jurkat cell line. While vepeside as well as quercetin initially induces apoptosis in both cell lines, the following survival of the exposed cells is essentially different. In resistant Jurkat/A4 cells, vepeside or quercetin treatment activates significantly less caspase-9 and -3 as compared with that in the parental cells. The expression of Bad and BNip1 proteins in Jurkat/A4 cells is lower than in the parental cell line. At the same time, XIAP and CAS levels in Jurkat/A4 cells increase. Upon apoptosis induction, XIAP and CAS levels in Jurkat cells decrease, this effect being negligible in resistant cells.** ***Conclusion:*** **Multidrug-resistant Jurkat/A4 cells exhibit reduced sensitivity to cytotoxic effects of quercetin. The expression profile of Jurkat/A4 cells is characterized by the increased levels of XIAP and CAS representing the endogenous inhibitors of apoptosis.**

Key Words: **quercetin, vepeside, T-cell acute lymphoblastic leukemia, apoptosis, cell cycle, caspase-9, caspase-8, caspase-3, PARP, CAS, XIAP, Bad, BNip1.**

Evasion of apoptosis is one of the hallmarks of human cancers contributing to tumor formation and treatment resistance. The alterations in apoptosis signaling pathway often occur in drug-resistant cancer cells. In particular, defective apoptosis signaling may be caused by an increase in content of anti-apoptotic molecules and/or by a decreased content or impaired function of pro-apoptotic proteins. Thus, identification of novel substances for overcoming the drug resistance has gained much attention in cancer therapy.

Chemosensitivity of cancer cells to cytotoxic drugs has been attributed partially to their ability to trigger apoptosis. The natural multi-targeted agents such as flavonoids are considered at present as the potential sensitizers for apoptosis induced by a variety of anticancer drugs, which might be advantageous for achieving better treatment outcome in cancer patients [1]. Quercetin, one of the most widely distributed flavonoids in nature, has been reported to induce cell cycle arrest and apoptosis in human leukemia and lymphoma cells [2–6]. Several mechanisms of the apoptogenic effects of quercetin have been suggested, with death receptor-5, anti-apoptotic Bcl-2 and Bcl-x_L proteins, caspases, Akt/PKB kinase, Cu-Zn superoxide dismutase and heat shock proteins being among the cellular targets [7, 8]. In particular setting, quercetin was demonstrated to modify the phenotype of drug resistance in leukemic cells [9, 10]. Nevertheless, little is known on the cross resistance between cytotoxic chemotherapeutical agents and such unrelated substances as natural flavonoids. Therefore, analysis of apoptosis induction by flavonoids in drug-resistant and drug-sensitive cells may be advantageous for better understanding of the mechanisms of drug resistance as well as providing the clues for developing approaches aimed at utilizing the potential of these substances in overcoming drug resistance.

The aim of the study was to compare the effects of the conventional cytotoxic drug, vepeside and natural flavonoid, quercetin in Jurkat cells and their multidrug resistant subline Jurkat/A4, in particular to analyze the effector mechanisms of apoptosis and the patterns of several pro- and antiapoptotic proteins in these cells upon exposure to vepeside or quercetin.

MATERIALS AND METHODS

Chemicals and antibodies. Quercetin purchased from Sigma (USA) was dissolved in 96% ethanol and diluted in culture medium, with final concentration of ethanol being less than 0.1%. Vepeside was purchased from Brystol-Myers Squibb SpA (Italy). Anti-caspase-8 (clone 5F7 recognizing the proform of caspase-8) and anti-caspase-9 (clone 5B4) monoclonal antibodies (mAbs) were obtained from Immunotech (France). Anti-caspase-3 (clone 84803.111) and anti-β-actin (clone AC-15) mAbs were from Sigma. Anti-BNip1 (Bcl-2 Nineteen kilodalton interacting protein 1; clone 5),

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Abbreviations used: CAS – Crk-associated substrate; PARP – poly(ADP-ribose) polymerase; XIAP – X-linked inhibitor of apoptosis protein.

anti-CAS (Crk-associated substrate; clone 24), anti-XIAP (X-linked inhibitor of apoptosis; clone 28) and anti-Bad (clone 48) mAbs were from BD Pharmingen Transduction Laboratories (USA). The HRP-conjugated goat anti-mouse antibody was from Promega (USA).

Cell culture and treatment. Jurkat cell line originally derived from the peripheral blood of a 14-year-old boy with acute lymphoblastic leukemia [11] was obtained from the National Collection of Cell Lines of the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). The CD95-deficient cell clone Jurkat/A4 was derived from the Jurkat cell line by serial treatment with apoptosis-inducing anti-CD95 mAb (clone IPO-4, class IgM) [12]. The cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2 as suspension cultures in RPMI-1640 medium supplemented with 10% fetal calf serum. The cultures were passaged every 3–4 days upon reaching maximum cell density. Quercetin was added to the cells at exponential growth phase. Cell growth and viability were assessed by direct counting of trypan blue dye-excluding cells.

Flow cytometric analysis. The cells were resuspended in hypotonic buffer containing 0.1% sodium citrate, 0.1% Triton X-100, 5 μg/ml propidium iodide. 250 μg/ml of RNAse A was added to each cell sample, and the cells were stained for 15 min at 37 °C. Flow cytometry was performed on a FACSCalibur automated system (Becton Dickinson, USA) and data were analyzed using CellQuest software package and ModFit LT 2.0 program. The dead cells and debris were eliminated from the analysis based on forward and sideways light scattering. The percentage of cells containing cleaved form of PARP (poly(ADP-ribose) polymerase) was assessed by flow cytometry using FITC-conjugated mAb (clone F21–852; BD Biosciences). This antibody reacts only with the 89-kDa fragment of human PARP-1 produced by caspase cleavage.

SDS-PAGE and Western blotting. All cells were washed twice with ice-cold PBS and then lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl with freshly added protease inhibitor mixture (Roche, Germany) for 30 min at 4 °C. Protein samples were collected from supernatant after centrifugation of the samples at 12,000 x g for 15 min. The aliquots of lysates were mixed with 5× sample buffer containing 2-mercaptoethanol and boiled for 5 min before loading on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE separation, proteins were transferred to PVDF membrane (Immobilon-P, Millipore, USA). The membrane was blocked for 1 h at RT with 5% skim milk in PBS containing 0.05% Tween 20. The blots were incubated for 1 h at RT with an appropriate dilution of primary antibodies followed by their incubation with HRP-conjugated goat anti-mouse antibody for 1 h. After washing four times for 10 min each by PBS-Tween 20, the proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, UK) and Agfa X-ray film. For each immunoblot, equal loading of protein was confirmed by reprobing it with anti-actin mAb.

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

Effects of quercetin on cell growth and apoptosis induction in Jurkat and Jurkat/A4 cells. In contrast to the parental Jurkat cell line, survival of Jurkat/A4 cells is only slightly changed after exposure to vepeside or quercetin (Fig. 1). Therefore, Jurkat/A4 cells demonstrate the resistance not only to vepeside but also to quercetin. When the fraction of hypodiploid cells is analyzed, both vepeside and quercetin, in fact, induces apoptosis in the initial Jurkat cells and in the resistant clone as well (Fig. 2, *a*). Nevertheless, apoptosis induction in a fraction of resistant cells does not affect the following survival of cells in contrast to their initial counterpart.

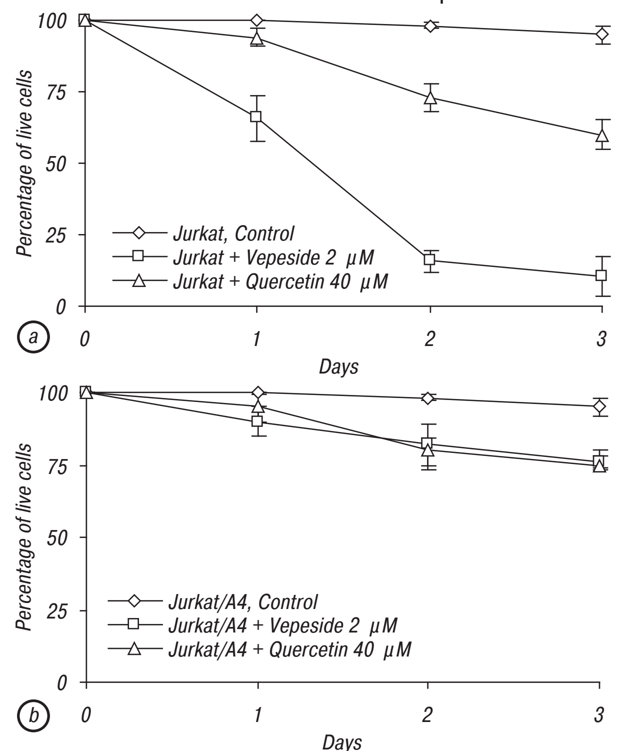

Fig. 1. Growth kinetics of Jurkat (*a*) and Jurkat/A4 cells (*b*) treated with vepeside or quercetin. Each time point represents the means ± S.D. of triplicate samples

Effects of quercetin on cell cycle progression in Jurkat and Jurkat/A4 cells. Both Jurkat and Jurkat/A4 cells show the similar patterns of redistribution in cell cycle phases upon the exposure to vepeside, namely the extensive cell arrest in G_0/G_1 is evident at the concentration of 2 μM (Fig. 3). Similarly, the effects of quercetin are essentially the same for both cell lines, namely the significant increase in cell population in G_2/M phase (see Fig. 3). This increase in G_2/M cell population is accompanied with a concomitant decrease in G_0/G_1-phase in Jurkat cells, while in Jurkat/A4 line both G_0/G_1- and S-phase percentage is reduced.

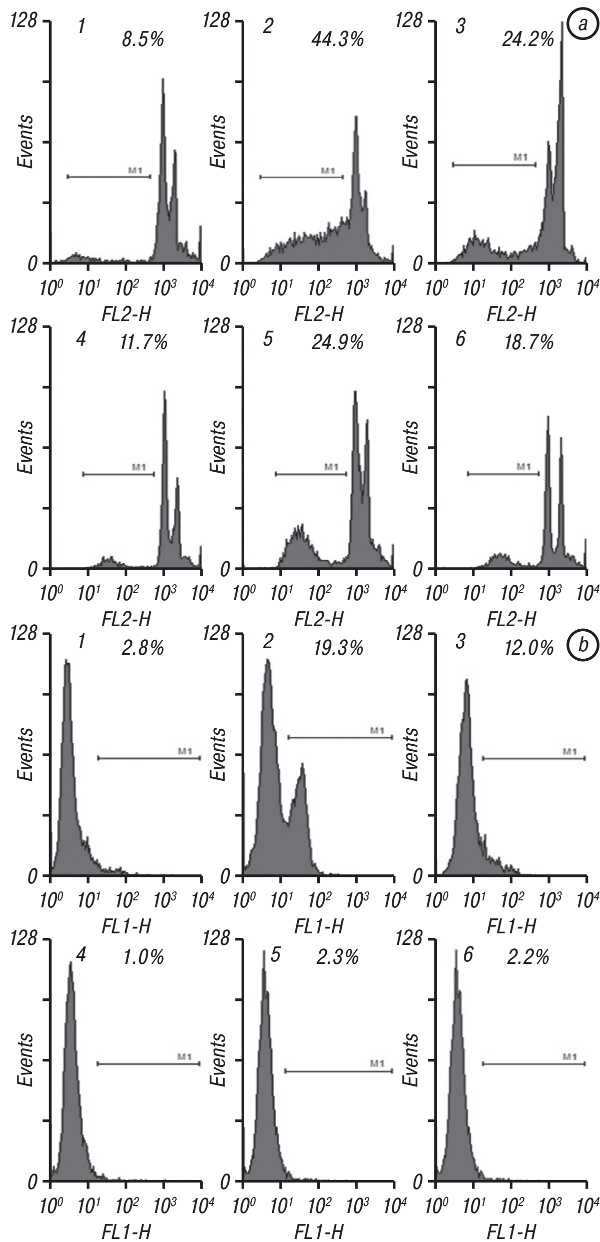

Fig. 2. Induction of apoptosis (*a*) and PARP cleavage (*b*) in Jurkat and Jurkat/A4 cells treated with vepeside or quercetin. *1, 4* — control; *2, 5* — 2 μM vepeside, 24 h; *3, 6* — 40 μM quercetin, 48 h. Each experiment was done in triplicate. The figures show a representative staining profile for 10,000 cells per experiment. M1 comprises the hypodiploid cells in (*a*) and cleaved PARP-positive cells in (*b*). The percentage of sub-G_1 cells or cleaved PARP-positive cells is given in each plot

Caspase-9 and -3 activation and PARP cleavage in Jurkat and Jurkat/A4 cells treated with vepeside or quercetin. The appearance of the active form of caspase-9 in Jurkat cells treated with vepeside or quercetin is evident (Fig. 4, *a*). Vepeside treatment activates reduced levels of caspase-9 in Jurkat/A4 cells as compared with that in parental Jurkat cells (see Fig. 4, *a*). The effect of quercetin on the activation of caspase-9 in resistant cells is negligible. In Jurkat cells, vepeside treatment results in decreasing level of procaspase-3 with accompanying appearance of the processed form (see Fig. 4, *a*). In Jurkat/A4 cells, in the immunoblot the slight band of the active form of caspase-3 is discernible. The treatment with vepeside or quercetin does not increase the intensity of the band in comparison with the initial one (see Fig. 4, *a*). As shown in Figure 2, *b*, treatment of Jurkat cells with vepeside or quercetin results in increased PARP cleavage. In comparison, no PARP cleavage is found in the Jurkat/A4 cells.

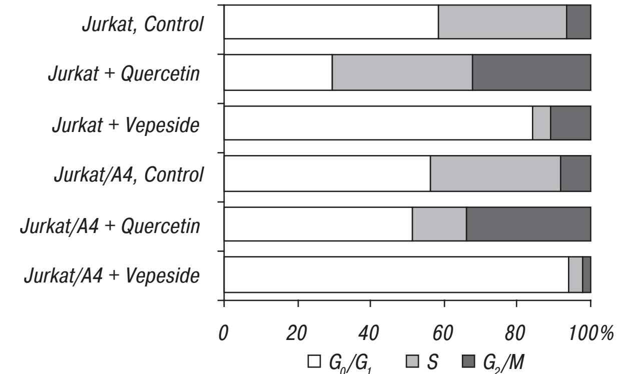

Fig. 3. Cell cycle distribution in Jurkat and Jurkat/A4 cells treated with vepeside or quercetin. After staining with propidium iodide, cells were analyzed by flow cytometry. The percentage of cells in G_1 (white bars), S (grey bars) or G_2/M phase (dark bars) are shown

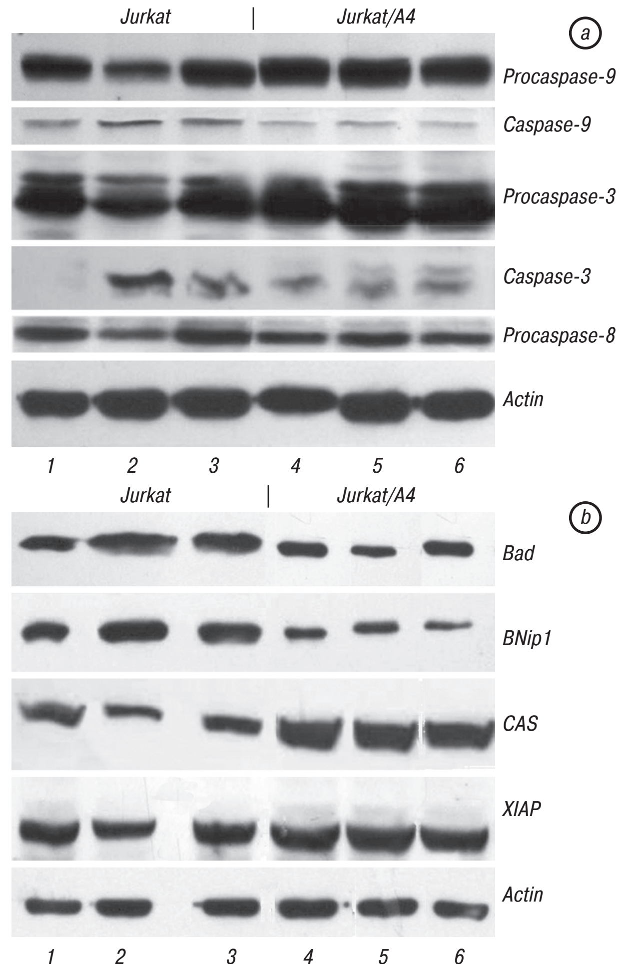

Fig. 4. Expression of caspases (*a*) and apoptosis-related proteins (*b*) in Jurkat and Jurkat/A4 cells treated with vepeside or quercetin. *1, 4* — control; *2, 5* — 2 μM vepeside; *3, 6* — 40 μM quercetin. Total cell lysates were prepared in SDS-Laemmli sample buffer and extracts were analyzed by Western blotting. Levels of β-actin are shown as the loading control

Procaspase-8 in Jurkat and Jurkat/A4 cells treated with vepeside or quercetin. The reduced ex-

pression of procaspase-8 is evident in Jurkat cells upon vepeside but not quercetin treatment. The effects of both vepeside and quercetin on the procaspase-8 level in Jurkat/A4 are insignificant (see Fig. 4, *a*).

Pro- and antiapoptotic proteins in Jurkat and Jurkat/A4 cells. The expression profiles of several apoptosis-related proteins were studied in Jurkat and Jurkat/A4 cells by Western blotting. The expression of Bad and BNip1 proteins in Jurkat/A4 cells is lower than in the parental cell line. At the same time, Bad and BNip1expression increases upon vepeside or quercetin treatment of Jurkat cells. Jurkat/A4 cells express more XIAP and CAS than parental Jurkat cells. Upon apoptosis induction with vepeside, XIAP and CAS levels in Jurkat cells decrease, this effect being negligible in resistant cells (see Fig. 4, *b*).

DISCUSSION

The serial treatment of Jurkat cells with the agonistic anti-CD95 mAb resulted in cross-resistance to cytotoxic substances [12], although other authors demonstrated that Jurkat cells resistant to CD95 and the parental cells were equally susceptible to anticancer drugs [13]. The appearance of drug resistance in cells selected for resistance to CD95-mediated apoptosis has been quite unexpectedly shown also in another T-cell line [14]. While CD95-deficient Jurkat/A4 cell line has been shown to possess cross-resistance to various unrelated cytotoxic agents including different anticancer drugs [15, 16], the underlying mechanisms of such multidrug resistance have not been completely elucidated. Several mechanisms have been proposed to be involved in the development of resistant phenotype of Jurkat/A4 as well as other resistant cell lines [16, 17]. It would be of interest to analyze the effector mechanisms of apoptosis and the profile of pro- and antiapoptotic proteins in the initial Jurkat cells and Jurkat/A4 cells with the phenotype of multiple drug resistance.

The typical mitochondrial apoptotic pathway is evident in Jurkat cells exposed to inhibitor of DNA topoisomerase II with activation of apical caspase-9 and effector caspase-3 and the cleavage of PARP as caspase substrate. The same features have been revealed in Jurkat cells treated with quercetin. Therefore, in addition to the cross-resistance of Jurkat/A4 cells to various unrelated chemotherapeutics demonstrated earlier [12], the present study reveales the resistance of Jurkat/A4 cells to the natural flavonoid. Quercetin as well as several other flavonoids is capable of inducing cytochrome *c*-dependent apoptosis with caspase-3 activation in variuos lines of leukemic cells [3, 4]. While in our study vepeside and quercetin initially induce apoptosis in both cell lines, the parameters of cytotoxicity and further viability of the parental and resistant cells differ drastically. In resistant cells, the increment in the percentage of hypodiploid cells upon the exposure to vepeside is less than in the initial Jurkat cells. Vepeside treatment activates reduced levels of caspase-9 and -3 in Jurkat/A4 cells as compared with that in parental Jurkat cells. The effect of quercetin on the activation of caspase-9 and -3 in resistant cells is negligible and the cleaved form of PARP is detectable in quercetin-treated Jurkat but not Jurkat/A4 cells.

The expression of CD95 in Jurkat/A4 cells, which are resistant to CD95-mediated apoptosis, is very low in comparison with the parental Jurkat cells (1–6% *vs* 70–80%) [12]. The processing of caspase-8 has been proved to accompany the mitochondrial pathway of apoptosis induced by various cytotoxic drugs, which is not necessarily required for drug-induced apoptosis *per se* [18]. In such setting, caspase-8 functions rather as an effector caspase in the mitochondrial pathway. We have shown the reduction in the content of 57-kDa proform of caspase-8 in Jurkat cells exposed to vepeside. This fact indirectly points to the processing of caspase-8 corroborating the data presented elsewhere [19]. Therefore, caspase-8 is activated by chemotherapeutic substances in the absence of a death receptor signaling. The level of procaspase-8 in resistant cells remains unchanged upon the exposure to vepeside. In contrast, the level of procaspase-8 remains unchanged in both Jurkat and Jurkat/A4 cells treated with quercetin. These results are in line with the absence of caspase-8 processing in the setting of quercetin-induced apoptosis in human hepatoma cells [20].

The drug resistance of cancer cells is a complex phenomenon comprising different intracellular processes. We have attempted to analyze the expression profile of several apoptosis-related proteins with the aim of evaluating the determinants of vepeside and quercetin resistance in Jurkat/A4 cells, in particular Bcl-2-related proteins, Bad and BNip-1, and the proteins with antiapoptotic properties, XIAP and CAS.

Bcl-2 family proteins are known to play a central role in apoptosis regulation. We have analyzed the expression of Bad as proapoptotic protein of Bcl-2 family and another proapoptotic protein BNip1. Both proteins belong to BH3-only proteins. The expression of Bad and BNip1 proteins in Jurkat/A4 cells turned out to be inferior to that in the parental Jurkat cell line. Therefore, the resistance phenotype in Jurkat/A4 cells is characterized by the reduced expression of both proapoptotic proteins. Upon the exposure both to vepeside and quercetin, the content of Bad and BNip1 proteins tends to increase in Jurkat cells while in Jurkat/A4 cells the levels of both proapoptotic proteins remain practically the same as in the cells without treatment.

On the other hand, expression profile of Jurkat/A4 cells is characterized by the increased level of XIAP representing one of the most potent endogenous inhibitor of caspases (both apical and effector ones). Our findings are in agreement to those of Wang *et al*. [21], who reported that drug resistant phenotype in U937 cells is associated with upregulation of XIAP. Increased CAS expression also is known to be associated with the resistance of cancer cells to chemotherapy (reviewed in [22]). In our study, the expression profile of Jurkat/A4 cells is characterized by the increase in CAS level.

To sum up, our data suggest that in Jurkat leukemia cells quercetin induces apoptosis mainly through a mi-

tochondrial-mediated pathway. Multidrug-resistant Jurkat/A4 cells are also resistant to cytotoxic effects of quercetin. The expression profile of Jurkat/A4 cells is characterized by the increased levels of XIAP and CAS representing the endogenous inhibitors of apoptosis. Further elucidation of the apoptotic pathways involved in resistance of Jurkat/A4 cells to chemotherapeutics will assist in designing more effective strategies to overcome drug resistance in cancer cells.

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