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Isothiocyanates Induce Cell Cycle Arrest, Apoptosis and Mitochondrial Potential Depolarization in HL-60 and Multidrug-resistant Cell Lines

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Abstract. *Isothiocyanates from cruciferous vegetables have been identified as potent anticancer agents in animal and human epidemiological studies. The present study compared the biological activities of six dietary isothiocyanates (ITCs), allyl-ITC (AITC), benzyl-ITC (BITC), phenethyl-ITC (PEITC), sulforaphane (SFN), erucin (ERN) and iberin (IBN), on cell cycle progression, apoptosis induction and mitochondrial transmembrane potential in multidrug-resistant HL60/ADR (MRP-1-positive) and HL60/VCR (Pgp-1-positive) cells in comparison to the parent cell line HL60. Multidrug-resistant HL60/ADR and HL60/VCR cells were less sensitive than the parental HL60 cells to all the six tested ITCs, since the medians of IC_{50} values were 2.8- and 2.0-fold higher. All the selected ITCs induced time- and dose-dependant G_2/M arrest, with the most effective AITC (10 μM, 24 h) inducing 52% G_2/M accumulation in HL60 cells. Apoptosis was determined by Annexin V-FITC staining, metabolic conversion of fluorescein diacetate and sub-G_1 population quantification. Cell cycle distribution and mitochondrial JC-1 aggregation were determined by flow cytometry. The effectiveness of ITCs in apoptosis induction and mitochondrial potential dissipation followed the order: BITC=PEITC>ERN=IBN>AITC>SFN. This study demonstrates that dietary ITCs are mitotic inhibitors and/or apoptosis inductors and suggests they could be chemotherapeutic agents in cells with multidrug resistance phenotypes.*

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Abbreviations: ITCs, isothiocyanates; AITC, allyl ITC; BITC, benzyl ITC; PEITC, phenylethyl ITC; SFN, sulforaphane; ERN, erucin; IBN, iberin; Pgp-1, P-glycoprotein-1; MRP-1, multidrug resistance associated protein-1; GSH, glutathione; MAPKs, mitogen-activated protein kinases; ROS, reactive oxygen species.

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Key Words: Isothiocyanates, multidrug resistance, cell cycle, apoptosis, mitochondrial transmembrane potential, HL60 cell line.

Many population-based studies have highlighted the ability of macronutrients and micronutrients in fruits and vegetables to reduce the risk of cancer (1). Several recent studies have focused on phytochemicals in the plant-based diet that possess cancer preventive properties and, among them, isothiocyanates (ITCs) are regarded as a significant dietary factor (2). ITCs are hydrolysis products of glucosinolates, which occur in large amounts in a variety of cruciferous vegetables. Upon plant tissue disruption during food processing, glucosinolates stored in the cell vacuole are released and hydrolyzed by the enzyme myrosinase to form ITCs (3). Additionally, human enteric microflora exhibit myrosinase-like activity and can convert a significant proportion of ingested unhydrolyzed glucosinolates to ITCs (4, 5). Studies showed that exposure of cells to ITCs resulted in their rapid accumulation at high levels in cells, which is critical for anticarcinogenic activity (6, 7). Although the principal driving force for cellular accumulation of ITCs is their reaction with glutathione (GSH), covalent protein modification has also been identified (8).

The cellular and molecular events affected or regulated by these chemopreventive phytochemicals include inhibition of phase I enzymes, involved in the bioactivation of carcinogens, and induction of phase II enzymes, functional in carcinogen detoxification (9-13). Recent studies have undertaken to delineate the influence of ITCs on the mechanisms of G_2/M checkpoint (14-17). Another cellular signaling pathway investigated is represented by the MAPK network (15, 18-21) and its connections to growth inhibition, ROS production and/or apoptotic death (12, 22-24). These effects of ITCs on most of the cell signaling cascades are ultimately linked to the modulation of cell cycle regulatory

molecules, leading to growth inhibition and/or apoptotic death of cancer cells (25-28). In this study, the effects of Pgp-1 and MRP-1 cell surface efflux pump expressions on ITCs-induced cell cycle modulation were examined, using both the parental HL60 cell line and its multidrug-resistant HL60/ADR and HL60/VCR sub-lines. In parallel, time-dependent changes in mitochondrial potential, sub-G_1 DNA fragmentation and appearance of apoptotic and necrotic cells induced by ITCs were also investigated.

Materials and Methods

Reagents. AITC, BITC and PEITC were purchased from Aldrich (Milwaukee, WI, USA). SFN was purchased from ICN Biomedical (Basingstoke, UK). ERN and IBN were purchased from LKT Laboratories (St. Paul, MN, USA). The Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences Pharmingen. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraetylbenzimidazolylcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR, USA). Propidium iodide (PI), fluorescein diacetate (FDA), RNA-se A and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell lines. The human myeloid leukemia HL60 cell line and its multidrug-resistant sublines, HL60/ADR (MRP-1-positive) and HL60/VCR (Pgp-1-positive), were obtained from Dr. P. Ujhazy (Roswell Park Cancer Institute, Buffalo, NY, USA) with consent of Dr. M. S. Center, Kansas State University (Manhattan, USA) and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as suspension cell cultures. All cells were maintained in 6-cm dishes in a humidified incubator at 37˚C with 5% CO_2.

Treatment with ITCs. Except for the cytotoxicity assay described below, in all other analyses, $1x10^6$ cells were grown on 6-cm dishes with 5 ml medium for 24 h and then treated with ITCs as indicated. The cells were exposed to various concentrations of ITC for 6 h and 24 h at 37˚C with 5% CO_2. Stock solutions of ITC were originally dissolved in DMSO, and an equal volume of DMSO (final concentration <0.1%) was added to the control cells.

Cell survival assay. The effect of ITCs on survival of cells was determined by a MTT assay (29). The cells ($5x10^3$ per well in 100 μl of medium) were seeded in a 96-well culture plate for 24 h. A test ITC was then added to each well with 100 μl medium. In every experiment, each dose of ITCs was tested in quadruplicate, and the cytotoxicity curve was constructed from at least six (0.5, 1, 2.5, 5, 10, 15 and 25 μM) different concentration of ITCs. After 72 h, the cells were incubated with 50 μl of MTT (1 mg/ml) in fresh media and left in the dark at 37˚C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals were dissolved in 200 μl of DMSO, and the absorbance was measured at 540 nm and 690 nm in a microplate reader (Dynatech Lab Inc., Chantilly, VA, USA). The concentration of drug that inhibited cell survival to 50% (IC_{50}) was determined by Calcusyn software.

Annexin V-FITC/PI staining. Apoptotic cells were quantified using the Annexin V-FITC apoptosis detection kit, according to the manufacturer's instructions. Briefly, the cells were collected by centrifugation at 700 x g for 3 min and washed twice with cold phosphate-buffered saline (PBS). Approximately $5x10^5$ cells were resuspended in 100 μl of manufacturer-supplied 1 x binding buffer, and mixed with 5 μl of Annexin V-FITC and 5 μl of PI. After 15-min incubation in the dark at room temperature, the cells were analyzed with a Coulter Epics Altra flow cytometer.

Fluorescein diacetate (FDA)/PI staining (30). Briefly, the cells were collected by centrifugation at 700 x g for 3 min and washed twice with cold PBS. Approximately $5x10^5$ cells were resuspended in 400 μl of PBS/0.2% BSA containing 10 nM of FDA (from a 5 mM stock in DMSO) for 30 min at room temperature. The cells were then cooled and 4 μl of PI (1 mg/ml) were added. Finally, after 15 min, the stained cells were analyzed with a Coulter Epics Altra flow cytometer.

Cytofluorimetric analysis of mitochondrial potential. Variations of mitochondrial membrane potential in the cells during apoptosis were studied using a JC-1 fluorescent probe. Cells with normal polarized mitochondrial membranes emit green-orange fluorescence, and the percentage of cells that emit only green fluorescence is attributable to depolarized mitochondrial membranes. For analyses of mitochondrial membrane potential ψ_m, the cells were collected by centrifugation at 700 x g for 3 min. Briefly, $5x10^5$ cells were washed twice with cold PBS and incubated in 400 μl of PBS/0.2% BSA containing 4 μM of JC-1 (from a 7.7 mM stock in DMSO) for 30 min at 37˚C. After 30 min of incubation in the dark at 37˚C, the cells were analyzed using a Coulter Epics Altra flow cytometer.

Cell cycle analysis. This assay was based on the measurement of the DNA content of nuclei labelled with propidium iodide. For flow cytometry analyses of DNA cell cycle profile, approximately $5x10^5$ cells were collected by centrifugation at 700 x g for 3 min. The cells were washed twice with cold PBS and resuspended in 0.05% Triton X-100 and 15 μl of RNA-se A (10 mg/ml) for 20 min at 37˚C. The cells were then cooled and incubated on ice for at least 10 min before PI (50 μg/ml) was added. Finally, after 15 min the stained cells were analyzed using a Coulter Epics Altra flow cytometer.

Flow cytometry measurements and data analysis. The Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser, and fluorescence emission was measured using a bandpass filter set 525, 575, 610, 675 nm with respective photomultipliers FL1-FL4 required for fluorochrome used as follows: Annexin V-FITC and PI (FL1, FL2), FDA and PI (FL1, FL2), JC-1 (FL1, FL2, ratio FL2/FL1), cell cycle (log FL2 – sub-G_1, lin FL3 – DNA cell cycle histogram, FL4 peak *vs.* integral for doublets discrimination). Forward/side light scatter characteristic was used to exclude the cell debris from the analysis. For each analysis, $1x10^4$ cells were acquired. Data were analyzed with WinMDI version 2.7 software (J. Trotter, Scripps Research Institute, La Jolla, CA, USA). The cell cycle calculations were performed with MULTI-CYCLE Software (Phoenix Flow System).

Results

Cytotoxicity of ITCs in HL-60, HL60/ADR and HL60/VCR cell lines. To assess the cytotoxic effect of ITCs, the human

Table I. *Cell survival analysis (72-h-treatment).*

	ITC, IC_{50} [μM]					
Cell line	AITC	BITC	PEITC	SFN	ERN	IBN
HL60	4.0±1.7	0.6±1.1	1.0±1.9	5.1±1.3	1.9±1.2	2.3±1.8
HL60/ADR	10.5±2.5	3.1±1.4	3.8±1.8	8.1±0.9	5.6±0.8	4.0±1.9
HL60/VCR	7.8±1.9	3.1±1.2	2.1±1.6	7.3±1.3	7.6±1.2	6.3±1.9
			relative IC_{50}*			
HL60/ADR	2.6	5.2	3.8	1.6	2.9	1.7
HL60/VCR	2	5.2	2.1	1.4	4	2.7

The IC_{50} values (the concentration of the ITCs that reduced cell survival by 50%) were determined by the MTT assay as described in "Materials and Methods". The HL60, HL60/ADR and HL60/VCR cells were treated with various concentrations of ITCs for 72 h in 96-well plates. Each treatment with a specific concentration of a compound was done in quadruplicate. The data presented are representative of results obtained from three replicate experiments; and the results are means±SE. *relative $IC_{50}=IC_{50(HL60/ADR)}/IC_{50(HL60)}$ or $IC_{50(HL60/VCR)}/IC_{50(HL60)}$, respectively.

myeloid leukemia HL60 cell line and its multidrug-resistant HL60/ADR and HL60/VCR sublines were treated with different concentrations of six ITCs. The concentration of each ITC that reduced cell survival by 50% (IC_{50}) was determined from cell survival plots, and the data are presented in Table I.

Marked differences in the resistance of the three cell lines to the various ITCs were observed. As shown in Table I, parental HL60 cells were relatively more resistant to AITC and SFN since the corresponding IC_{50} were ≥4 times higher than those of the BITC and PEITC treatments (72 h). Moreover, the SFN sulfide analog, ERN, and sulfoxide analog, IBN, were approximately 2 times more effective than SFN in HL60 cells. The lowest IC_{50} value was from BITC (0.6 μM) in HL60 cells. Both multidrug-resistant cell lines tested were less sensitive to the cytotoxic effect of ITCs. Among all the tested ITCs, the least difference was seen in SFN treatments, with IC_{50} values of 5.1, 8.1 and 7.3 μM in HL60, HL60/ADR and HL60/VCR, respectively. Most of the relative IC_{50} values were within the range of 2 – 4-fold, with the exception of BITC (5.2-fold) being higher in multidrug-resistant cell lines than in parental HL60 cells. In comparison to parental HL60 cells, the overall resistance of HL60/ADR cells to the ITC panel was higher than the resistance of HL60/VCR cells, with median relative IC_{50} values of 2.8- and 2.0-fold, respectively.

Induction of apoptosis in HL-60, HL60/ADR and HL60/VCR cell lines.

Annexin-V staining: To determine whether reduced cell survival of HL60, HL60/ADR and HL60/VCR by ITCs was associated with apoptosis or necrosis induction, cells were treated with 5, 10 and 20 μM ITCs for 6 and 24 h. Transmembrane externalization of phosphatidylserine is a recognized early event of apoptosis, which was detected by the widely used flow cytometry-based Annexin V staining. Propidium iodide, which does not enter cells with intact membranes, was used to differentiate between early apoptotic (Annexin V^+ and PI^-) and late apoptotic or necrotic cells (Annexin V^+ and PI^+). Flow cytometric analysis of Annexin V staining revealed a dose-dependent increase of both early apoptotic and late apoptotic/necrotic cells (Figure 1A). Approximately 1/2 and 1/3 of the cells were viable (bottom left quadrant) after 5 and 10 μM of BITC treatment, respectively. Although distinctive apoptotic changes were detectable in parental HL60 cells treated with all ITCs tested for 6 h (Figure 1B), maximal induction was observed after 24-h treatment (Figure 1C). Flow cytometric analysis of the sensitive HL60 cells indicates that ITCs treatment increased Annexin V^+/PI^- and Annexin V^+/PI^+ cells in the order of potency: BITC=PEITC>ERN=IBN>AITC>SFN (Figure 1C). The highest concentration (20 μM) of the most effective ITCs was very toxic (data not shown), while less toxic AITC and SFN at 20 μM induced 55% and 20% HL60 cell death, respectively.

Annexin V staining of HL60/ADR (Figure 2A) and HL60/VCR cells (Figure 2B) treated with ITCs for 24 h confirmed the MTT data, that both cell lines have a more resistant phenotype compared to parental HL60 cells (Figure 1C), *i.e.* parental HL60 cells were the most sensitive cells to ITCs, followed by HL60/VCR cells; the HL60/ADR cells were the least sensitive cells. Aromatic ITCs (BITC, PEITC) were the most effective compounds, and SFN was the least effective ITC in all 3 cell lines studied.

FDA/PI staining is another method for apoptosis monitoring. Vital cells take up the fluorogen FDA, activate it by non-specific esterases and retain the free fluorescein in their cytoplasm. Apoptotic and dead cells show reduced or no uptake, and the turnover of FDA and counterstaining with PI identifies dead cells. As shown in Figure 3A, flow cytometric analysis of the HL60 cells treated with 5 and 10 μM of BITC revealed 13.0 and 22.2% apoptotic cells

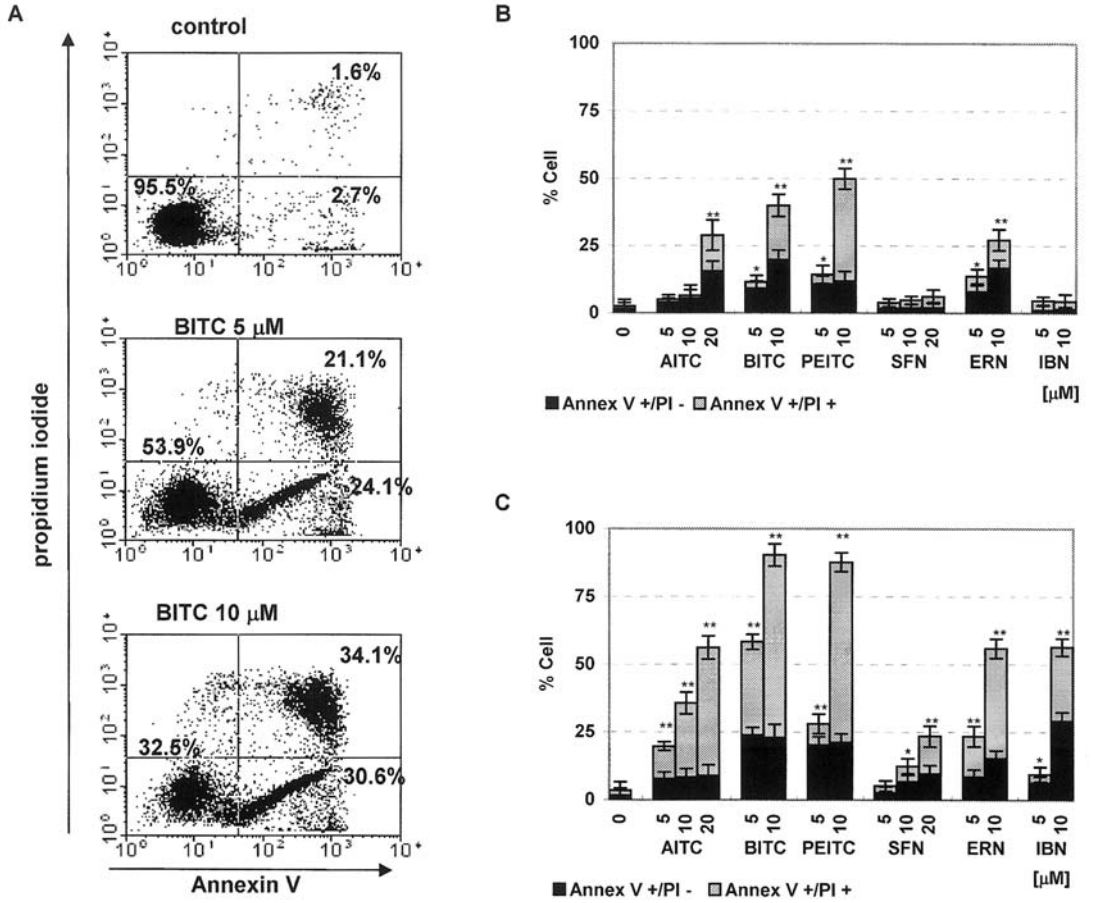

Figure 1. *Effect of ITCs treatment on apoptosis and necrosis induction in HL60 cells using Annexin V/PI staining. (A) The Pgp-1-expressing HL60/VCR cells were treated with 5 and 10 μM of BITC and stained with Annexin V-FITC and PI. Numbers in the bottom right quadrant of each dot blot represent the percentage of cells in early apoptosis (Annexin V-FITC$^+$/PI$^-$) for untreated and BITC-treated cells. Numbers in the top right quadrant of each dot blot represent the percentage of cells in late apoptosis/necrosis (Annexin V-FITC$^+$/PI$^+$ double-positive). The data shown are representative of three independent experiments. The HL60 cells were exposed to either DMSO (control) cells or different concentrations of ITCs for 6 h (B) and for 24 h (C). The percentage of apoptotic (Annexin V-FITC$^+$/PI$^-$) and late apoptotic/necrotic (Annexin V-FITC$^+$/PI$^+$ double-positive) cells are shown. Each column represents the mean±SE of the data obtained from three independent experiments. Significant difference from the controls, *p<0.05; **p<0.01.*

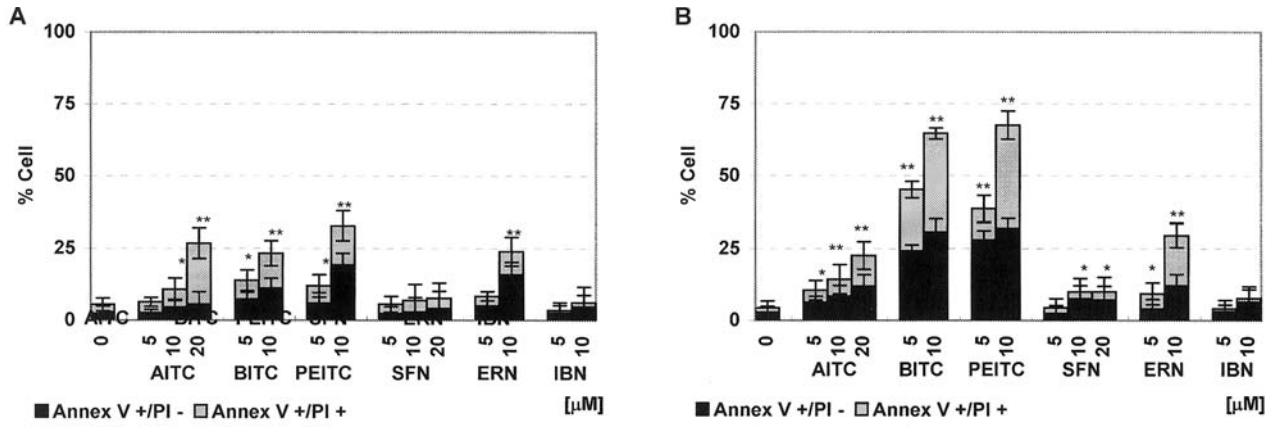

Figure 2. *Effect of ITCs treatment on apoptosis and necrosis induction in multidrug-resistant sublines HL60/ADR and HL60/VCR using Annexin V-FITC/PI staining. (A) The MRP1-expressing HL60/ADR cells and (B) Pgp-1-expressing HL60/VCR cells were exposed to different concentrations of ITCs for 24 h. An equal volume of DMSO was added to the controls. Apoptotic cells were quantified by flow cytometry after staining with Annexin V-FITC and PI. Percentages of apoptotic (Annexin V-FITC$^+$/PI$^-$) and late apoptotic/necrotic (Annexin V-FITC$^+$/PI$^+$ double-positive) cells are shown. Each column represents the mean±SE of the data obtained from three independent experiments. Significant difference from the controls, *p<0.05; **p<0.01.*

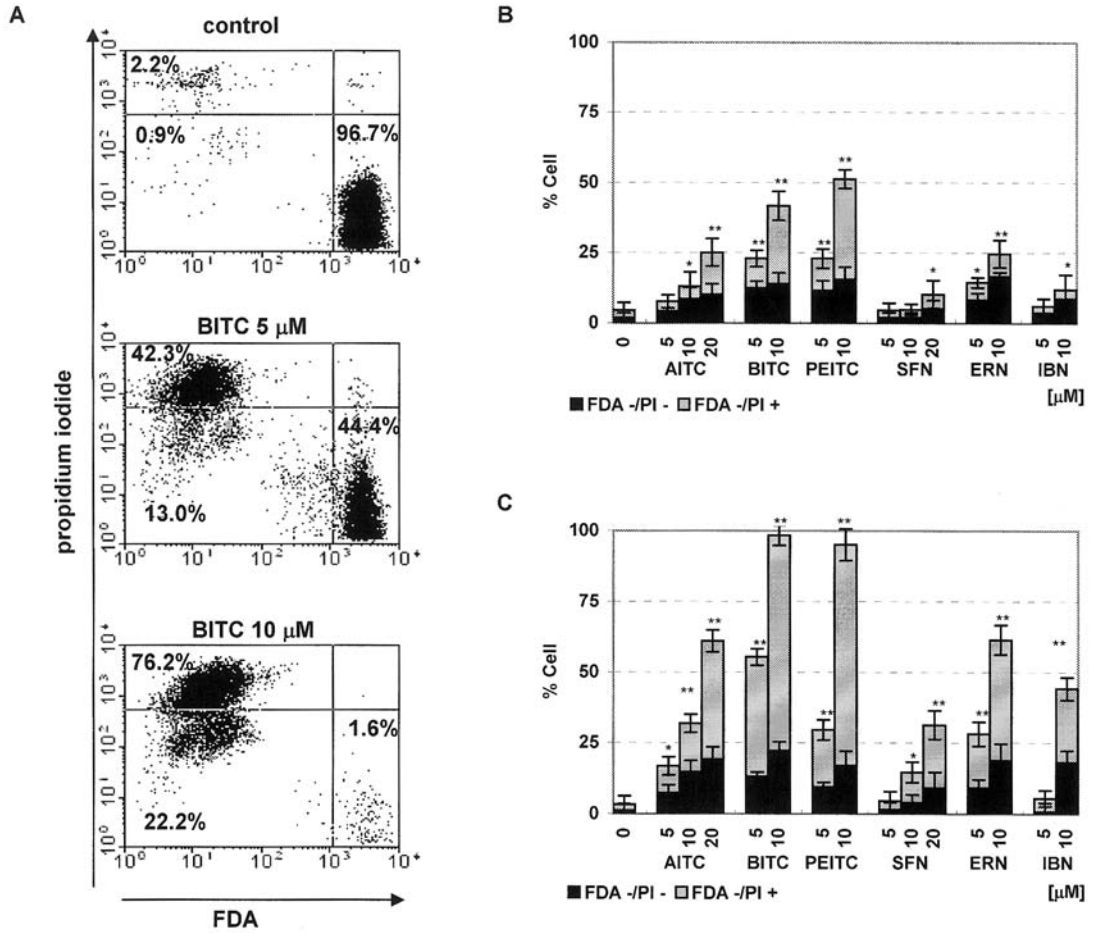

Figure 3. *Effect of ITCs treatment on induction of apoptosis and necrosis in parental HL60 cell line using FDA/PI staining. (A) The HL60 was treated with 5 and 10 μM BITC and stained with FDA and PI. Numbers in the bottom right quadrant of each dot blot represent the percentage of viable cells (FDA^+/PI^{low}) and bottom left quadrant of each dot blot represent the percentage of cells in early apoptosis (FDA^-/PI^{low}) for untreated and BITC-treated cells. Numbers in the top left quadrant of each dot blot represent the percentage of cells in late apoptosis/necrosis (FDA^-/PI^{high}). The data shown are representative of three independent experiments. The cells were exposed to either DMSO (control cells) or different concentrations of ITCs (B) for 6 h and (C) for 24 h. The data presented is from the three independent experiments; and the results are means±SE. Significant difference from the controls, *p<0.05; **p<0.01.*

(FDA^-/PI^{low} - bottom left quadrant) and 42.3 and 76.2% of dead or necrotic cells with FDA^-/PI^{high} characteristic staining (top left quadrant), respectively. Both dose- and time-dependent increases, corresponding to Annexin V/PI staining, of FDA^-/PI^{low} and FDA^-/PI^{high} positive cells were observed in the range: BITC=PEITC>ERN=IBN>AITC >SFN (Figure 3B and 3C). The extent of HL60/ADR cell death (both apoptotic and necrotic) after 24-h treatment (Figure 4A) was similar to the effects seen in parental HL60 cells after 6-h treatment (Figure 3B). The resistance of HL60/VCR (Figure 4B) was similar to HL60/ADR cells, with the exception of aromatic PEITC and BITC treatment. These ITCs induced more profound apoptotic and necrotic effects in HL60/VCR cells in comparison to HL60/ADR cells, in which necrotic, *i.e.* FDA^-/PI^{high} positive, cells were the dominant fraction.

DNA fragmentation: Flow cytometry was used for the quantification of cells with sub-G_1 staining patterns which represent DNA fragmentation, a hallmark event in apoptotic cells. As shown in Figure 5, ITCs induced dose- and time-dependent DNA fragmentation. A higher dose (10 μM) of BITC, PEITC and ERN produced 42.2, 41.2 and 36.8% cells with sub-G_1 pattern after 6-h treatment in HL60 cells. However, in the presence of AITC, SFN or IBN, no increase in percentage of sub-G_1 cells was found. Extended treatment for 24 h caused clear DNA fragmentation at 5 μM ITCs treatment, with the exceptions of SFN and AITC (Figure 5A). Nevertheless, increased concentration of both ITCs produced significant DNA fragmentation. In both drug-resistant cell lines HL60/ADR and HL60/VCR treatment with BITC, PEITC or ERN for 6 h resulted in enhanced sub-G_1 fraction, but no increase in the number of apoptotic cells

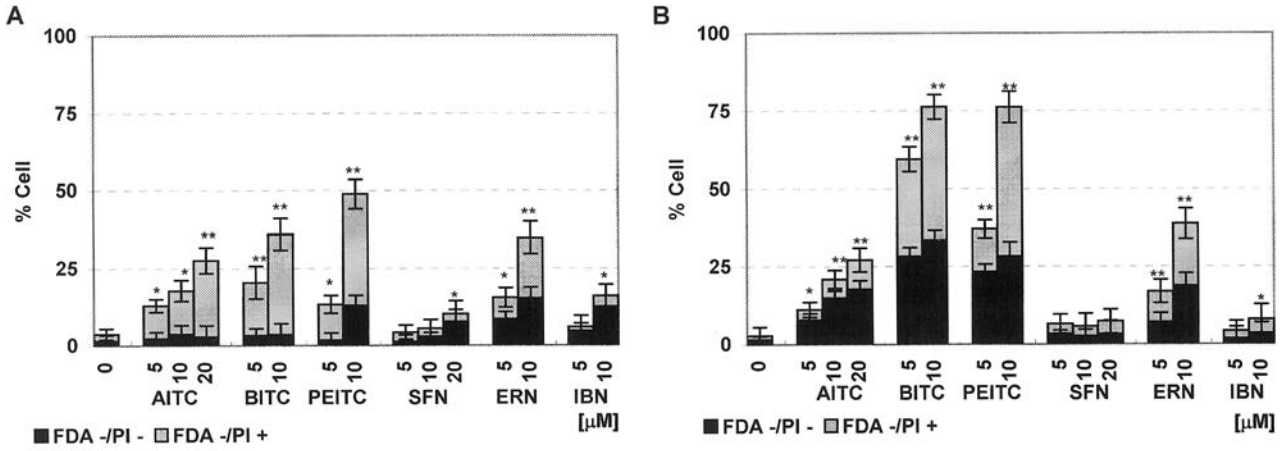

Figure 4. *Effect of ITCs treatment on apoptosis and necrosis induction in multidrug-resistant sublines HL60/ADR and HL60/VCR using FDA/PI staining. (A) The MRP1-expressing HL-60/ADR cells and (B) Pgp-1-expressing HL-60/VCR cells were exposed to either DMSO (control cells) or different concentrations of ITCs for 24 h. Percentages of apoptotic (FDA^{-}/PI^{low}) and late apoptotic/necrotic (FDA^{-}/PI^{high}) cells are shown. Each column represents the mean±SE of the data obtained from three independent experiments. Significant difference from the controls, *p<0.05; **p<0.01.*

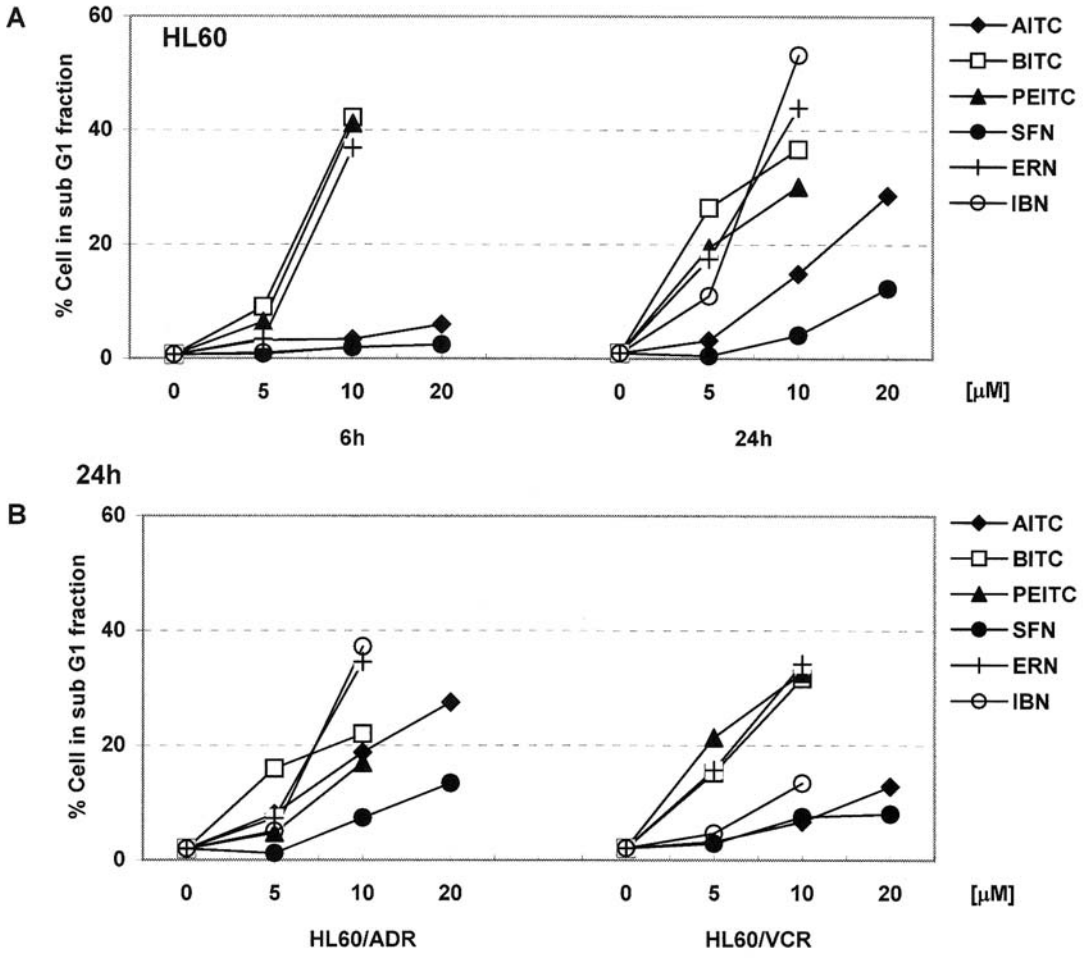

Figure 5. *Effect of ITCs on sub-G_1-phase of the cell cycle in HL60 cells and its multidrug-resistant sublines HL60/ADR and HL60/VCR. The HL60 cells were exposed to DMSO (control) or different concentrations of ITCs for 6 h and for 24 h. (B) The HL60/ADR and HL60/VCR cells were exposured to ITCs for 24 h. The cells were collected, detergent permeabilized and stained with 50 μg/ml concentration of PI in the presence of RNA-se A. The percentage of the sub-G_1 fraction was obtained from analysis of SSC versus log FL2 dot plot using WinMDI software. The representative data presented are from the three independent experiments.*

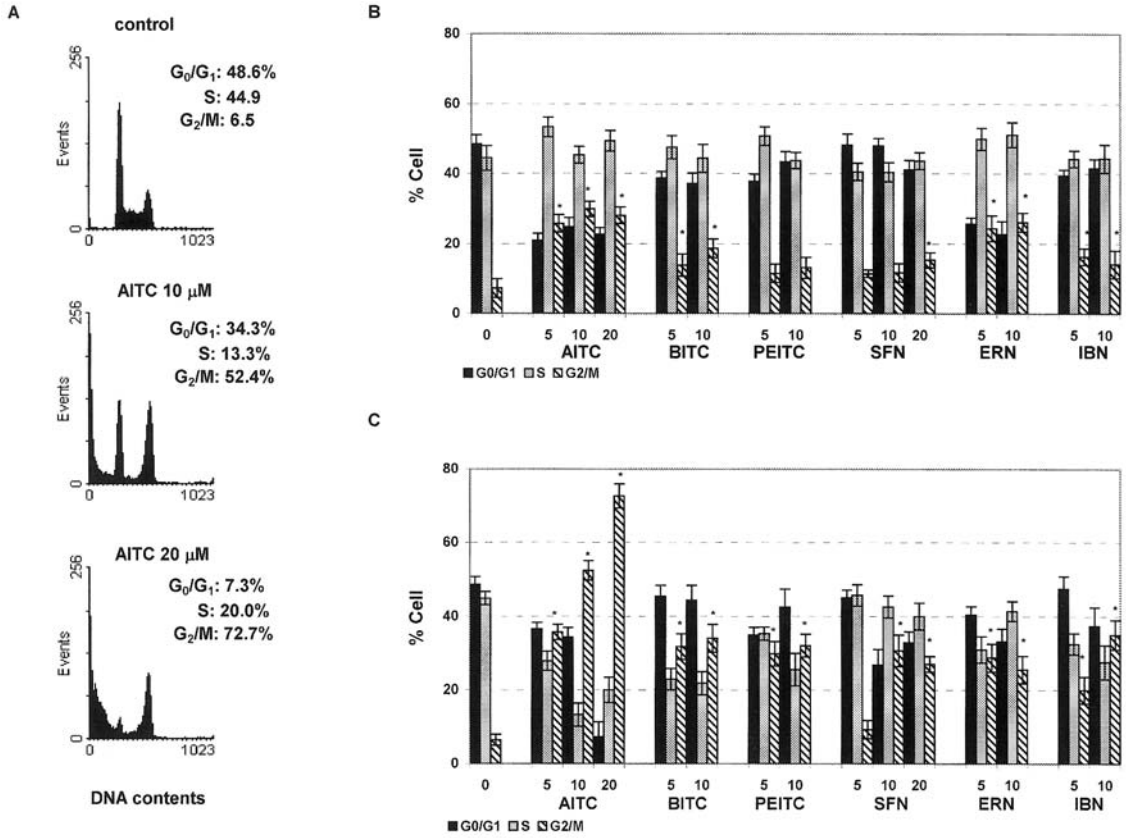

Figure 6. *Effect of ITCs on the cell cycle progression in HL60 cells. (A) Effect of 10, and 20 μM AITC on the cell cycle progression of HL60 cells for 24 h. The HL60 cells were exposed to DMSO (control) or different concentrations of ITCs (B) for 6 h and (C) for 24 h. The cells were collected, detergent permeabilized and stained with 50 μg/ml concentration of PI in the presence of RNA-se A. The distribution of cells in G_0/G_1-, S- and G_2/M-phases was analyzed by flow cytometry and Multi-cycle software. One experiment representative of three is reported (A). Three independent experiments were performed and mean±SE are presented. Significant difference from the controls, *p<0.01.*

was observed following exposure to AITC, SFN or IBN (data not shown). However, there was a significant increase in DNA fragmentation after 24-h treatment (Figure 5B). In comparison with parental HL60 cells, both multidrug-resistant sublines were more resistant to ITCs treatment.

ITCs and cell cycle arrest in HL60, HL60/ADR and HL60/VCR cell lines. The effect of ITCs on cell proliferation was evaluated by measuring the distribution of cells in the different cell cycle phases. The most profound effect on cell cycle was seen in HL60 cells (*i.e.* 52.4 and 72.7% of cells in G_2/M-phase) after 24-h treatment with 10 and 20 μM AITC (Figure 6A). Analysis of parental HL60 cells exposed to AITC for 6 h showed that the lower (5 μM) concentration of AITC increased the number of cells in the S- and G_2/M-phases of the cell cycle as 1.2- and 3.6-fold, respectively (Figure 6B). A similar increase of G_2/M cells at the expense of a decrease in G_0/G_1 cells was found in ERN-treated cells. A smaller but significant G_2/M increase with concomitant decrease of G_0/G_1 cells occurred in IBN- and BITC-treated cells. There was no significant difference in cell cycle phase distribution after 6-h treatment of 5 μM or 10 μM ITCs. The cell cycle distribution pattern after 24-h treatment is complex (Figure 6C). AITC induced the highest G_2/M accumulation and both SFN and IBN showed dose-dependent increase of G_2/M, in the case of SFN concurrently with decrease of G_0/G_1 cells. As shown in Figure 7A, flow cytometric analysis of the HL60/ADR cells treated with ITCs for 24 h revealed an increase of G_2/M-phase. In addition, the percentage of cells in S-phase was increased in PEITC- and IBN-treated cells. AITC treatment of the HL60/VCR cell line caused cell accumulation in G_2/M-phase (Figure 7B). Cell cycle analysis revealed a similarity of HL60 and HL60/VCR cells in comparison to HL60/ADR cells. The pattern of G_0/G_1 cell changes was similar in HL60 and HL60/VCR cells at all ITC concentrations tested (Figures 6B and 7B).

Effects of ITCs on mitochondrial membrane potential disruption. An involvement of mitochondria in the mechanisms of ITCs cytotoxicity was determined from a ratio of orange

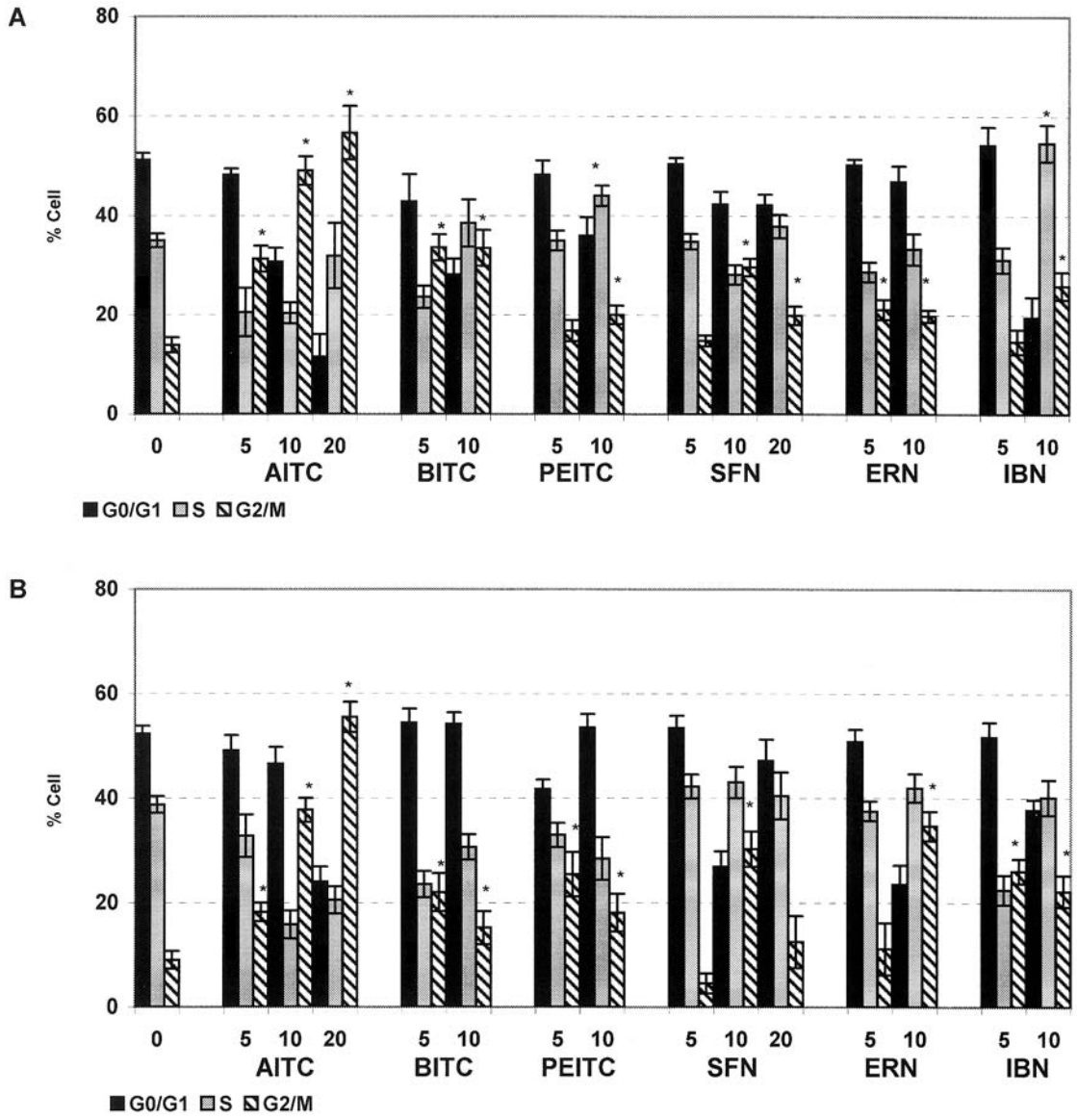

Figure 7. *Effect of ITCs on the cell cycle progression in multidrug-resistant sublines HL60/ADR and HL60/VCR. (A) The MRP1-expressing HL60/ADR cells and (B) Pgp-1-expressing HL60/VCR cells were exposed to DMSO (control) or different concentrations of ITCs for 24 h. The cells were collected, detergent permeabilized and stained with 50 μg/ml concentration of PI in the presence of RNA-se A. The distribution of cells in G_0/G_1-, S- and G_2/M-phases was analyzed by flow cytometry and Multi-cycle software. Three independent experiments were performed and mean±SE are presented. Significant difference from the controls, *p<0.01.*

fluorescence of JC-1 aggregates and green fluorescence of JC-1 monomers. The aggregation of monomers is directly correlated to mitochondrial membrane potential ψ_m, and their breakdown in dying cells results in increase of green fluorescence. The representative dot plots are shown in Figure 8A, where JC-1 aggregates accumulated in control cells and displayed high orange signal (top right quadrant). Treatment of cells with 5 μM BITC for 24 h resulted in a shift to higher green fluorescence with concomitant decrease of orange fluorescence intensity (65.2% of cells in bottom right quadrant). An induced decrease of ψ_m by ITCs treatment for 6 h was clearly visible in the HL60 cell line, to a lesser extent in the HL60/ADR cells and not observed in HL60/VCR cells (Figure 8B). The extent of ψ_m modulation was time- and concentration-dependent (Figure 8C), because treatment for 24 h caused more intensive fluorescence ratio decrease in comparison to 6-h treatment. Both aromatic ITCs (PEITC and BITC) easily diminished ψ_m, while SFN and AITC were less effective. Finally, the results showed that ITCs disrupted the mitochondrial potential of the cell lines according to the

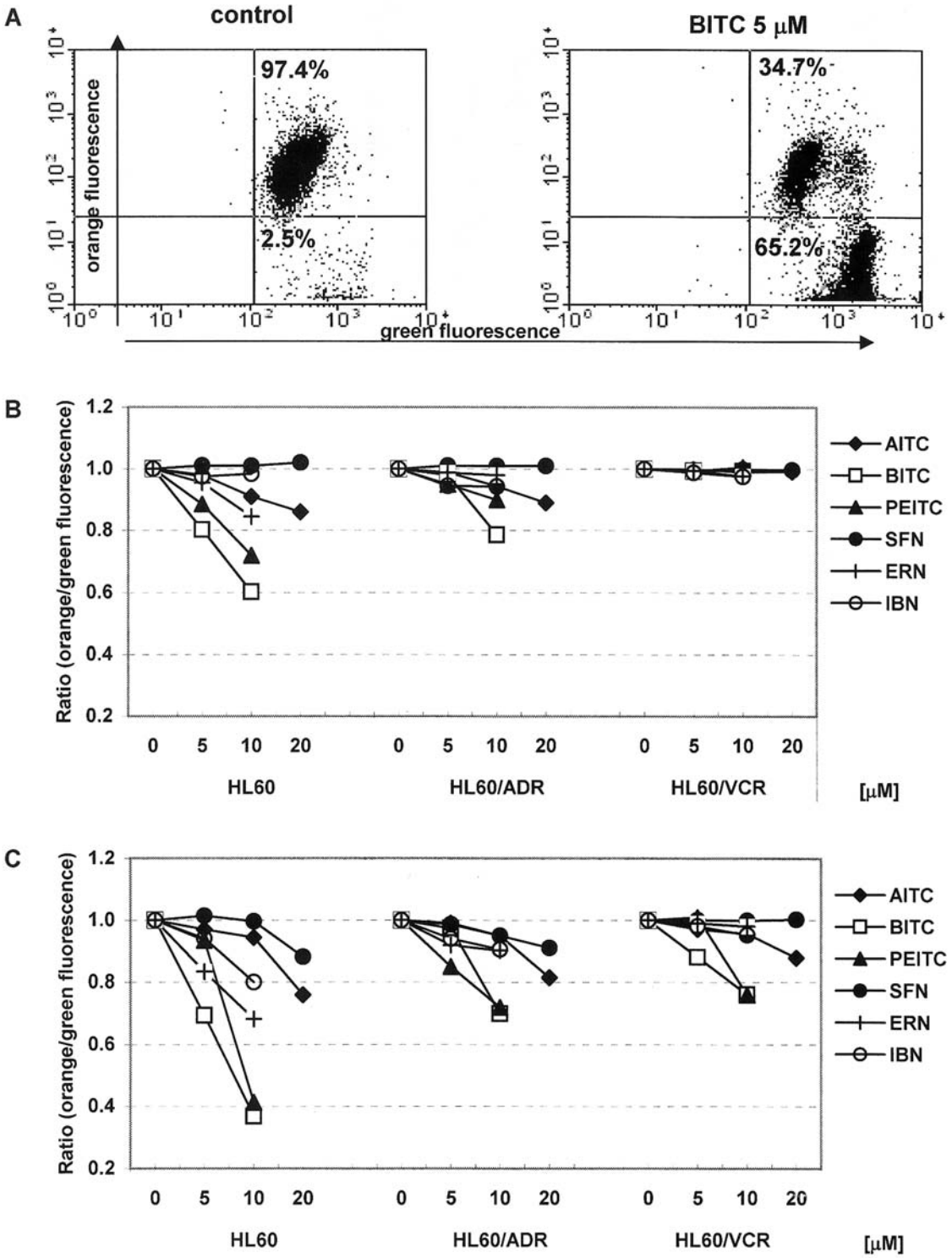

Figure 8. *ITCs induce depolarization of mitochondrial membranes in HL60, HL60/ADR and HL60/VCR. (A) The control HL60 cells (left dot blot) and cells treated with 5 μM BITC (right dot plot) for 24 h and stained with the mitochondria selective JC-1 dye. Cells with polarized mitochondrial membranes emit green-orange fluorescence (top right quadrant). The number in the bottom right quadrant of each dot plot represents the percentage of cells that emitted only green fluorescence, attributable to depolarized mitochondrial membranes. One experiment representative of three is shown. ITCs induced depolarization of mitochondrial membranes in HL60, HL60/ADR and HL60/VCR cells treated for 6 h (B) and for 24 h (C). Variations of the orange/green (FL2/FL1) fluorescence ratio as a function of the ITCs concentration are shown. The cells were exposed to either DMSO (control cells) or different concentrations of ITCs (5, 10 and 20 μM), stained with JC-1 dye and analyzed using a Coulter Epics Altra flow cytometer. The results shown are representative of at least three independent experiments. Data normalized against control=1.*

order of susceptibility: HL60>HL60/ADR>HL60/VCR and the effectiveness of ITCs followed the order: BITC=PEITC>ERN=IBN>AITC >SFN, the same order in observed potency in apoptosis induction.

Discussion

It is widely accepted that an imbalance between cell death and proliferation may result in tumor formation. Moreover, that tumor cells attempt to evade apoptosis can play a significant role in their resistance to treatment. In this study, the effects of six ITCs were studied, including the extent of cell cycle alterations, apoptosis induction and mitochondrial potential modulation. Three different methods, Annexin V staining, accumulation and metabolic conversion of FDA and appearance of cells with a sub-G_1 population, were used to confirm apoptotic cell death. Although Annexin-V and FDA staining measures different cellular events, the percentage of apoptotic and necrotic cells were similar independent of the method used. Generation of the sub-G_1 population is a later event in cell death, thus a lower number of cells in this phase were found at similar times. Short, 6-h treatment of parental HL60 cells was sufficient to induce apoptosis at the ITCs concentration used. Extension of the treatment for 24 h increased the percentage of cells in late apoptotic/necrotic phase. The IC_{50} values of particular ITCs for both multidrug-resistant cell lines were at least 4-times higher in comparison to the IC_{50} value for parental HL60 cells. Such an increase of IC_{50} values for ITCs in multidrug-resistant cells is consistent with recently published data (28). This relatively small difference of IC_{50} suggests the possible involvement of MRP-1 or Pgp-1 in resistance to ITCs since, in our hands, the resistance to paclitaxel or daunomycin was 2-3 logs higher for HL60/ADR and HL60/VCR cells in comparison to parental HL60 cells (31). It is known that MRP proteins transport drugs, either unmodified or conjugated, to anionic ligands such as GSH, glucuronate, or sulphate, while P-glycoproteins are thought to transport drugs in an unmodified form (32, 33). Initial cellular accumulation in the order AITC<PEITC<BITC, which did not exceed factor 4 between the highest concentration in parental cells and the lowest concentration in resistant cells, was followed by transporter-mediated export of ITCs in the form of dithiocarbamates (34). These findings are consistent with our survival IC_{50} data, Annexin V and FDA staining, DNA fragmentation and resulting increase of cell resistance to all ITCs tested in the order: HL60<HL60/VCR<HL60/ADR. Recent data have suggested that at least PEITC and BITC are not substrates for Pgp-1, but that their glutathione conjugates are probably substrates of MRP-1 (35).

There are several reports that ITCs induce cell cycle arrest in the G_2/M-phase, which is correlated with an increased expression of cyclins A and B1, followed by cell death. On the other hand, treatment of the prostate cell line LNCaP and phytohemagglutinin-stimulated human T lymphocytes resulted in G_0/G_1 cell phase arrest (18, 25). A significant number of possible molecular targets of ITCs treatment have been proposed, and the list of potential signaling pathway nodules involves Chk2, cdc2, cyclin B1, cdc25B and cdc25C (36, 37), $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ (38, 39), Bax and Bcl2 levels (16), Nrf2 (40, 41), GADD gene members (42), tubulin polymerization (15) and histone deacetylase inhibition (43). Flow cytometric study of parental HL60 cells and multidrug-resistant HL60/ADR and HL60/VCR cell lines showed significant accumulation of cells in the G_2/M-phase. We observed a dose-dependent increase of G_2/M content after ITCs treatment, with the exception of high concentrations of aromatic ITCs. This treatment (20 μM) induced early damage of the cell membrane, allowing propidium iodide permeation, and increased both forward and side scatters in cytometric dot plots and dissipation of mitochondrial potential; a typical pattern of toxic cell attack (data not shown). Despite aliphatic ITCs being less effective in apoptosis induction, they were equally potent or more potent compounds (AITC) relative to aromatic ITC in the induction of G_2/M block. In contrast to Zhang *et al.* (28), we did not observe AITC-induced G_0/G_1 block in parental HL60 cells. In our hands, short, 6-h AITC treatment increased the number of G_2/M-arrested cells, an effect that was extended more markedly after 24 h treatment in parental and both resistant cell lines. This is in accordance with recently published data (24). Our results showed that, after accumulation in the G_2/M cell cycle phase, the proportion of cells with sub-G_1 DNA content started to grow. The induction of cell cycle arrest is not a separate event; rather the cell cycle arrest leads to apoptotic cell death. Indeed, in this study, apoptotic cell death is preceded by an arrest of the cell cycle and accumulation of cells in the G_2/M-phase at the expense of the G_0/G_1-phase.

Analysis of the effect of ITCs in the human leukemia cell lines revealed that these agents induced changes in mitochondrial potential leading to progressive depolarization culminating in apoptosis (12, 44, 45). Disruption of normal ψ_m presumably will compromise ATP synthesis in cells and may be responsible for caspase-9 activation, which is mediated by mitochondria (46). The parental HL60 cells treated with ITCs for 24 h lost mitochondrial potential. The same effect was observed in multidrug-resistant HL60/ADR cells. On the other hand, no significant changes were visible in HL60/VCR cells treated for 6 h. Although the JC-1 mitochondrial probe is a substrate for Pgp-1 protein, extended treatment for 24 h allowed us to observe the mitochondrial potential changes. Probably, a limited availability of ATP in late apoptotic/necrotic cells is responsible for the lower activity of Pgp-1 protein.

Finally, the relative potency of ITCs to decrease mitochondrial potential followed the same order as their ability to induce apoptosis, as measured by Annexin V staining: BITC=PEITC>ERN=IBN>AITC>SFN for both parental and multidrug-resistant cell lines. ITCs, particularly sulforaphane, are perceived as promising compounds effective in *Helicobacter pylori* eradication (47), in protecting the retina from photoreceptors against oxidative stress (48) and in cancer prevention (all ITCs) (49-52). ITCs possess mitotic inhibitor and/or apoptosis inducer capabilities, together with ability to induce detoxification enzymes, thus indicating potential applications as chemotherapeutic agents in cancer treatment. Such effects might be quite relevant and merit further study.

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