Flavonoid quercetin, but not apigenin or luteolin, induced apoptosis in human myeloid leukemia cells and their resistant variants*

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Received November 23, 2004

Flavonoids and their *in vivo* metabolites are neuroprotective, cardioprotective and chemopreventive agents acting as hydrogen-donating antioxidants or modulators functioning at protein kinase and lipid signaling pathways. In presented study treatments of human leukemia cells HL60 and their MDR-1 resistant subline HL60/VCR by flavonoids apigenin (API), luteolin (LUT), quercetin (QU) and anticancer drug doxorubicin (DOX) are reported. Of all flavonoids used only QU treatments led in both cell lines to DNA fragmentation, cleavage of poly (ADP-ribose) polymerase (PARP), up-regulation of proapoptotic Bax and posttranslational modification (phosphorylation) of antiapoptotic Bcl-2. Cytochrome *c* and $p21^{WAF1/CIP1}$ levels remained unchanged in these cells. Furthermore, treatments of both cell lines by QU and in its combined application with DOX increased phosphorylation of ERK, while Akt-1 and phosphorylated Akt-1 levels were not changed. All these events resulted in effective induction of apoptosis associated with down-regulation of P-glycoprotein (P-gp) in resistant cells. Presented results suggest that in human leukemia cells QU is a potent regulator of the cell apoptotic program associated with the modulation of several signaling molecules.

Key words: dietary flavonoids, quercetin, human leukemia cells, P-glycoprotein, apoptosis, flow cytometry, western blotting

Epidemiological studies have shown that the consumption of flavonoids is associated with a low risk of cardiatic and cancer diseases. Protective role of flavonoids is probably realized by inhibiting the activities of certain P450 isoenzymes against the induction of cellular damage by the activation of carcinogens [37]. Alternatively, their chemopreventive effects may result from induction of phase II metabolizing enzymes by which carcinogens are detoxified and eliminated from the body [3, 36]. As natural antioxidants flavonoids act against cancer through the serving hydroxyl groups and by scavenging superoxide anion, singlet oxygen, lipid pyroxi-radicals and/or stabilizing free radicals [11, 15].

Flavonoids are also effective at inhibiting and stimulation signal transduction enzymes, such protein tyrosine kinase (PTK), protein kinase C (PKC), phosphoinosite 3-kinases (PIP3) and MAP/MEK-1-ERK signal transduction pathways which are involved in the regulation of cell proliferation [12, 21, 25, 28].

Certain flavonoids have been considered to possess potent inhibitory activity against the drug exporting function of MDR1 (P-gp), MRP1 or MRP2 that extrude cytotoxic drugs at the expense of ATP hydrolysis by: a) inhibiting the overexpression of MDR-1, MRP1 or MRP2 genes, b) direct binding to nucleotide-binding domains, c) inhibiting ATP-ase activity, d) MDR1 independent mechanism. These events are often accompanied with the induction of apoptosis [10, 16, 17, 32].

We demonstrate here that of all flavonoids studied in both sensitive and multidrug-resistant leukemia cells only inhibition of cell proliferation by QU led to the induction of effective apoptotic events associated with DNA fragmentation, altered expression of some Bcl-2 family of proteins, PARP cleavage and down-regulation of P-gp in resistant cells. These events were linked with a fosforylation of signaling molecules ERK. Presented results showed the potential of QU, as an effective dietary compound, to cause events associated with programmed cell death.

*This work was supported by the Grant 2/4069/2004 from VEGA Slovakia.

Material and methods

Compounds. Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) and flavonoids (API, LUT and QU) were from Sigma. Protein inhibitor set was purchased from Boehringer. Rabbit polyclonal anti-Bcl-2, anti-Bcl-x_L, anti-Bax, anti-PARP, anti-$p21^{WAF/CIP}$, anti-cytochrome *c*, anti-ERK-1, anti p-ERK-1, anti-Akt-1, anti-p-Akt-1, anti-MDR-1, anti-actin IgGs, goat anti-rabbit-IgG HRP and the ECL chemiluminiscence reagent were purchased from Santa Cruz Biotechnology, CA, USA.

Cell culture. Human leukemia cell line HL60 and its multidrug-resistant subline HL60/VCR (with MDR-1 gene coding for P-gp) obtained from Dr. P. Ujhazy (Roswell Park Cancer Institute, Buffalo, USA) were grown in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, 100 µg/ml streptomycin and 2 mM L-glutamine in an atmosphere of 5% CO_2 in humidified air at 37 °C. In all experiments exponentially growing cells were used.

Cell viability assay. Cell viability was assayed in triplicate using MTT assay (24). Briefly, exponentially growing cells ($1.5x10^5$ cells/ml) were incubated in 96-well microtiter plates with medium containing various concentrations of drugs in the final volume of 200 µl. After 72 h drug incubation at 37 °C, 50 µl of MTT (1 mg/ml, Sigma) was added to each well followed by 4 h incubation at 37 °C. Cells were centrifuged, the supernate was discarded and the pellet was dissolved in DMSO (150 µl). $OD_{540\ nm}$ was determined by Microplate Autoreader (Labsystem Multiscan Multisoft, Finland) and Genesis software provided by the producer. MTT data were used to calculate efficiency of combined treatments with different flavonoids and DOX with the aid of CalcuSyn version 1.1 statistical software, when combination indexes (CI) represent synergistic (CI<1), additive (CI=1) and antagonistic (CI>1) effects of the drugs in combinations (flavonoid and DOX).

Flow cytometric analysis. Detergent-permeabilized cells (DNA analysis, apoptosis) or non-permeabilized (viability), as well as treated cells for FDA analysis [2] were measured by Altra Flow Cytometer, as described previously [30, 31].

DNA fragmentation. Harvested cells were lysed in digestion buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 0.5% Triton X-100 and 10 µg/ml proteinase K), at 37 °C for 1 h and then heated at 70 °C for 10 min. After RNA digestion with RNAse (100 µg/ml), at 37 °C for 1 h, lysates were cleared by centrifugation at 10,000 x g for 15 min. The DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1) for 1 min. and then centrifuged at 12,000 x g, at 4 °C for 10 min. The aqueous phase was precipitated with 2 volumes of ethanol overnight. Unfragmented DNA was discarded (12,000 x g, 10 min) and after adding of 3 volumes of 70% ethanol to the supernatant DNA was again centrifuged. The final DNA pellet was evaporated and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Extracted DNA was finally analyzed by electrophoresis in a 2% agarose gel in Tris-phosphate buffer (100 mM Tris, pH 8.0, 1.5 mM EDTA, 10 mM H_3PO_4). DNA fragments were visualized with ethidium bromide under UV light.

Western blot analysis. Cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.02% SDS, 2 mM sodium orthovanadate, 2 mM sodium fluoride and a mixture of protease inhibitors (1 mM PMSF, 1 mM pefabloc, 1 µM leupeptin, 1 µM pepstatin, 0.3 µM aprotinin). The lysates were then collected by microcentrifugation at 10,000 x g, at 4 °C for 10 min and used for SDS-PAGE. Equal amounts of lysate protein (50 µg/lane) were run in 12.5% SDS/PAGE gels and electrophoretically transferred to nitrocellulose. Blots were incubated with the corresponding rabbit polyclonal IgGs and antibody reaction with anti-rabbit IgG-HRP was visualized by chemiluminescence reagent (ECL).

Results

For the time-course and dose-dependent experiments human parental HL60 and MDR-1 resistant HL60/VCR cells were treated with API, LUT or QU and chemotherapeutical DOX used alone or in combination with flavonoids. Cell viability was assessed by the MTT assay. Figure 1 (a, b, c) shows

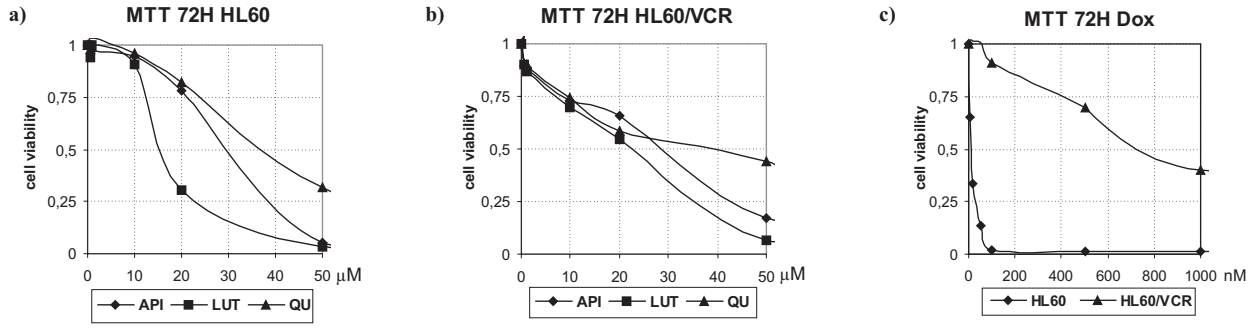

Figure 1. Influence of flavonoids (API, LUT and QU) and DOX on viability of HL60 (a) and HL60/VCR (b, c) cells. Cells were treated 72 hours with drugs at the concentrations indicated. Cell viability was measured with MTT assay as described in Material and methods.

that all flavonoids and DOX caused a dose-dependent reduction of cell viability with different cytotoxic effect upon the examined sensitive and resistant cells. By comparing IC_{50} of parental and resistant cells, both were similarly sensitive to all flavonoids used, however, MDR-1 positive cells exerted approximately 70 times higher resistance to DOX. Concurent treatment of the cells with API or LUT with DOX exerted no effect on cytotoxicity in both cell variants, while cotreatment of QU with DOX slightly decreased DOX cytotoxicity in parental cells (CI>1), but partially impaired survival (CI<1) in multidrug-resistant cells (data not shown).

Figure 2 (representative for QU and DOX) shows cytofluorometric measurements (Fig. 2a; side scatter SSC versus FL2 logarithmic scale) of parental and resistant cells treated with QU or in combined application with QU and DOX for 48 hours. These results indicate that both cell lines exerted increased portion of apoptotic cells after single treatment with QU, however co-treatment of flavonoid with DOX slightly reduced the portion of HL60 apoptotic cells, but not the portion of HL60/VCR apoptotic cells. Alternative cytofluorometric method, FDA staining, for detection of vital, apoptotic and death cells (shown equally suitable as Annexin-V staining) confirmed SSC results (Fig. 2b; PMT2 logarithmic scale versus PMT2 logarithmic scale). Cell cycle analysis (Fig. 2c; cell number versus FL3 linear scale) determined the presence of QU- or QU plus DOX-induced apoptotic cells (appearance of hypodiploid cells with subG0) and G2/M arrest in both cell variants.

Because apoptosis in mammalian cells has been shown to be regulated by Bcl-2 family of proteins and resulting in PARP cleavage and caspase activation, we were trying to determine whether flavonoid- or flavonoid- and DOX-induced apoptosis in HL60 and HL60/VCR cells was also associated with alterations in these molecules. To test this possibility, cell lysates from both sensitive and resistant cells were examined by western blot analysis (Fig. 3). By using anti-Bcl-2 family- and anti-PARP antibodies we analyzed whether apoptosis machinery was activated by single drug treatment or in their combinations wit DOX. We determined that of all flavonoids used in both cell lines, an 85 kDa cleaved PARP fragment appeared only after treatment with QU and also after co-treatment with QU and DOX. We showed further that QU or QU combined with DOX induced slight up-regulation of pro-apoptotic Bax, when compared with both parental and resistant cells. By inspecting of anti-apoptotic Bcl-2 and Bcl-x_L signals, an additional Bcl-2 band with slightly slower electrophoretic mobility appeared in all cells corresponding to apoptosis status, while anti-apototic Bcl-x_L profiles remained throughout unchanged. Furthermore, chemiluminescence signals with $p21^{WAF1/CIP1}$ and cytochrome *c* showed that these molecules remained at basal levels in all parental and resistant cells without respect to apoptotic status. Apoptotic events were in resistant HL60/VCR cells accompanied with a significant down-regulation of P-gp.

As QU induced accumulation of cells in G2/M and apoptosis in both cell lines examined (API or LUT did not, data not shown) possible activation of ERK, as downstream target MEK1/2, and Akt-1 kinase, as downstream substrate of PI-3 kinase, were examined. For investigation, cell lysates were subjected to western blot analysis using anti-ERK/anti--phospho-ERK and anti-Akt-1/anti-phospho-Akt-1 antibodies to detect basal or phosphorylated ERK and Akt-1 levels. Results of these studies showed unchanged expression of ERK in all non-apoptotic and apoptotic cells, however, a slight ERK phosphorylation appeared in sensitive apoptotic cells and a stronger in resistant cells treated with QU or QU combined with DOX. Interestingly, a sligh ERK phosphorylation also appeared only in resistant cells treated with API, LUT, DOX, as well as in their combinations with DOX. On the other hand, in both cell variants no observable changes in Akt-1 kinase were detected, either on the level of Akt-1 basal expression or Akt-1 phosphorylation. The above findings were supported by DNA electrophoretic analysis showing DNA fragmentation in all apoptotic cells.

Discussion

Several studies indicate that vegetables and fruits contain components that exert antiproliferative, chemopreventive and antineoplastic properties. The anthracycline antibiotic DOX is one of the most effective chemotherapeutic agents against a wide variety of cancers. In combined chemotherapy, to attenuate side effects of DOX, several antioxidants, inclusively flavonoids, are used [35]. In our experiments the only supplementation of QU or QU combined with DOX resulted in apoptosis in both cell types. In combined treatments with QU and DOX, a slight decrease of parental apoptotic cells, but not in resistant cells was observed. These findings correspond with our previous observations in murine leukemia cells [6]. On the other hand, combined treatment with QU and cis-Pt led in HL60 cells to a strong synergistic effect resulting in an effective DNA fragmentation [7].

In this study we showed that of all flavonoids studied, in both leukemic sensitive and resistant cells, only QU alone or in combination with DOX inhibited cell proliferation with the accumulation of cells in G2/M of the cell cycle and induced apoptosis. DNA fragmentation was accompanied by limited PARP cleavage but in corresponding apoptotic cells no cytochrome *c* release and alterations in regulation of $p21^{WAF1/CIP1}$ were detected. These observations are partially in discrepancy with those found in human LNCaP prostate carcinoma cells, where increased levels of $p21^{WAF1/CIP1}$, induced by API and LUT but not by QU, were detected [18]. On the contrary, in human melanoma cells QU up-regulated $p21^{WAF1/CIP1}$ which correlated with G1 cell cycle arrest [4]. Growth inhibitory effect by API in human DU145 prostate carcinoma cells was associated with $p21^{WAF1/CIP1}$ increase and G1 arrest. These events were independent of p53 and resulted in alteration in Bax/Bcl-2 ratio in favour of apoptosis associated with the release of cytochrome *c*, PARP cleavage and

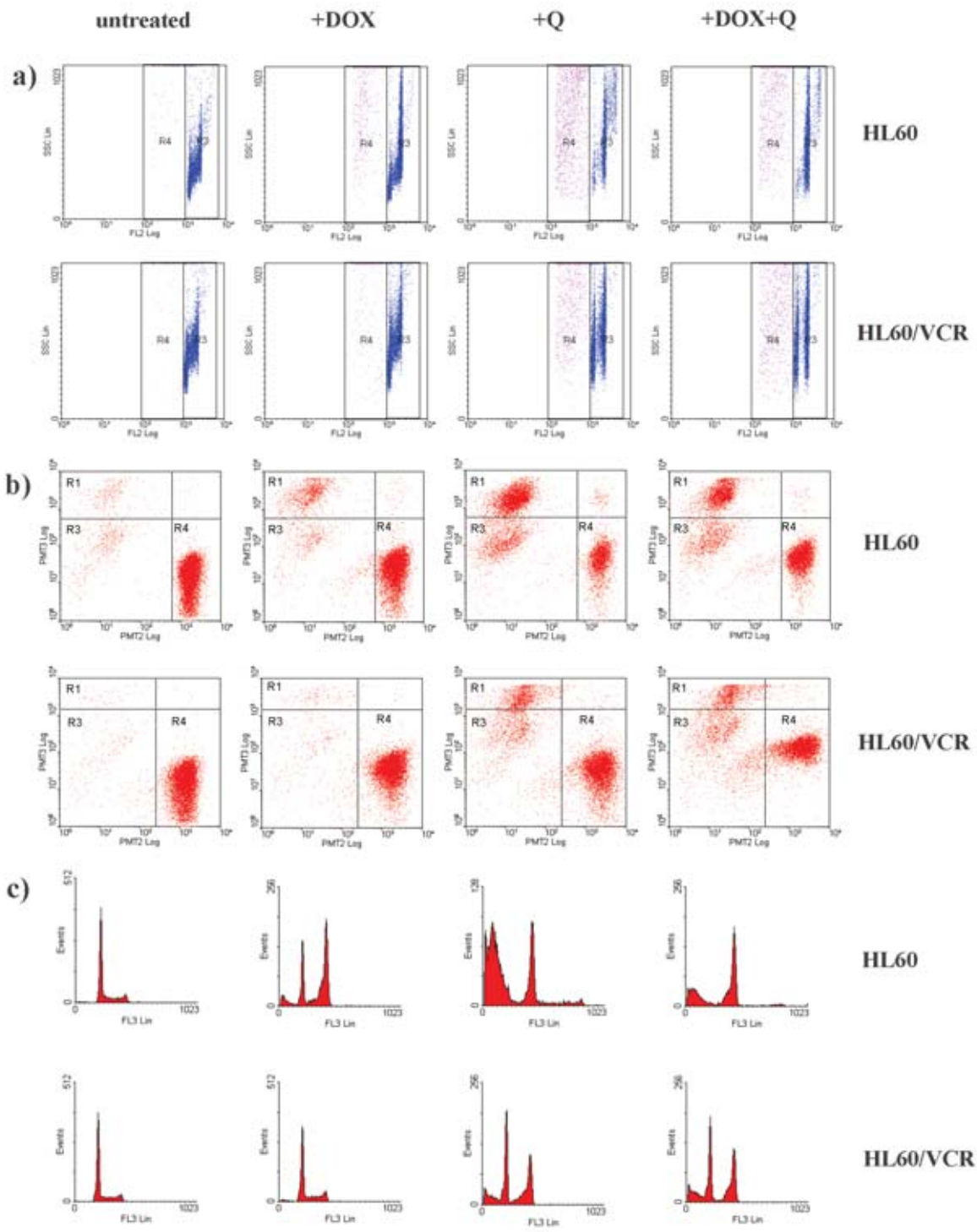

Figure 2. a) Cytofluorometry (representative for QU and DOX) of detergent-permeabilized, PI-stained HL60, HL60/VCR cells treated with drugs in concentrations: QU (35 µM for HL60; 40 µM for HL60/VCR), DOX (10 nM for HL60; 700 nM for HL60/VCR), or in combined applications (QU plus DOX). Abscissa: FL2-PI staining, DNA content, logarithmic scale. Ordinate: SSC (side scatter), R4 – apoptotic cells, R3 – cells in the cell cycle.
b) Dot plot analysis of FDA stained cells. R1 – necrotic cells (FDA-/PI+), R3 – apoptotic cells (FDA-/PI-), R4 – viable cells (FDA+/PI-). Abscissa: PMT2 logarithmic scale. Ordinate: PMT3 logarithmic scale.
c) Cytofluorometry of cells in cell cycle. Abscissa: FL3 (red fluorescence of incorporated PI in detergent-permeabilized cells on linear scale. Ordinate: relative number of cells.

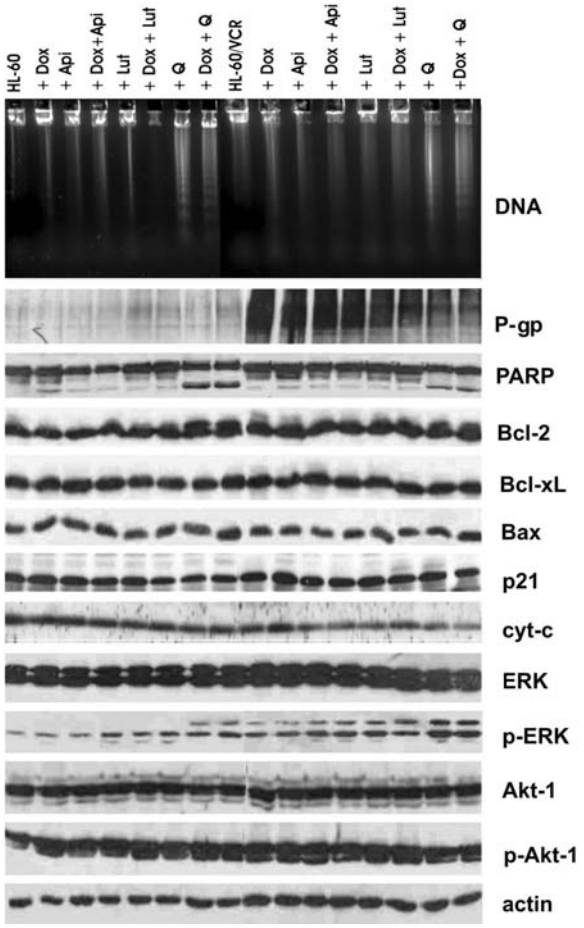

Figure 3. Western blotting analysis of apoptotic/antiapoptotic proteins and DNA fragmentation of HL60 and HL60/VCR cells. Cells were treated with flavonoids API (30 μM for both HL60 and HL60/VCR), LUT (15 μM for HL60, 25 μM for HL60/VCR) and QU (35 μM for HL60, 40 μM for HL60/VCR), DOX (10 nM for HL60, 700 nM for HL60/VCR), or in combined applications of flavonoids and DOX. Cell lysates were prepared and handled as described in Material and methods. Rabbit polyclonal anti-actin IgG was used as reference antibody.

DNA fragmentation [13, 34]. In our experimental conditions administration of QU resulted in a slight up-regulation of Bax, unchanged expression of Bcl-x_L, while the expression Bcl-2 protein was accompanied by the appearance of an additional protein with slightly slower electrophoretic mobility apparently corresponding to phosphorylated Bcl-2. These results are partially in contrast with experiments where leukemia cells HL60 treated with QU exerted apoptotic characteristics including an increase of DNA ladder intensity, but no changes in Bax, Bcl-2 an Bcl-x_L levels were observed [32]. Furthermore, in human A549 lung cancer cells the treatment with QU led to a PARP cleavage, and increase in Bax and in Bcl-x_L but a decrease in Bcl-2 [25]. Interestingly, in HeLa cells QU was able to block taxol-induced phosphorylation of Bcl-2 [22]. We speculate that phosphorylation of Bcl-2 by QU allow less Bcl-2:Bax complex and Bad would become bound to Bcl-2 that could lead to sequestering Bcl-2 and Bcl-x_L. The result of the net effect would be more free Bax and its translocation to mitochondrial membrane [5].

To demonstrate whether tested flavonoids influenced apoptosis signaling, ERK and PI-3 signal transduction pathways were studied. Presented data showed unchanged expression of basal ERK levels in all non-apoptotic and apoptotic cells treated with flavonoids and DOX. However, a slight ERK phosphorylation appeared in HL60 apoptotic cells and a stronger in apoptotic and non-apoptotic HL60/VCR cells treated with API, LUT, QU and DOX, as well as in those combined with DOX. Akt-1 kinase, shown as downstream substrate of PI-3 kinase, regulates Bcl-2 family of proteins, presumably Bad [9, 25]. In the present study, in sensitive or resistant cells no observable alterations of Akt-1 kinase in all experiments with flavonoids and DOX, either at the level of basal expression or phosphorylation, were detected. In human A549 lung cancer cells QU induced apoptosis associated with phosphorylation of ERK, inactivation of Akt-1 and down-regulation of Bcl-2 [25]. Interestingly, it has been shown that ERK activation may exert either anti-apoptotic or pro-apoptotic effect depending upon the cellular context [1, 23].

Several approaches were proposed for multidrug resistance reversal including targeting apoptosis pathways. Here we provided the evidence that of all flavonoids used only administration of QU or its co-treatment with DOX decreased basal levels of P-gp in resistant cells assumed as an apparent consequence of apoptotic events. Various and contradictory results have been reported for flavonoid effects upon multidrug resistance reversal depending upon the type of cancer cells and chemotherapeutical drugs used. In this context, some results showed that QU restored sensitivity to DOX in multidrug resistant MCF-7 cells and other cell lines [8, 27, 29] or inhibited the Pg-p-mediated transport of the fluorescent probe, at least in part by inhibiting the ATP-binding site at the nucleotic binding site NBD2 [32]. QU was also reported to suppress multidrug resistance via P-gp-independent mechanism when MDR1 promoter in P388/M resistant cells has been shown to contain heat shock element (HSE) and showed constitutively activated heat shock factor (HSF) suggesting that HSF might be an important target for reversal the MDR1 resistance [16]. It has also been documented that some QU derivates appeared more effective in MDR1 modulation. Pentamethyl-pentaalyl QU derivates effectively reversed P-gp resistance in DOX-resistant K562/ADM cells [14]. Further, experiments in human breast cancer cells showed that QU affected MRP-1 transport activity, but it did not affect MRP-2-mediated transport activity [38]. In other experiments QU stimulated MRP-1-mediated GSH transport [19] or reduced vincristine resistance in MRP-1-transfected HeLa cells and in human pancreatic Panc-1 cells expressing MRP-1 protein [20, 26].

The presented data in human leukemia cells support a con-

siderable role of QU in growth inhibition and in regulation of apoptosis involving a set of cellular genes including Bcl-2 related family members and genes representing signaling pathways family, as well as the possibility of QU for using either as a single agent or in combination with other chemotherapeutic agents in cancer chemoprevention and anticancer therapy.

The excellent technical cooperation of J. CHOVANCOVA is greatly appreciated.

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