Diverse resveratrol sensitization to apoptosis induced by anticancer drugs in sensitive and resistant leukemia cells*

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Naturally occuring dietary compound resveratrol (RES), possessing chemopreventive and cytostatic properties, has been shown as potent sensitizer for apoptosis induced by a variety of anticancer drugs. Cell cycle analysis in sensitive promyelocytic leukemia HL60 cell line and its multidrug-resistant variant HL60/VCR (P-gp positive) treated with RES resulted in cell cycle arrest in S-phase in both cell variants. Flow cytometry measurements showed diverse activities of RES in combination with anticancer drugs doxorubicin (DOX), cycloheximide (CHX), busulfan (BUS), gemcitabine (GEM) and paclitaxel (PTX), in some cases resulting in apoptosis induction, preferentially at the expense of S-phase. Thus, RES could become a candidate to enhance the efficacy of combination anticancer therapy in a variety of human cancer cells inclusive leukemias.

Key words: human leukemia cells, resveratrol (RES), anticancer drugs, combination treatments, cell cycle, flow cytometry

Resveratrol (3,4',5-trihydroxystilbene, RES), a natural polyphenol compound found in fruits and red wine, has been regarded as a promising candidate for clinical management of cancers [1, 2]. It has been suggested that RES may be a main compound involved in the "French paradox", the inverse correlation between red wine consumption and the incidence of cardiovascular diseases [3].

RES has been shown to exhibit estrogenic [4], antiplatelet aggregation activity [5], anti-inflammatory [6] and neuroprotective effects in mammals [7]. As a naturally occuring antioxidant, free-radical scavenger, RES has gained considerable attention because of its chemopreventive [8], cardiopreventive, canceropreventive or direct antitumor activity [9] and cytostatic properties in a variety of human tumors, where it causes cell cycle arrest in the S- and G2/M phases [10, 11]. RES exhibited its genotoxic activity in some cells by inhibiting microtubule assembly resulting in mitotic alterations [12].

Several studies revealed that RES is capable to promote differentiation and committ to Fas-independent apoptosis [13]. Furthermore, the compound was shown as a potent sensitizer for anticancer drug-induced apoptosis in a variety of human tumor cell lines by inducing cell cycle arrest in a p53-independent manner [14].

Current attempts to improve the survival of cancer patients largely depend on strategies to overcome cell drug resistance. In search for strategies to enhance efficacy of anticancer drugs CHX, BUS, GEM, DOX and PTX, respectively, to induce apoptosis and to overcome resistance in cancer cells, we tested antitumor activities of RES alone or with cooperation with these anticancer drugs in human leukemic cell line HL60 and in multidrug-resistant subline HL60/VCR. We found that RES mediated cell cycle arrest in S-phase and, in some cases, it was a prerequisite for anticancer drug-induced apoptosis in studied cells. Various aspects of interactions of RES with selected anticancer drugs in studied leukemia cell variants are discussed.

Material and methods

Cell culture. Human leukemia cell line HL60 and its multidrug-resistant subline HL60/VCR (with *MDR-1* gene coding for P-gp) 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. Cells in concentration of $5x10^5$ cells/ml were

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cultured in 96, 24- or six well plates. In all experiments exponentially growing cells were used.

Determination of cell viability and proliferation. Cells were preincubated with RES for 24 h and then treated with CHX, BUS, GEM, DOX and PTX (all from Sigma), respectively, at indicated concentrations for 48 h. Cell survival was assayed in triplicates using MTT assay [15]. 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 of drug incubation at 37 °C, 50 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, 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 cell pellet was dissolved in DMSO (150 µl). OD_{540nm} was determined by Microplate Autoreader (Labsystem Multiscan Multisoft, Finland) and Genesis software provided by the producer. Mean ±S.D. at least tree experiments were used.

Flow cytometric analysis. Detergent-permeabilized cells propidium iodide (PI, Sigma) stained nuclei (DNA analysis, apoptosis) or non-permeabilized (viability) were measured with the aid of Altra Flow Cytometer, as described [16]. Mean ±S.D. at least of two experiments were used.

Results

Incubation of the sensitive HL60- or resistant HL60/VCR cells with RES resulted in inhibition of proliferation in dose-dependent manner, determined as relative cell viability calculated from MTT assay (Fig. 1). Concentrations of 15- or 20 µM RES for parental or resistant cells (concentrations that at the time 48 h were mildly cytotoxic but significantly inhibited cell growth) were selected and used in all combination experiments with RES and/or anticancer drugs here reported.

Whether RES can induce apoptosis in both cell variants, 0–50 µM RES concentrations for 48 h were used to evaluate subG1 cell populations and cell cycle kinetics. As shown in Figure 2, RES in concentrations ≥15- (for HL60) or ≥20 µM (for HL60/VCR) induced an increase (>5%) of the hypodiploid cells accumulated in subG1 phase in dose dependent manner. Since differential sensitivity to apoptosis has been linked to distinct phases of the cell cycle, we analyzed in more detail the effect of RES on the cell cycle. Cells were stained with propidium iodide for cell cycle analysis or apoptosis (hypodiploid subG1 cells) and analyzed by flow cytometry. RES, in dose dependent manner, caused in both cell types cell cycle phase distribution with an increase of cells in S-phase associated with a concomitant reduction of the cell portion predominantly in G0/G1 phase.

Finding the relatively low cytotoxicity of RES as a single agent at selected concentrations 15- or 20 µM in studied cells, we tested RES in combination with conventional anticancer compounds which belong to diverse classes of anticancer drugs. We used the compounds related to antifungal antibiotics (CHX), alkylating agents (BUS), antimetabolites (GEM), anthracyclines (DOX) and taxanes (PTX), in concentrations calculated from MTT assay (data not shown). Dose response experiments presented in Figure 3 (selected representative drug concentrations) or in Figure 4 (all concentrations of anticancer drugs used) revealed that pre-incubation of cells with RES for 24 h followed with selected anticancer agents up to 48 h led to diverse levels of sensitization in HL60- or HL60/VCR cells resulting in some cases in apoptosis.

Parental cells and their resistant variants exerted similar relatively low sensitivity to antifungal antibiotic CHX. In con-

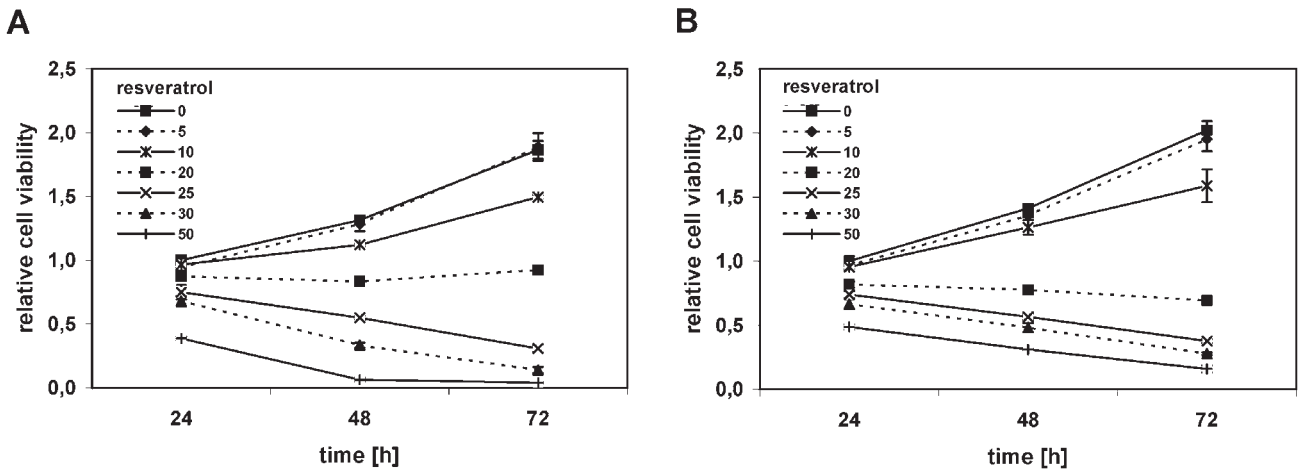

Figure 1. Relative cell viability of (A) HL60- and (B) HL60/VCR cells after RES (0–50 µM) treatments. Cells were seeded at $5x10^3$ cells/well in 96-well TC-microplates and treated for 72 hours with RES at the concentrations indicated. Relative cell viability was measured spetrophotometrically with MTT assay. Data represent mean ±SD of tree replicate experiments.

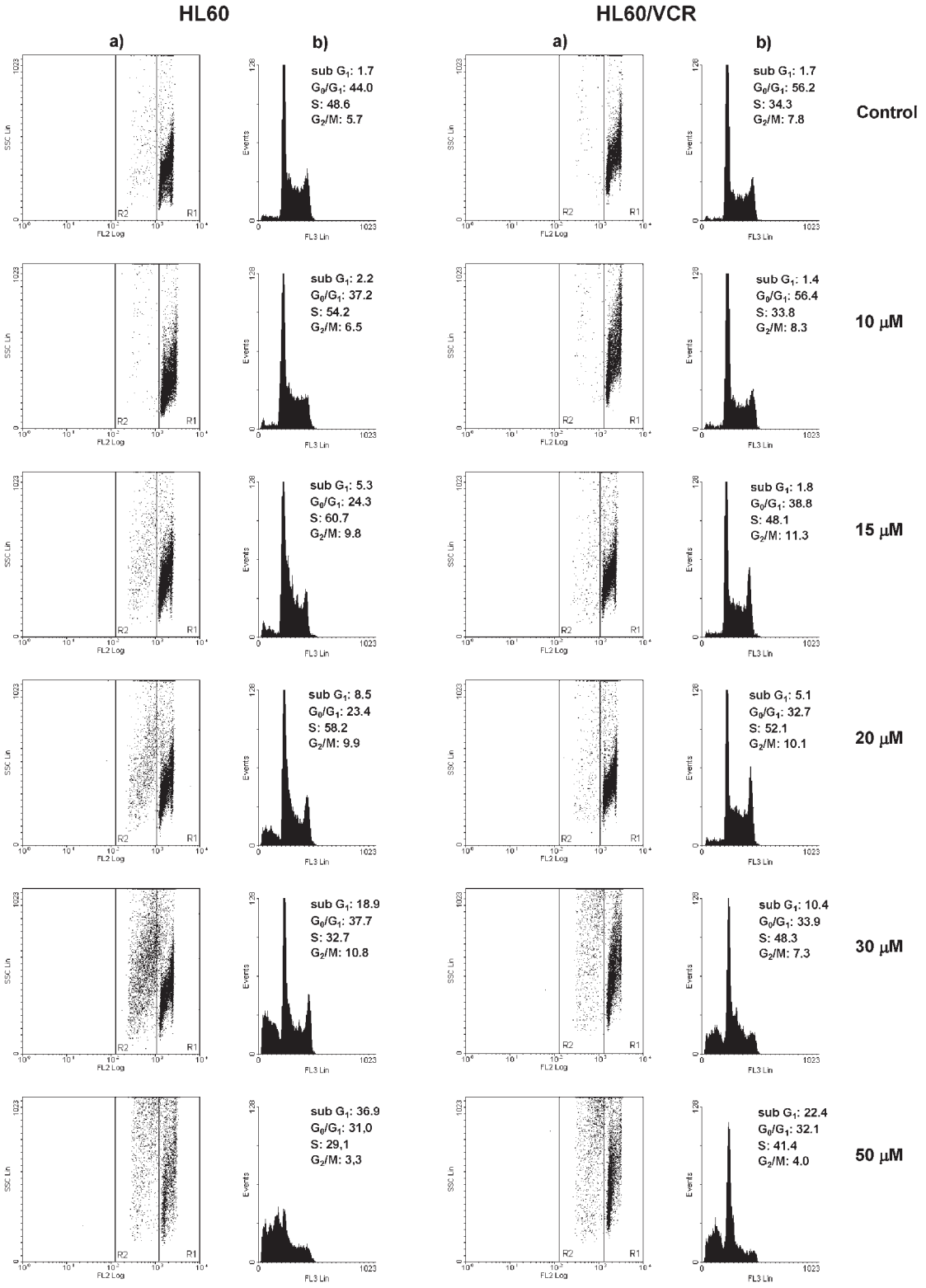

Figure 2. Effect of RES (0–50 μM) on cell cycle and apoptosis (subG1) of HL60- and HL60/VCR cells measured after 48 h by flow cytometry of propidium-iodide (PI) stained DNA (representative results of the two experiments are shown): a) abscissa: FL2-PI staining, DNA content, logarithmic scale; ordinate: SSC (side scatter); Gate: R1 – cells in the cell cycle; R2 – apoptotic cells; b) abscissa: FL3 (red fluorescence of incorporated PI in detergence-permeabilized cells on linear scale); ordinate: relative cell number of detergent permeabilized, PI – stained cells.

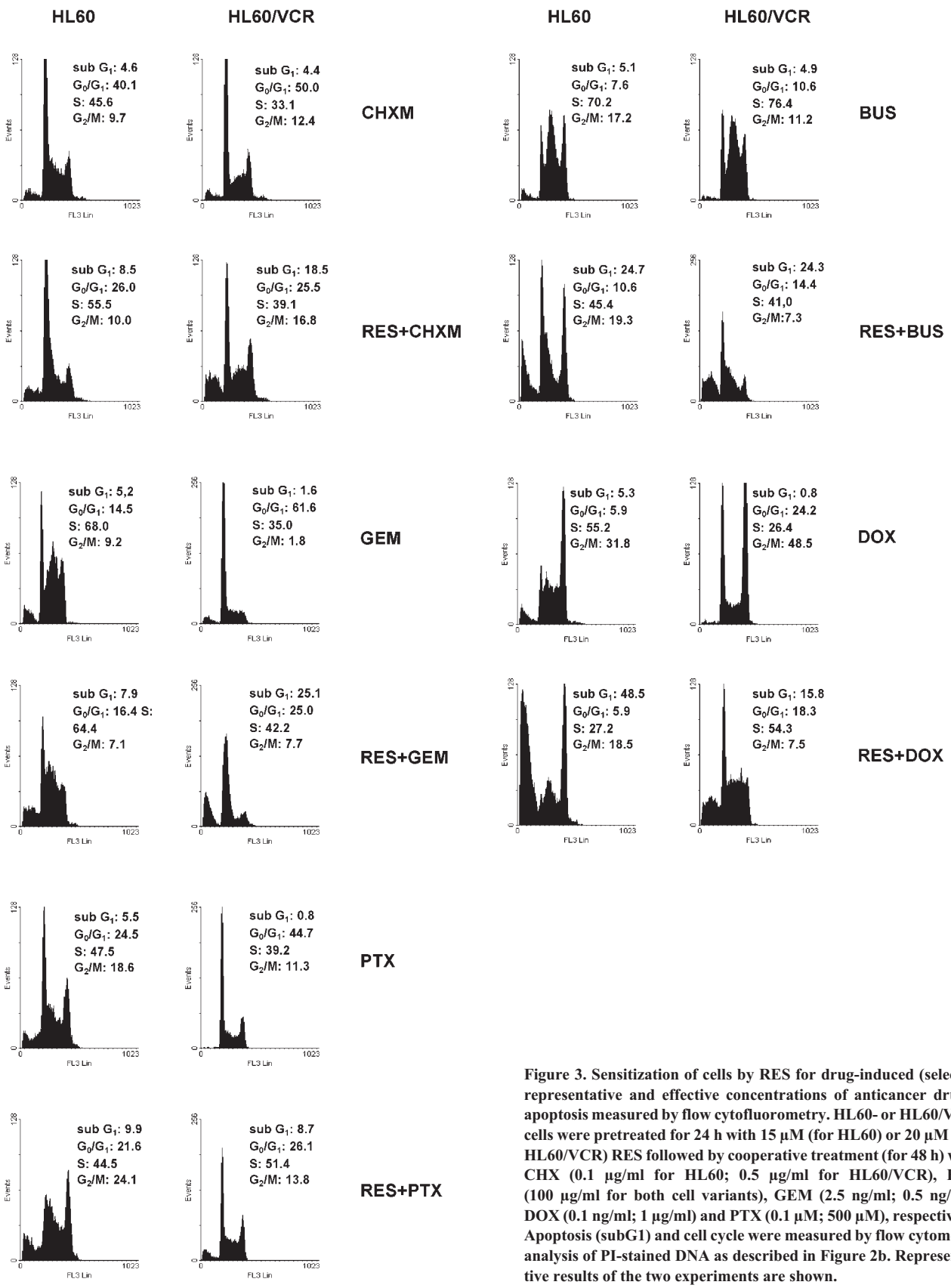

Figure 3. Sensitization of cells by RES for drug-induced (selected representative and effective concentrations of anticancer drugs) apoptosis measured by flow cytofluorometry. HL60- or HL60/VCR cells were pretreated for 24 h with 15 μM (for HL60) or 20 μM (for HL60/VCR) RES followed by cooperative treatment (for 48 h) with CHX (0.1 μg/ml for HL60; 0.5 μg/ml for HL60/VCR), BUS (100 μg/ml for both cell variants), GEM (2.5 ng/ml; 0.5 ng/ml), DOX (0.1 ng/ml; 1 μg/ml) and PTX (0.1 μM; 500 μM), respectively. Apoptosis (subG1) and cell cycle were measured by flow cytometry analysis of PI-stained DNA as described in Figure 2b. Representative results of the two experiments are shown.

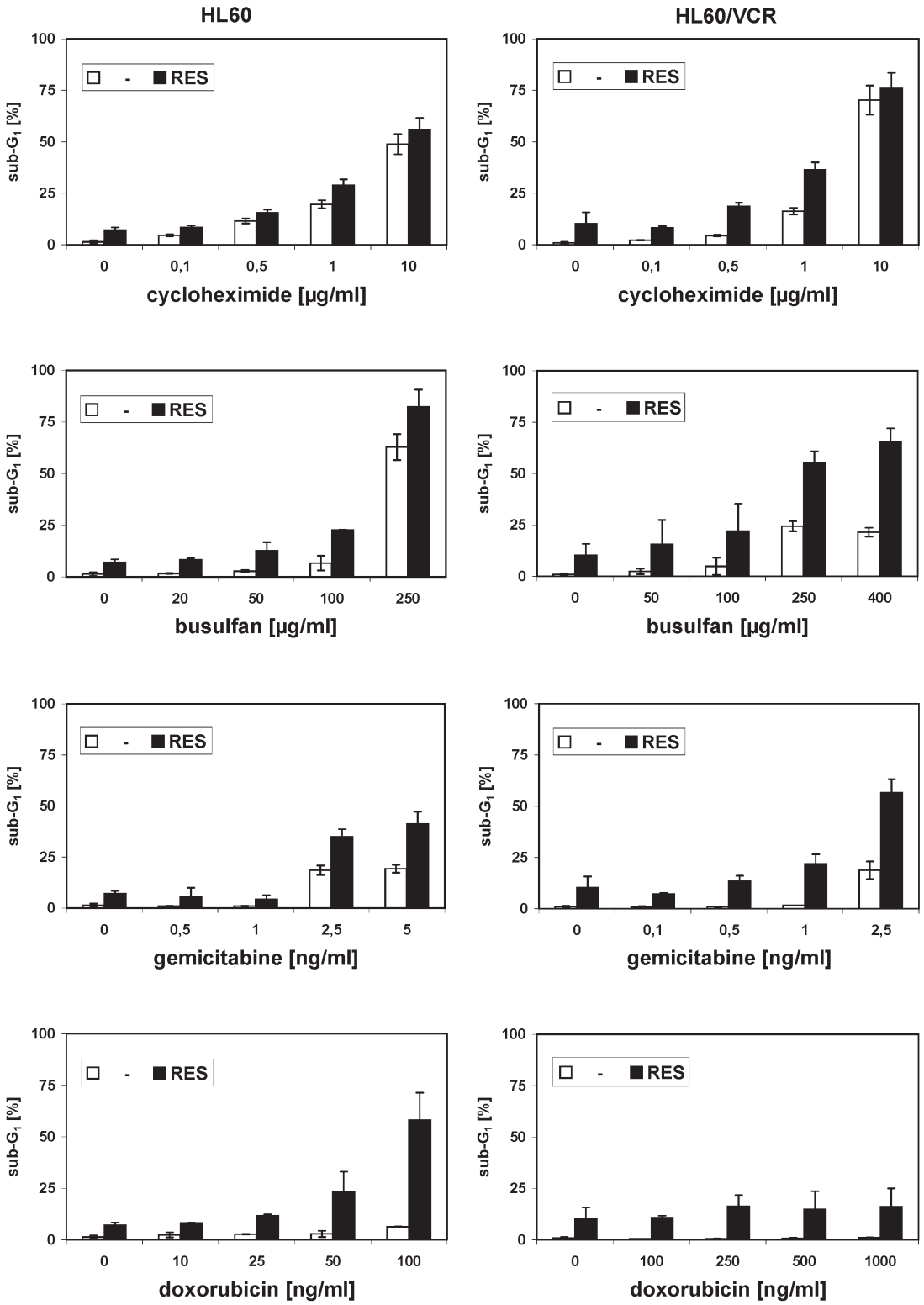

Figure 4. Sensitization of cells for drug-induced apoptosis by RES measured by flow cytofluorometry. HL60- or HL60/VCR cells were pretreated for 24 h with 15 µM (for HL60) or 20 µM (for HL60/VCR) RES followed by co-treatment (for 48 h) with CHX, BUS, GEM, DOX and PTX, respectively, with indicated concentrations. Percentages of apoptosis (subG1) were calculated from flow cytometry measurements of PI-stained DNA as described in Figure 2b. Data represent mean ±SD of at least two experiments.

centrations 0.1–10 μg/ml, CHX caused in both cell variants a proportional accumulation of cells in G0/G1 and S-phase. Single treatment with CHX or cooperation of RES with CHX created a decrease in G0/G1 and S-phase cell portions compensated with a partial enhancement in subG1 cells in dose-dependent manner. However, no significant RES potentiation in subG1 for CHX was observed in both cell lines.

Both cell lines tested exerted relatively low sensitivity to alkylating agent BUS. In the range of concentrations 20–250 μg/ml (for HL60)- and 50–400 μg/ml (for HL60/VCR) cells, treatments with BUS caused effective accumulation of sensitive- as well as resistant cells preferentially in S-phase. By using of single BUS or RES and BUS in combination, increased percentages of subG1 cells followed by concomitant cell decrease predominantly in S-phase, were observed. Similar effects of the drugs were determined also in resistant cells.

Sensitive and resistant cells used exhibited relative high sensitivity to antimetabolite GEM, when resistant cells were approximately two-fold more sensitive in comparison with sensitive variants. Even 0.5–5 ng/ml drug concentrations induced accumulation of cells in G0/G1 and S-phase of the cell cycle and, in dose-dependent manner, an appearance of apoptosis followed a decrease of cells predominantly in S-phase. Cooperative treatment of RES and GEM caused significant sensitization indicated predominantly in resistant cells accompanied with an increased appearance of subG1 cells at the expense of cells accumulated in S-phase.

Anthracycline DOX at concentration 10–100 ng/ml accumulated sensitive HL60 cells in S-phase and, to a lesser extent, also in G0/G1. At these concentrations no accumulation of cells in subG1 phase was determined. However, cooperation of RES and DOX caused dramatic changes in the cell cycle distribution resulting in significant increase of apoptotic subG1 cells. Multidrug-resistant HL60/VCR cells exerted no sensitivity either to 10-fold higher DOX concentrations (0.1–1 μg/ml) then those used in sensitive cells. Combination of RES and DOX also exerted no appearance of any portion of subG1 apoptotic cells.

PTX concentrations in sensitive cells (0.1–1 nM) and in resistant cells (0.05–1 μM) were used. Former accumulated the sensitive cells in the G2/M phase in dose- dependent manner and increased concentrations of PTX reduced G2/M portion of sensitive cells and proportionally enhanced the number of cells in subG1. In combined treatments of RES and PTX, the percentage of cells arrested in G2/M was not significantly changed and no substantial increase in subG1 was observed. Resistant cells exerted no sensitivity even to relatively high PTX concentrations (0.05–1 μM) and also cooperative treatments with PTX and RES had no influence on these cells.

Discussion

RES has been shown to induce dose-dependent cell cycle arrest, growth inhibition or apoptosis in several human cell lines [8, 9]. Previous studies demonstrated the ability of RES to block the S-G2/M transition resulting in accumulation of cells in S or G2/M phase [10]. The S-phase arrest has been explained by inhibition of ribonucleotide- reductase or DNA polymerase and can be exploited with selected anticancer drugs for cancer therapy [17].

In search for strategies to sensitize and/or to overcome P-gp resistance in leukemic cells, we studied activities of a single RES or in combination with selected anticancer drugs in human sensitive P-gp-negative leukemia HL60- and P-gp-positive HL60/VCR cells.

Both cell types used exerted relatively low sensitivity to translational inhibitor CHX resulting in accumulation of cells predominantly in S-phase. Cooperative treatment with RES and CHX led in both cell variants to a slight additive increase of apoptotic subG1 cell populations in dose-dependent manner. Several effects are referred with CHX on diverse cancer cells. CHX reversed resistance to Fas-mediated apoptosis along with caspase-8 activation [18], up-regulated TRAIL-induced apoptosis in human oral cancer cells [19] and prevented VP-16-induced apoptosis [20].

Alkyl sulfonate BUS is, in combination with other anticancer drugs, widely used in conditioning to bone marrow transplantation of patients with acute myeloid leukemia, myelodisplastic syndrome and multiple myeloma. Both cell lines used were relatively low sensitive to BUS and accumulated the cells in S-phase. Co-administration of RES with BUS caused an appearance of subG1 cells at the expense of S-phase. There are no experimental data on BUS co-treatment with flavonoids. Various types of human cancer cells are *in vivo* markedly resistant to this agent. BUS, in combination with dihydroxyvitamin D2, produced additive killing effect in human breast cancer cells [21]. Its analog, treosulfan, exerted anti-leukemic effect followed by pro-apoptotic effect mediated in part by activation of PKC isoforms [22].

Pyrimidine nucleoside analog of cytosine-arabinoside, GEM, is commonly used as anti-tumor drug in several human solid tumors used *in vivo* and *in vitro*, predominantly in pancreatic cancer and neuroblastoma. In our experimental system, both cell variants exerted relatively high sensitivity to GEM, and, interestingly, resistant cells were more sensitive than parental cell variants. The drug activity resulted in accumulation of cells in S- and G0/G1 phase and cooperative treatments with RES caused synergistic effect and led to an appearance of subG1 cells presumably at the expense of S-phase. These observations are in accordance with those, when GEM down-regulated P-gp resistance in human ovarian cancer cells [23]. GEM is also reported as potent sensitizer of apoptosis-associated genes and their translational products in cooperation with diverse anticancer drugs inclusively fludarabine [24], cytosine-arabinoside [25], taxanes [26], cisplatin [27] and other drugs such as topoisomerase-I- [28], proteasome- [29], cdk- [30] and PI-3/Akt-kinase inhibitors [31], as well as isoflavones [32].

Anthracycline antiobiotic, DOX, showed in our experi-

ments high cytotoxicity against sensitive cells but not against the resistant cell counterparts. Co-administration of RES and DOX synergistically potentiated DOX cytotoxicity and apoptosis in sensitive cells but resistant cell culture remained refractory to the combination treatment with RES. These observations indicate that leukemia cell DOX-resistance does not allow RES to potentiate the cells to apoptosis. Several cases of combination chemotherapy with DOX and other anticancer drugs to stimulate cytotoxic effects, modulate multidrug resistance or regulate apoptotic signaling pathways are referred, i.e. with taxanes [33], cisplatin or carboplatin [34], proteasome inhibitors [35], Bcl-2 anti-sense oligonucleotids [36], apoptosis inhibitors [37], histone deacetylase inhibitors [38], mitochondria-targeting agents [39], TNF-related apoptosis-inducing ligand [40], as well as flavonoids [9, 41, 42].

Anticancer agent, PTX, stabilizes microtubules leading to G2/M cell cycle arrest, sensitization of cells to drug or radiation resulting in apoptosis. PTX-resistance appeared as mediated by increased cell P-gp expression. The drug has been reported to interact with a variety of drugs with the objective to overcome multidrug resistance and potentiate their growth inhibition, to increase cytotoxicity, and, in some cases, resulting in the induction of apoptosis. P-gp resistance to PTX is referred to be inhibited by a variety of unrelated drugs such as proteasome inhibitors [43], EGFR tyrosine kinase inhibitors [44], histidine kinase inhibitors [45], or flavonoids [46]. PTX also can overcome cisplatin resistance in ovarian cancer cells [47]. In our experimental model, after PTX treatment, we found accumulation of sensitive cells in G2/M and slight increased appearance of subG1 cells in dose-dependent manner. Interestingly, combination treatment of RES and PTX resulted in a slight inhibition of sensitive cells to generate subG1 cell population. The resistant cells, even at 10-fold higher concentrations of PTX than those in sensitive variants, did not exhibit any drug chemosensitivity and no cell cycle redistribution. This attests the fact that PTX is in HL60/VCR cells a substrate for P-gp transporter. In fact, some experimental data showed that co-administration of RES and PTX acted in an anti-apoptotic manner presumably through its active metabolites such as piceatannol which is also able to alter cell cycle progression [48, 49].

In conclusion, these results found in sensitive- and multidrug-resistant human leukemia cells show the potential of RES to interfere with the cell cycle and to exert various activities in cooperated treatments with selected anticancer drugs resulting in some cases in the cell death induction. Thus, these data speak in favor of potential implications of RES in chemoprevention and in combination chemotherapy.

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