Overcoming Multidrug Resistance in Human Lung Cancer with Novel Benzo[*a*]quinolizin-4-ones

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Abstract. *Aim: To investigate the ability of synthetic benzo[a]quinolizin-4-one derivatives to reverse multidrug resistance (MDR) in lung cancer cells. Materials and Methods: A cell line with MDR, A549RT-eto, was established by exposure to 1.5 μM etoposide. Cytotoxic activity was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) method. The mechanism of drug resistance was studied by real-time PCR, Western blot analysis, and flow cytometry. Benzo[a]quinolizin-4-one derivatives were synthesized and tested for cytotoxic activity and ability to modulate MDR. Results: A549RT-eto cells had an IC_{50} for etoposide of 176 μM, 28-fold higher than parental cells, due to increased levels of MDR1 gene and P-glycoprotein (P-gp), resulting in greater drug efflux. Three benzo[a]quinolizin-4-ones reduced etoposide IC_{50} from 176 μM to 22.4 μM -24.7 μM. This resulted from increased drug accumulation without altering P-gp expression at the transcription or translation level. Conclusion: Non-toxic concentrations of benzo[a]quinolizin-4-one derivatives can reverse drug resistance of A549RT-eto by increasing the intracellular drug accumulation.*

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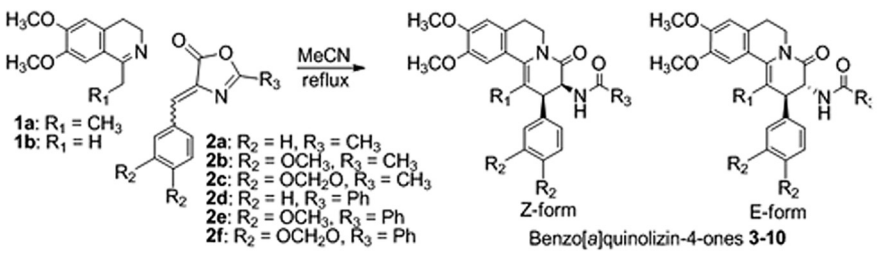
Multidrug resistance (MDR) in tumor cells is a major problem in the success of chemotherapy for many types of cancers. Of the various mechanisms proposed for drug resistance in cancer cells (1), a major mechanism involves the increase of drug efflux out of cells mediated by P-glycoprotein (P-gp). P-gp is a member of the ATP-binding cassette (ABC) superfamily of transporter proteins and utilizes the energy released from ATP hydrolysis to pump out cytotoxic drugs from cancer cells, leading to lower intracellular concentrations of chemotherapeutic drugs (1).

Drug resistance can be overcome by co-administering substances that inhibit transporters together with anticancer drugs to increase the efficiency of chemotherapy. Inhibition of P-gp as a way of reversing MDR has been extensively studied (2). Verapamil, cyclosporin, and taxoxifen have been shown to modulate the P-gp transporter (3, 4). Unfortunately, these reversing agents produced disappointing results *in vivo* because of their low binding affinities, so that reversal of MDR requires higher concentrations resulting in unacceptable toxicity. Thus, verapamil, a first-generation P-gp inhibitor, has effective concentrations in the range of 5 to 50 μM, which is cytotoxic to normal cells, resulting in clinical toxicity *e.g.* arterio-ventricular block, hypotension and cardiac toxicity at concentrations required to inhibit drug resistance (3). More recently, several natural products and synthetic compounds, such as valspodar (PSC833) (5), biricodar (VX-710) and elacridar (GF120918) (6), have been reported to overcome drug resistance through inhibition of P-gp activity *in vitro*. However, some compounds showed characteristics that limit their clinical usefulness, for example, significantly



3(Z)	H	H	Ph
3(E)	H	H	Ph
4(Z)	CH_3	H	Ph
4(E)	CH_3	H	Ph
5(Z)	CH_3	H	CH_3
5(E)	CH_3	H	CH_3
6(Z)	H	OCH_2O	CH_3
6(E)	H	OCH_2O	CH_3
7(Z)	H	OCH_3	CH_3
7(E)	H	OCH_3	CH_3
8(Z)	H	OCH_3	Ph
8(E)	H	OCH_3	Ph
9(Z)	CH_3	OCH_3	CH_3
9(E)	CH_3	OCH_3	CH_3
10(Z)	H	OCH_2O	Ph
10(E)	H	OCH_2O	Ph

Figure 1. *Schematic representation of synthesis of benzo[a]quinolizin-4-one and molecular structure of benzo[a]quinolizin-4-one derivatives 3-10.*

inhibiting metabolism *in vivo* or exhibiting severe side-effects (7-8). Therefore, new P-gp modulators with lower toxicity and greater potency are still needed.

The benzo[*a*]quinolizine ring system is an important basic structure found in many biologically active compounds including berberine, emetine and related ipecac alkaloids. These compounds have been reported to have interesting biological activities such as anti-neoplastic, antimicrobial and anti-depressant activities, as well as lowering cholesterol and blood sugar levels (9). Recently, our group has reported the synthesis of novel derivatives of benzo[*a*]quinolizin-4-ones (10). In the present study, some benzo[*a*]quinolizine derivatives were screened for cytotoxic activity and for ability to reverse MDR using a newly developed etoposide-resistant non-small cell lung carcinoma cell line (A549RT-eto) as an *in vitro* model. These derivatives showed moderate inhibition of P-gp-mediated efflux and reversed P-gp-dependent drug resistance at non-toxic concentrations.

Materials and Methods

Chemicals. Etoposide, doxorubicin, cisplatin, colchicine, vinblastine, 5-fluorouracil (5-FU), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT), calcein-acetoxymethylester (calcein-AM), verapamil, ProteoPrep Membrane Extraction Kit and P-glycoprotein antibody, C219, were purchased from Sigma, St. Louis, MO, USA. RNeasy kit, DNaseI and QuantiTect SYBR Green PCR master mix were purchased from Qiagen, (KJ Venlo, the Netherlands). Bradford reagent and taxol were purchased from Biorad Laboratories (Hercules, CA, USA) and Bristol Myers Squibb (New York, NY, USA), respectively. SuperscriptIII was obtained from Invitrogen (San Diego, CA, USA). All other chemicals were analytical grade. Non-small cell lung adenocarcinoma cell line, A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Rabbit anti-mouse immunoglobulin G (IgG) was obtained from Dako Cytomation, (Glostrup, Denmark). KB-V1 was kindly provided by Professor Gottesman MM (Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, MD, USA)

Development of drug-resistant lung cancer cells. To develop etoposide resistant cells (A549RT-eto), parental A549 cells were continuously exposed to increasing concentrations of etoposide up to 1.5 μM over a period of 18 months. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics at 37˚C in the presence of 5% CO_2. Resistant cells were maintained in 1.5 μM etoposide. Morphology of both etoposide-resistant and the parental lung cancer cell lines was studied using a Nikon TMS inverted microscope (Tokyo, Japan).

Synthesis of chemomodulator benzo[a]quinolizin-4-one derivatives 3-10. The tricyclic benzo[*a*]quinolizin-4-ones were prepared as described previously (10). The reaction of azlactones 2a-f (1 mmol) with various 1-substituted 3,4-dihydroisoquinolines 1a-b (1.5 mmol) containing methylene moiety by refluxing in acetonitrile (10 ml) for 2 h gave the corresponding benzo[*a*]quinolizine-4-ones 3-10 as a mixture of *cis*/*trans* (*Z*/*E*) form. The solvent was evaporated to dryness under vacuum. The crude product was purified by PLC on silica gel using 50% ethyl acetate in hexane as an eluent to give diastereoisomer of benzo[*a*]quinolizin-4-ones (*Z*/*E*) in moderate to good yield (Figure 1).

Cytotoxicity assay. The sensitivities of A549 and A549RT-eto cells to various chemotherapeutic drugs; etoposide, doxorubicin, cisplatin, taxol, colchicine, 5-FU, vinblastin and benzo[*a*]quinolizin-4-one derivatives were determined by MTT assay as previously described (11). Cells were cultured in drug-free medium for at least one week before the experiments were performed. Briefly, cells suspended in culture media were seeded at 5×10^3 cells per well into a 96-well plate, and incubated in humidified atmosphere, 5% CO_2 at 37˚C. After 24 hours, the cells were then cultured for 72 hours in the presence of chemotherapeutic drugs. Thereafter, the media were removed and the fresh media with MTT were added to each well. The plates were incubated at 37˚C for 2 hours. The dark blue formazan crystals formed by viable cells were dissolved in dimethyl sulfoxide (DMSO) solution. Absorbance of individual samples was determined at 550 nm using a Spectra Max Plus 384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The concentrations required to inhibit growth by 50% (IC_{50} values) were calculated. Each drug concentration was tested in at least three independent experiments and average values±S.D. were calculated. Relative resistance was calculated as the ratio of the IC_{50} value of A549 cells to the IC_{50} value of A549RT-eto cells.

Chemosensitivity assay. The effect of MDR-reversing agent of benzo[*a*]quinolizin-4-one derivatives on A549RT-eto cells to etoposide was also determined using MTT assay as described above. Non-cytotoxic benzo[*a*]quinolizin-4-one derivatives were selected and used to treat cells at final concentration of 10 μM in combination with several concentrations of etoposide. Reversing index was calculated as the ratio of the etoposide IC_{50} value towards A549RT-eto in the absence and the presence of test compounds.

Real-time PCR studies. Quantitative analysis of mRNA expression for drug resistance related genes in A549 and A549RT-eto cells was performed by real-time PCR. Total cellular RNA was isolated from cells using RNeasy kit. QuantiTect SYBR Green PCR master mix was used with 2 μl of cDNA and 10 pmol of primers: *MDR1* F: 5'GTCTTTGGTGCCATGGCCGT3', R: 5'ATGTCCGGTCGGGTG GGATA3' *MRP1* F: 5'CTGACAAGCTAGACCATGAATGT3', R: 5'C CTTTGTCCAAGACGATCACCC3'*MRP2*F: 5'GCCAGATTGG CCCAGCAAA3', R: 5'AATCTGACCACCGGCAGCCT3', *MRP3*F: 5'GGGACCCTGCGCATGAACCTG3', R: 5'TAGGCAAGTCCAGC ATCTCTGG3', BCRPF: 5'TGGCTGTCATGGCTTCAGTA3', R: 5'GCCACGTGATTCTTCCACAA3', *LRP*F: 5'GAGCAGTTCACA GTGTTGTCC3', R: 5'AAAGCCAAAGACAGCAGTGCG3', *GST*F: 5'TACGGGCAGCTCCCCAAGTT3', R: 5'TGCCCGCCTCATAGTT GGTG3', *TOPO2A*: 5'GGCTCGATTGTTATTTCCAC3', R: 5'ATGG TTGTAGAATTAAGAATAGC3', *TOPO2B*: 5'GCTGTGGATGAC AACCTC3', R: 5'GCTGTGGATGACAACCTC3', *actin*: 5'GACCT GACTGACTACCTCATGA3', R: 5'AGCATTTGCGGTGGACGA TGGAG3'. Real-time PCR was performed in capillary glass tubes on a LightCycler (Roche Applied Science, Indianapolis, IN, USA). During the amplification, SYBR green binds to double strand PCR products, so the fluorescence signal increases with increasing amounts of products. An initial activation step at 95˚C for 15 minutes was followed by 35 cycles comprising denaturation at 94˚C for 15 seconds, annealing at the respective temperature 55˚C for 30 seconds and extension at 72˚C for 30 seconds. Melting curve analyses and gel electrophoresis of products were used to validate the reactions. Reactions were photographed with Gene Genius Bio-Imaging system (Syngene, Cambridge, UK) and revealed single amplification products with the predicted sizes. The ratios of gene expression values were normalized using that of a housekeeping gene (β-actin as internal control) and calculated using the following formula:
Ratio of target gene expression=Fold change in target gene expression (A549RT-eto/A549)/Fold change in reference gene expression (A549RT-eto/A549)

Western blot analysis. For the investigation of the changes in protein expression, cells were treated as described above. Membrane proteins were extracted using ProteoPrep Membrane Extraction Kit. Protein concentrations were determined by Bradford assay. Solubilized membrane proteins (40 μg) were boiled in sodium dodecyl sulfate (SDS) sample buffer at 100˚C for 5 minutes and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 10% non-fat dried milk in TBS-T buffer (20 mM Tris buffer saline, pH 7.6 containing 0.1% Tween-20) overnight, membrane was probed with 1:1,000 diluted human P-gp monoclonal antibody C219 for 1 hour. Then, the membrane was incubated in 1:5,000 diluted secondary antibody linked with horseradish peroxidase for visualization by chemiluminescence (ECL Plus detection system with high-performance film; GE Healthcare, Waukesha, WI, USA).

Calcein-AM efflux assay. Evaluation of P-gp activity was performed by measuring the fluorescence due to intracellular accumulation of calcein produced by ester hydrolysis of the P-gp substrate calcein-AM. The transport capacity of P-gp is inversely proportional to the intracellular accumulation of fluorescent calcein (12). The ability of modulators to inhibit P-gp mediated efflux was investigated. Briefly, 1×10^6 cells cells were incubated with 0.5 μM of calcein-AM at 37˚C in the absence and presence of 10 μM benzo[*a*]quinolizin-4-one derivatives or P-gp inhibitor, verapamil for 1 hour. After incubation, cells were washed, resuspended and then calcein accumulation was measured using a FACSCalibur™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) with excitation wavelength of 488 nm and emission of 530 nm.

Statistical analysis. Statistics were performed with the Unpaired *t*-test, one-way ANOVA and Tukey–Kramer multiple comparisons test using GraphPad Instat (GraphPad software, San Diego, CA, USA). Values significantly different from the control are shown by $*p<0.05$, $**p<0.01$ and $***p<0.001$.

Results

Development of drug-resistant lung cancer cells. An etoposide resistant cell line (A549RT-eto) was established over a period of 18 months. There was no significant difference in growth curve and doubling time between the A549 parental cells and the A549RT-eto resistant cells (data not shown). However, the two cell lines showed differences in morphology. A549 cells showed an epithelioid-like shape and adhered to the dish, whereas morphology of A549RT-eto cells changed to a spindle-like shape, varying in size and having unclear cell borders (Figure 2).

Cytotoxicity assay. Cytotoxic effects of etoposide on the lung cancer cell lines were studied by measuring cell viability using the MTT assay. Dose–response curves were obtained after 72 hours of exposure of A549 and A549RT-eto cells to various concentrations of etoposide. The concentration of etoposide inhibiting growth of A549RT-eto cells by 50% (IC_{50}) was 176±9.5 μM, or 28-fold higher than that for A549 cells, which had an IC_{50} of 6.33±1.46 μM (Table I). Moreover, the resistant cells exhibited cross-resistance to the structurally unrelated antitumor agents, doxorubicin, taxol, colchicine and vinblastin, but still remained sensitive to cisplatin and 5-FU, as shown in Table I.

Mechanism of drug resistance. In order to analyze changes in gene expression associated with drug resistance in A549RT-eto cells, real-time PCR was used to compare the expression of drug resistance related genes: P-glycoprotein (*MDR1*), multidrug resistance related protein (*MRP1, MRP2* and *MRP3*), breast cancer resistance protein (*BCRP*), lung resistance-related protein (*LRP*), glutathione-*S*-transferase π (*GSTP*) and topoisomeraseII (*topoIIa* and *topoIIb*) in resistant cells compared with parental cells. A549RT-eto cells

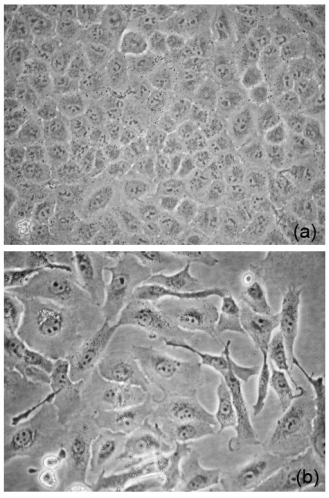


Figure 2. *Morphologies of parental (A549), and etoposide-resistant (A549RT-eto) cells shown under ×400 magnification by inverted microscopy.*

Table I. *IC_{50} and resistance index of A549 cells and A549RT-eto cells to chemotherapeutic drugs. Each IC_{50} value is an average value±S.D. from three independent experiments. Resistance index represents the ratio of IC_{50} for A549RT-eto cells to the IC_{50} for A549 cells. IC_{50} values differing significantly between A549RT-eto cells and A549 cells by unpaired t-test are shown by *p<0.05, **p<0.01 and ***p<0.001.*

Anticancer drug	IC_{50}		Resistance index
	A549	A549RT-eto	
Etoposide (μM)	6.33±1.46	176±9.5	27.7***
Doxorubicin (μM)	0.32±0.10	1.57±0.15	4.90**
Taxol (nM)	3.50±1.00	12.2±1.0	3.48***
Colchicine (nM)	18.2±10	56.3±8.0	3.09*
Vinblastin (nM)	0.20±0.15	0.80±0.10	4.00*
Cisplatin (μM)	8.09±4.63	12.2±4.7	1.50
5FU (μM)	30.7±2.98	34.0±4.5	1.11

Table II. *Ratios of target gene expression in etoposide-resistant A549RT-eto cells compared with that in parental A549 cells using real-time PCR.*

Gene	Ratio of target gene expression
MDR1	16.0
MRP1	1.82
MRP2	0.52
MRP3	0.65
BCRP	0.50
LRP	1.51
GSTP	1.13
TOPO2A	0.26
TOPO2B	0.31

expressed *MRP1, MRP2, MRP3, BCRP, LRP, GSTP, TOPO2A* and *TOPO2B* at comparable levels to A549 cells (Figure 3A). In contrast, the expression level of *MDR1* in resistant cells was dramatically increased by 16-fold compared to the parental cell line (Table II). The results suggest that the major mechanism of acquired etoposide resistance in the A549 cell line involves the up-regulation of *mdr1* gene, which encodes for a drug efflux transporter, P-gp. P-gp expression of the resistant cell line was confirmed by Western blot analysis using C219 antibody. P-gp (170 kDa) was highly expressed in A549RT-eto resistant cells, whereas no detectable P-gp could be observed in A549 parental cells (Figure 3B). This indicates that resistance to etoposide is related to the overexpression of P-gp in A549RT-eto cells. A functional study of P-gp was performed by calcein accumulation using flow cytometry. Calcein-AM becomes fluorescent after cleavage by cellular esterase producing a fluorescent derivative calcein. P-gp actively extrudes the calcein-AM, but not the fluorescent calcein. Therefore, calcein-AM efflux measured by flow cytometric analysis can be used to assess the function of the P-gp pump. Increased accumulation of calcein is detectable as a shift in the fluorescence peak to the right. As shown in Figure 3C, calcein was accumulated at higher levels in A549 cells than in A549RT-eto cells, suggesting that A549RT-eto cells showed higher activity of the efflux pump transporting calcein-AM out of the cells. Moreover, the addition of established P-gp inhibitor, verapamil, resulted in a substantial increase in the accumulation of calcein compared to untreated A549RT-eto cells. The data strongly confirm that the acquired resistance in A549RT-eto cells is associated with overexpression of P-gp which lowers the intracellular drug accumulation.

Reversal of etoposide resistance by benzo[a]quinolizine-4-one derivatives 3-10. Fifteen synthetic benzo[*a*]quinolizine-4-one derivatives, *Z*- and *E*-isomers of compounds 3-10, and *Z*-isomer only of compound 6 were investigated for their ability to inhibit proliferation of A549RT-eto cells using the MTT assay. The cytotoxicity of these derivatives on the multidrug resistant cells, A549RT-eto, is shown in Table III. Four of the synthetic compounds, 3(*Z*), 4(*Z*), 4(*E*) and 8(*E*), displayed cytotoxic activity towards the A549RT-eto cell line with an IC_{50} in the range of 30-40 μM, while 11 of the synthetic compounds did not show cytotoxicity at the concentrations tested (IC_{50} more than 100 μM).

The IC_{50} of etoposide alone towards A549RT-eto cells was 176 μM. The ability of the eleven non-cytotoxic compounds to restore drug sensitivity of A549RT-eto cells towards etoposide was investigated by determining the IC_{50} of etoposide towards A549RT-eto in the presence of non-cytotoxic concentrations (10 μM) of these compounds. As shown in Table III, only 3 compounds, 5(*Z*), 7(*Z*) and 9(*Z*)

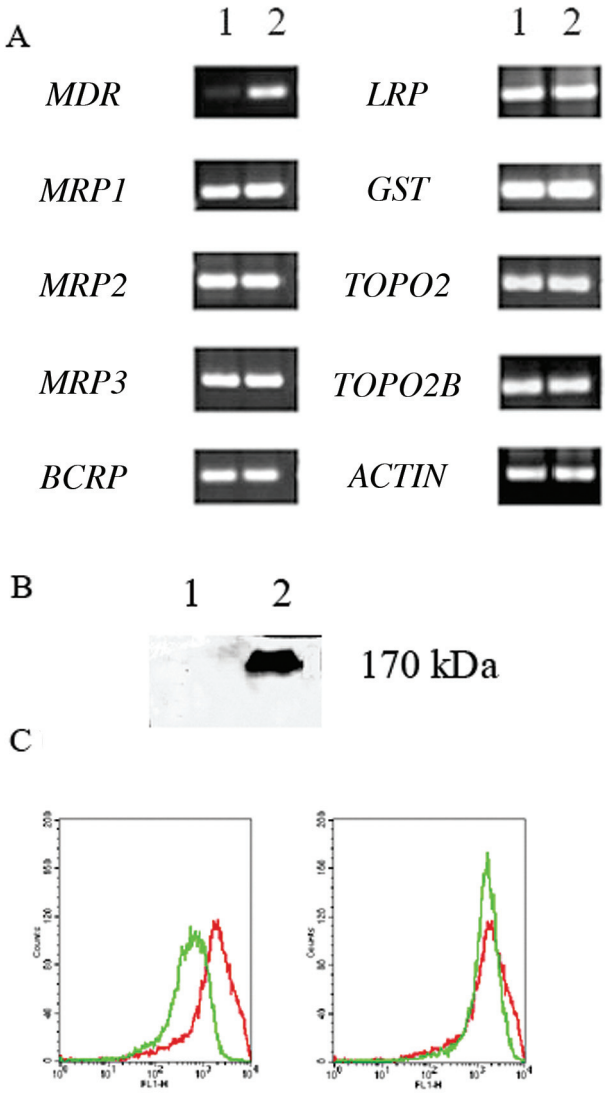


Figure 3. *Expression and activity of drug-resistance genes and proteins. A) RT-PCR characterization of drug resistance-related genes (MDR1, MRP1, MRP2, MRP3, BRCP, LRP, GST, TOPO2A, TOPO2B) and control (actin) in parental A549 (lane 1) and (lane 2) resistant A549RT-eto cells. B) P-gp expression in parental A549 cells (lane 1) and resistant A549RT-eto cells (lane 2) by immunoblotting. C) Accumulation of calcein-AM analyzed by flow cytometry. Left: Accumulation of calcein-AM in A549 cells (red line) compared to that with resistant A549RT-eto (green line) cells. Right: Accumulation of calcein-AM in A549 cells (red line) compared to that of resistant A549RT-eto cells in the presence of P-gp inhibitor, verapamil (green line).*

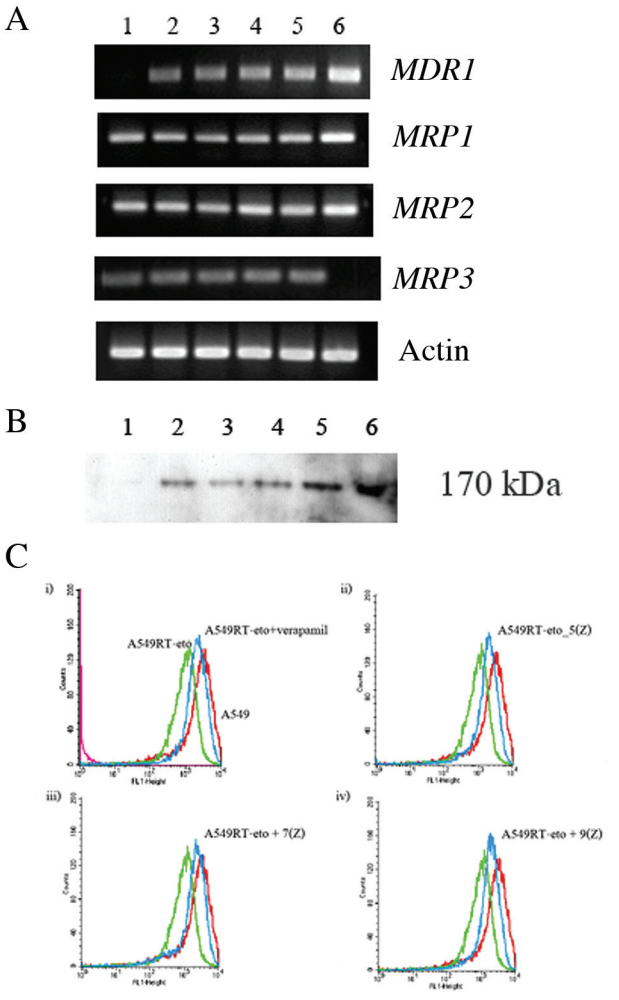


Figure 4. *Expression and activity of ABC transporter genes and proteins. A) Expression of genes (MDR1, MRP1, MRP2, MRP3) in lane 1: parental A549 cells; lane 2: resistant A549RT-eto cells; lanes 3-5; resistant A549RT-eto cells treated with 5(Z), 7(Z) and 9(Z) respectively; lane 6: positive control, KB-V1. B) protein expression of P-gp by immunoblotting in lane 1: parental A549 cells; lane 2: resistant A549RT-eto cells; lanes 3-5; resistant A549RT-eto cells treated with 5(Z), 7(Z) and 9(Z) respectively; lane 6: positive control, KB-V1. (C) Intracellular accumulation of calcein-AM by flow cytometry in A549 cells (red) compared to that of resistant A549RT-eto cells (green) and resistant A549RT-eto cells in the presence of the selected compounds (blue) namely i) verapamil or benzo[a]quinolizin-4-one derivatives (ii) 5(Z), (iii) 7(Z) and (iv) 9(Z).*

showed the ability to partially reverse drug resistance in A549RT-eto, by reducing the IC_{50} of etoposide to 22.6, 24.7 and 22.4 μM respectively.

Possible mechanism of action of potential reversing agents, 5(Z), 7(Z) and 9(Z) in sensitizing A549RT-eto cells. First, the expression of drug resistance-related genes was studied by real-time PCR analysis in A549RT-eto cells treated with reversing agents 5(*Z*), 7(*Z*) and 9(*Z*) and in untreated A549RT-eto cells. Comparison of untreated and reversing agent-treated cells showed no significant difference in the expression levels of any of the drug resistance-related genes, namely the ABC transporters, *MDR1, MRP1, MRP2* and *MRP3* (Figure 4A). Similarly, immunoblotting of P-gp showed that the up-regulation of P-gp levels in A549RT-eto

Table III. *Effect of benzo[a]quinolizin-4-one derivatives on the sensitivity of A549RT-eto cells. Each IC_{50} value is an average value±S.D. from three independent experiments.*

Compound name	Cytotoxicity IC_{50} (μM) of compound alone	IC_{50} (μM) for etoposide in the presence of compound	Reversing index
Etoposide	176		
Verapamil	>100	36.4±8.80	4.79
3(*Z*)	40	Nd	Nd
3(*E*)	>100	153±5	1.14
4(*Z*)	35	Nd	Nd
4(*E*)	30	Nd	Nd
5(*Z*)	>100	22.6±7.0	7.74***
5(*E*)	>100	178.5±10.0	0.98
6(*Z*)	>100	118.9±5.7	1.47*
6(*E*)	ND	Nd	Nd
7(*Z*)	>100	24.7±6.4	7.10***
7(*E*)	>100	128±15	1.37
8(*Z*)	>100	68.0±10.6	2.57**
8(*E*)	38	Nd	Nd
9(*Z*)	>100	22.4±4.6	7.80***
9(*E*)	>100	153±10	1.14
10(*Z*)	>100	153±7	1.14
10(*E*)	>100	153±10	1.14

Cytotoxicity of each derivative was determined and derivatives showing an IC_{50} greater than 100 μM were studied for their ability to affect the IC_{50} of etoposide. Reversing index is defined as the ratio of IC_{50} for etoposide in the absence and in the presence of added compound. Statistics were performed using one-way ANOVA and Tukey-Kramer Multiple Comparisons Test. Significantly different values are shown by *p<0.05, **p<0.01 and ***p<0.001. Nd, not determined since test compounds were toxic to cells.

cells was not diminished by treatment with benzo[*a*] quinolizine-4-one derivatives (Figure 4B). These results indicate that 5(*Z*), 7(*Z*) and 9(*Z*) do not inhibit expression of the drug transporter genes, including P-gp at either the gene expression level (Figure 4A) or the protein level (Figure 4B). The ability of the 5(*Z*), 7(*Z*) and 9(*Z*) to affect intracellular drug accumulation in A549RT-eto resistant cells was further investigated. Flow cytometric analysis of their action on reversal of drug resistance demonstrated that the addition of all modulating compounds caused a significant increase in accumulation of calcein in A549RT-eto (Figure 4C). In contrast, the tested substances did not cause any significant increase in the intracellular calcein levels of parental A549 cells (data not shown). Therefore, the calcein accumulation study was consistent with the reversal of drug resistance evaluated by the MTT assay.

Discussion

In the present study, an etoposide-resistant A549RT-eto cell line was developed and used as an *in vitro* model of screening for inhibitors of P-gp. The resistant cells showed change in morphology from epitheloid shape to spindle-like shape. Morphological changes have also been reported in several studies of resistant cell lines (13, 14). Thus, cisplatin-resistant neuroblastoma cell lines demonstrated alterations in their morphology without changes in cell growth (13). Moreover, a correlation between the epithelial to mesenchymal transition (EMT) and drug resistance has been reported by Arumugam (15). Morphological changes in cells may also be caused by alterations of the actin cytoskeleton or by mutations of the *c-KIT* gene (16). In an adriamycin-resistant leukemia cell line, morphological changes have been reported to be associated with the tumor cell environment rather than the abundance of P-glycoprotein in the plasma membrane (14). However, the relationship between the changes in morphology and changes in gene expression of the A549RT-eto resistant cell line is still unclear.

The mechanisms of etoposide-resistance in A549 cells have been shown to vary. Long *et al*. demonstrated that the mechanism of acquired etoposide-resistance in A549 cell lines was likely to relate to decreased topoisomeraseII expression (17). On the other hand, Trussadi *et al*. reported that etoposide resistance in A549 cells paralleled the increased expression of the *MRP1* gene and decreased expression of the *LRP* gene (18). However, our studies by real-time PCR, Western blotting and drug efflux pump analysis clearly show that the etoposide resistance of A549RT-eto cells involves up-regulation of the *MDR1* gene, resulting in overexpression of P-gP.

To overcome multidrug resistance in human cancer cell, a number of natural or synthetic compounds have been discovered that exhibit MDR activity (19-21). For example, a benflumetrol derivative, LY980503, has been reported to inhibit P-gp activity in vincristine-resistant gastric cancer cells and increase drug sensitivity by about 6-fold (20).

Benzo[*a*]quinolizin-4-ones exhibit the common structure of most P-gp inhibitors in having a methoxy phenol group and a basic nitrogen atom, as reported by Pajeva (22). Therefore, a series of benzo[*a*]quinolizin-4-one derivatives was synthesized having substituents differing in molecular size, hydrophobicity and *E*- and *Z*- stereoisomer at C2-C3 position (10). These synthesized compounds were initially tested for their ability to inhibit cell proliferation. Certain derivatives showed cytotoxic activity. Thus only 11 compounds, which had IC_{50} values higher than 100 μM, were further investigated for their ability to reverse MDR and potentiate etoposide cytotoxic activity in A549RT-eto resistant cells. The data demonstrated that three benzo[*a*]quinolizin-4-one derivatives of *Z*-isomer, 5(*Z*), 7(*Z*) and 9(*Z*), were able to

increase the sensitivity of A549RT-eto to etoposide. Interestingly, none of the E-isomers was effective, indicating the stereospecificity of the target site. The benzo[*a*]quinolizin-4-one derivatives appear to reverse MDR by enhancing drug accumulation in A549RT-eto cells without altering the levels of the transcription of the *MDR1* gene or the level of P-gp expression. Thus, the action of the active benzo[*a*]quinolizin-4-one derivatives may be due to direct binding to P-gp and competition with drugs, such as etoposide, thereby enhancing intracellular drug accumulation.

Thus, our studies show that derivatives of benzo[*a*] quinolizine-4-one can sensitize drug-resistant A549RT-eto cells apparently by decreasing the activity of P-gp at non-toxic concentrations (10 μM). Thus benzo[*a*]quinolizine-4-one derivatives may be promising lead compounds for developing new drugs which reverse MDR *in vivo*. Future studies are required in order to investigate the action of these compounds *in vivo*, as well as to develop more effective compounds by modifying their structures.

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