รายงานวิจัยฉบับสมบูรณ์

โครงการ : การหาสารประกอบจากธรรมชาติที่มีฤทธิ์ยับยั้งการดื้อยาของเซลล์มะเร็ง

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สัญญาเลขที่ MRG5080092

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สถาบันวิจัยจุฬาภรณ์

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Project code: MRG5080092

Project Title: Screening for Inhibitors of Multidrug Resistance from Marine and Plant Sources using Drug Resistant Cancer Cell Lines

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Project Period: 2 years

The development of drug resistance is one of the major obstacles to effective cancer chemotherapy. In order to understand the mechanism of drug resistance, a resistant lung cancer cell line A549RT-eto were established by continuous exposure of A549 cell line to increasing concentrations of etoposide. This A549RT-eto cell line was approximately 28-fold more resistant to etoposide when compared to parental A549 cell line. Moreover, A549RT-eto resistant cells were cross resistant to doxorubicin but not to taxol and cisplatin. To investigate the drug resistance mechanism, the expression levels of drug resistance-related genes were determined using real-time PCR. Of all the genes investigated, only *mdr1* transcript levels were drastically increased in A549RT-eto cells compared to the parental cells. Since the *mdr1* gene encodes for P-glycoprotein (P-gp), a membrane protein involved in drug efflux pumps, the expression of P-gp and its transport activity were studied. Western blot analysis showed higher P-gp expression in the resistant cells compared to A549 cells. As determined by flow cytometry, the levels of calcein transported in the drug resistant cells were also enhanced, which correlates with the increase in P-gp expression shown in the western blot. Therefore, these studies strongly suggest that expression of *mdr1* induced by etoposide plays a major role in the development of drug resistance in A549RT-eto cell line. In the future, this cell line may be used as an *in vitro model* for the screening of novel P-gp inhibitors to overcome drug resistance in human cancer cells.

Keyword: Multidrug resistance, P-glycoprotein, Flow cytometry, Lung cancer cell

บทคัดย่อ

รหัสโครงการ: MRG5080092

ชื่อโครงการ: การหาสารประกอบจากธรรมชาติที่มีฤทธิ์ยับยั้งการดื้อยาของเซลล์มะเร็ง

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ระยะเวลาโครงการ: 2 ปี

ปัจจุบันนี้ปริมาณผู้เสียชีวิตจากการป่วยเป็นมะเร็งสูงขึ้นทุกปี การรักษาด้วยยาเคมีบำบัด เป็นทางเลือกหนึ่ง แต่เนื่องจากปัญหาการดื้อยาของเซลล์มะเร็งเป็นอุปสรรคสำคัญต่อประสิทธิภาพการรักษามะเร็งด้วยวิธีนี้ งานวิจัยครั้งนี้จึงมุ่งเน้นที่จะเข้าใจถึงกลไกการดื้อยาของเซลล์มะเร็ง ในการทดลองครั้งนี้ได้ทำการพัฒนาเซลล์มะเร็งปอดดื้อยา (A549RT-eto) โดยการเลี้ยงเซลล์มะเร็งปอด (A549) ในอาหารที่มียา etoposide ในปริมาณต่ำๆ และ ค่อยๆเพิ่มปริมายาขึ้นจนถึง 1.5 ไมโครโมลาร์ หลังจากนั้น นำเซลล์มะเร็งปอดดื้อยา และเซลล์มะเร็งปอด ไปหาค่า IC_{50} ต่อยา etoposide doxorubicin taxol และ cisplatin พบว่า เซลล์มะเร็งปอดดื้อยามีความสามารถต้านทานยาฆ่ามะเร็ง etoposide และ doxorubiciin ได้มากกว่าเซลล์มะเร็งปอดถึง 28 และ 5 เท่า ตามลำดับ เพื่อที่จะศึกษาถึงกลไกการดื้อยาของเซลล์มะเร็งในระดับอาร์เอ็นเอ การทดลองต่อไปจึงได้ทำการสกัดอาร์เอ็นเอของทั้งสองเซลล์ และเปรียบเทียบการแสดงออกของยีนที่เกี่ยวข้องกับการดื้อยาของเซลล์มะเร็ง พบว่า เซลล์มะเร็งปอดดื้อยามีการแสดงออกของ ยีน mdr1 สูงกว่าเซลล์มะเร็งปอด ในขณะที่ทั้งสองเซลล์มีการแสดงออกของยีนอื่นๆ ใกล้เคียงกัน การศึกษาระดับโปรตีน ยีน mdr1 encodeได้พีไกลโคโปรตีน การทดลองขั้นต่อไปจึงได้ทำการศึกษาการแสดงออกระดับโปรตีนของพีไกลโคโปรตีนในทั้งสองเซลล์ พบว่ามีการแสดงออกของโปรตีนพีไกลโคโปรตีนสูงในเซลล์มะเร็งปอดดื้อยา ซึ่งสอดคล้องการการศึกษาการทำงานของพีไกลโคโปรตีนด้วยวิธีโฟลไซโตเมทรี เซลล์มะเร็งปอดดื้อยา สามารถขับยาออกจากเซลล์ได้มากกว่า เซลล์มะเร็งปอด การทดลองครั้งนี้จึงสรุปได้ว่า การดื้อยาของเซลล์มะเร็งปอดดื้อยาเกิดจากการแสดงออกของยีน mdr1 ซึ่งแสดงออกเป็นพีไกลโคโปรตีน และมีหน้าที่ขับยาออกจากเซลล์

Keyword: ดื้อยา, พีไกลโคโปรตีน, โฟลไซโตเมทรี, มะเร็งปอด

Executive Summary

Cancer is considered to be the leading cause of illness and mortality in Thailand. The incidence of cancer has dramatically increased and currently, approximately 60,000 people die of cancer each year in Thailand. Cancer occurs when cells progressively accumulate mutations and undergo a transformation resulting in uncontrolled division. Chemotherapy is the treatment of choice for approximately 50% of all cancers. Nevertheless, this therapeutic approach has offered only limited success so far since it is generally complicated by multidrug resistance, designated MDR. Exposure of the tumor cells to the chemotherapy drug can confer resistance, and MDR is considered to be a major obstacle to the success of chemotherapy. Some cancers such as non-small cell lung cancer and rectal cancer show primary resistance or natural resistance in which they do not respond to standard chemotherapy drugs from the beginning. On the other hand, many types of cancers respond well to chemotherapy drugs initially but develop resistance later.

Several mechanisms are known to be responsible for the MDR process. One involves increased drug efflux out of cells mediated by ATP-binding cassette (ABC) superfamily of transporter proteins, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP). 170-kDa P-gp, 190-kDa MRP and 72-kDa BCRP proteins are products of the *MDR1*, *MRP* and *BCRP* genes, respectively. These transporters utilize the energy that is released from ATP hydrolysis to drive the transport of various molecules across the cell membrane. These proteins are expressed in normal tissues and are often dramatically over-expressed in human cancer (Borges WM et al., 2003, Gottesman et al., 2002, Long BH et al., 1991, Trussardi A et al., 1998, Chen L et al., 2000, Martin M et al., 2004, Higgins CF et al., 1992). Over-expression of transporter proteins can result in increased pumping out of cytotoxic drugs from the cancer cell leading to lower intracellular concentration of chemotherapeutic drugs (Gottesman MM et al., 2002, Ambudkar et al., 1999). The drug-resistance pattern generated by MRP overexpression is similar but not identical to that of P-gp. However, it has been reported that MRP tends to transport anionic and neutral drugs conjugated to acidic ligands, such as glutathione (GSH) (Brost P et al., 2000, Gottesman MM et al., 2002).

Another mechanism involves vault protein, a complex ribonucleoprotein particle that plays a role in redistribution of drugs away from their targets. Vaults are thought to participate in transporting substrates between nucleus and cytoplasm. It has been reported that overexpression of 110-kDa Lung resistance related protein (LRP), the major vault protein, has been linked directly to MDR in cancer cells (Kedersha and Rome, 1986, Scheffer et al., 1995).

The other model of MDR development relates to detoxifying enzymes i.e. glutathione-S-transferases (GSTs). The GSTs are a large family of phase II enzymes which facilitate the detoxification of various carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress. The enzymes protect cells against toxicants by conjugating the thiol group of glutathione to electrophilic xenobiotics and thereby protecting cells against the mutagenic, carcinogenic and toxic effects of the compound. Although GSTs are involved in protecting an organism from toxic and mutagenic xenobiotics, GSTs are also involved in the development of natural and acquired resistance towards xenobiotic compounds such as chemotherapy agents. In particular, the overexpression of GSTs in tumors appears to be a factor in the development of acquired resistance towards anti-cancer drugs (Akiyama SI et al., 1999, Panasci L et al., 2001, Bredel M et al., 2001). Hence GSTs might be the potential targets for ameliorating the chemotherapeutic treatment.

Resistance to chemotherapy appears to be the major hindrance to chemotherapy of lung cancer, the leading cause of cancer death in both women and men in Thailand. Thus, inhibition of proteins mediating MDR may serve as an alternative approach to reverse the MDR process and, in turn, increase the efficiency of chemotherapeutic treatment, leading to an increase in the survival rate of patients. In order to understand drug resistance mechanisms, the expression of proteins involved in MDR will be studied using the small-cell lung carcinoma (SCLC) H69 cell lines and non-small cell lung carcinoma (NSCLC) A549 cell lines.

Thailand is endowed with a great diversity of indigenous medicinal plant species and marine organisms. Plants have provided the basis of traditional medicine for many years. However, in recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the marine organisms. Plant and marine worlds offer an extremely rich resource for novel compounds and represent a great challenge that requires input from various scientific areas to make the best use of plant and marine chemical diversity for therapeutic applications.

In this study, Doxorubicin-resistant H69AR small-cell lung carcinoma (SCLC) cell lines and Etoposide-resistant A549 non small-cell lung carcinoma (NSCLC) cell lines will be used for screening the MDR protein inhibitors from natural plant and marine compounds.

Objective

The aim of this study is

- To develop and elucidate the mechanism of Etoposide-resistance in A549 lung cancer cell lines.

Material and Method

Development of drug resistant lung cancer cells

To develop etoposide resistant cells A549RT-eto, the parental cells, A549, were continuously exposed to increasing concentrations of etoposide up to 1.5 μM for 18 months. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 10% Fetal bovine serum (FBS, Hyclone, UT) and 0.5% penicillin-streptomycin at 37°C in the presence of 5% CO_2. Resistant cells were maintained in the indicated concentration of etoposide.

Cytotoxicity assay

The sensitivities of resistant and parental cells to various chemotherapeutic drugs were determined by MTT assay. Cells were cultured in drug-free medium for at least 1 week before the experiments were performed. Briefly, cells suspended in RPMI-1640 containing 10% FBS were seeded at 5 x 10^3 cells (100 μl) per well in a 96-well plate, and incubated in humidified atmosphere, 5% CO_2 at 37°C. After 24 hours, additional media (100 μl) containing various concentrations of chemotherapeutic drugs (etoposide, doxorubicin, cisplatin and taxol) were added and plates were incubated for 72 hours. Thereafter, the media were removed by aspiration, and 100 μl of fresh media with MTT was added to each well. The plates were incubated at 37°C for 2 hours. The dark blue formazan crystals formed by live cells were dissolved in 100 μl of DMSO. Absorbances of individual samples were determined at 550 nm using Spectra Max Plus 384 microplate spectrophotometer (Molecular Devices). The concentration required to inhibit growth by 50% (IC_{50} values) were calculated. Data were obtained from at least five independent experiments. Resistance index (RI) equals the ratio of IC_{50} values of resistant to sensitive cells.

Elucidation into the mechanism of multidrug resistance using real time PCR

The mRNA gene expression of *mdr1, mrp1, mrp2, mrp3, bcrp, lrp, gst-π, topoisomeraseIIa and topoisomeraseIIb* in the resistant cells, A549RT-eto, were compared with those of parental A549 cell line using real-time PCR.

Total cellular RNA was isolated from cells using RNeasy kit. Total RNA preparations were treated with DNase I for 15 minutes at room temperature. For cDNA synthesis, 1 μl of 10 pmol oligo dT primer was added to 2 μg RNA in 20 μl reaction and incubated for 10 minutes at 70°C. 200 units of SuperScript III reverse transcriptase was added to the mixture and incubated first for 50 minutes at 42°C and then for an additional 15 minutes at 70°C. QuantiTect SYBR Green PCR master mix was used with

2 μl of cDNA and 10 pmol of specific primers. PCR was performed on a LightCycler (Roche Diagnostics) in capillary glass tubes. During the amplification, SYBR green binds to double strand PCR products and so the fluorescence signal increase with the increasing amount of product. Primer sequences were designed based on the reported sequences shown in Table 1 to amplify a portion of exons in the relevant genes related to drug resistance and β-actin gene as an internal control. An initial activation step at 95°C for 15 minutes was followed by 35 cycles comprising of a denaturation step at 94°C for 15 seconds, annealing at the respective T_a (shown in Table 1) for 30 seconds and extension at 72°C for 30 seconds. Melting curve analyses and gel electrophoresis of products validated 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) and calculated using the following formula:

$$\text{Ratio target gene expression} = \frac{\text{Fold change in target gene expression (A549RT-eto/A549)}}{\text{Fold change in reference gene expression (A549RT-eto/A549)}}$$

Western Blot Analysis

Membrane proteins were extracted using ProteoPrep Membrane Extraction Kit. Protein concentrations were determined by Bradford assay. Solubilized membrane proteins (40 μg) were boiled in SDS sample buffer at 100°C for 5 minutes and separated by electrophoresis on 10% SDS-PAGE, then transferred to a nitrocellulose membrane. After blocking with 10% non-fat dried milk in TBS/T buffer (20 mM Tris buffered saline pH 7.6 containing 0.1% Tween-20) for 1 hour at room temperature, the membrane was probed with 1:100 diluted specific monoclonal antibody C219 and incubated at 4°C overnight. After three washing steps in TBS/T buffer, the membrane was incubated in 1:5000 rabbit anti-mouse immunoglobulin G for 1 hour. After washing, the reaction was developed using ECL plus detection system with high-performance film (Hyper-film ECL; GE Healthcare).

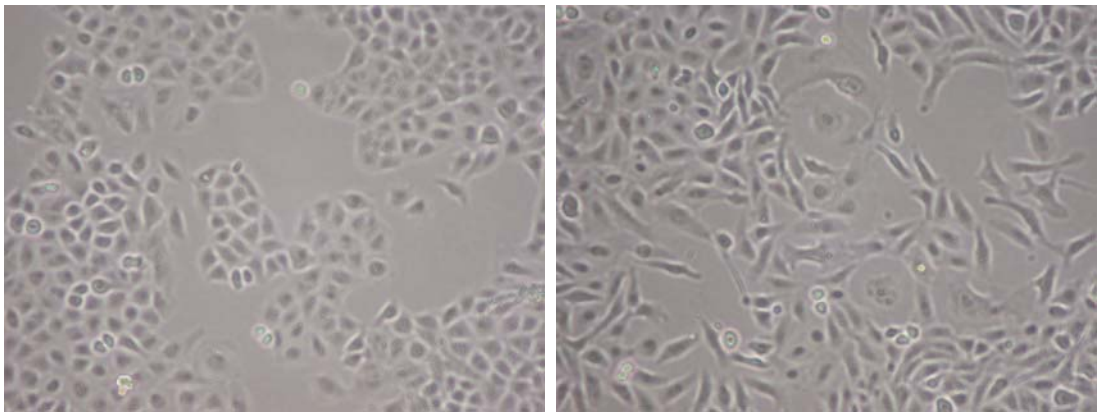
Measurement of cellular accumulation of calcein AM

Evaluation of P-gp activity was performed by the fluorescence measurement of intracellular accumulation of calcein produced by the ester hydrolysis of the P-gp substrate calceinAM. The transport capacity of P-gp is inversely proportional to the intracellular accumulation of fluorescent calcein. Briefly, cells were counted at $1x10^6$ cells and were incubated with 0.5 μM of calceinAM at 37°C in the absence and

presence of 10 μM P-gp inhibitor, verapamil for 1 hour. After incubation, cells were washed twice with cold phosphate buffer saline (PBS), resuspended in ice-cold Hank's buffered salt solution and then calcein accumulation was measured using FACSCaliber™ flow cytometer (Becton Dickinson) with excitation wavelength of 488 nm and emission of 530 nm).

Result

We established the etoposide resistant cell line (A549RT-eto) over a period of 18 months. There was no difference in doubling time between the A549 parental cells and the A549RT-eto resistant cells. However, differences in the morphology of the two cells were observed with A549 cells showing epitheloid-like shape whereas A549RT-eto cells spindle-like under a Nikon TMS inverted microscope (Figure 1).



A549 ***A549RT-eto***

Figure 1 Morphologies of parental (A549), and etoposide resistant (A549RT-eto) cell lines shown under 400X magnification by inverted microscope.

Cytotoxicity assay

Cytotoxic effects of etoposide on the two lung cancer cell lines were evaluated by measuring cell viability using MTT assay. Dose response curves were obtained after 72 hours of exposure of A549 and A549RT-eto to increasing concentrations of four chemotherapeutic drugs (Figure 2). The concentration of etoposide inhibiting cell growth by 50% (IC_{50}) of A549RT-eto was 103.3 $\pm$ 5.6 μg/ml or greater than 28-fold more resistant than A549 as shown in Table 2. Moreover, to investigate whether etoposide exposure causes cross-resistance to other MDR-related chemotherapeutic drugs, doxorubicin, cisplatin and taxol were also studied. The resistant cells were highly cross-resistant to doxorubicin with IC_{50} of 0.91 $\pm$ 0.09 μg/ml, but not to cisplatin and taxol.

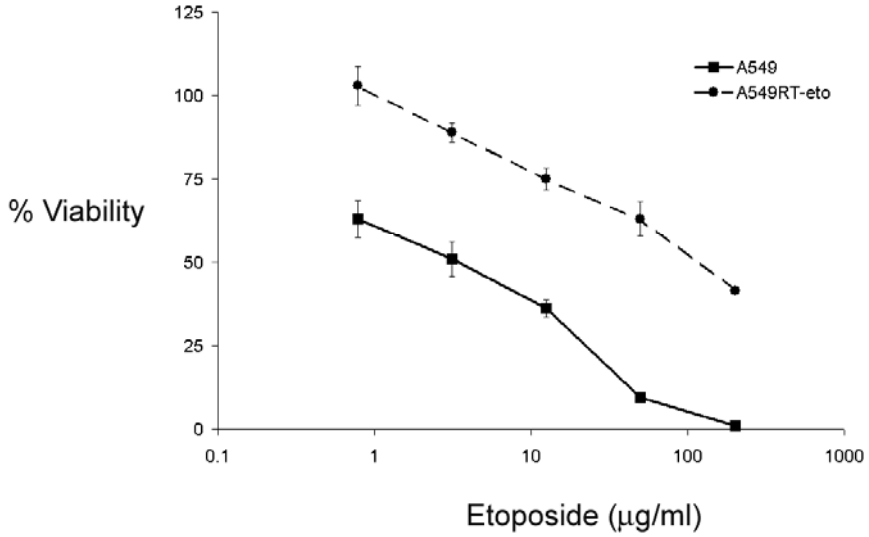


Figure 2 Cytotoxicity of A549 and A549RT-eto cells by MTT assay.

Table 2 **Cytotoxicity of anticancer drugs towards A549 and A549RT-eto cell lines**

Drugs	IC*$_{50}$		Resistance Index*
	A549	A549RT-eto	
Etoposide (μg/ml)	3.73 ± 0.86	103.33 ± 5.59	28
Doxorubicin (μg/ml)	0.19 ± 0.06	0.91 ± 0.09	5
Taxol (ng/ml)	3.02± 1.08	3.43 ± 1.10	1.1
Cisplatin (μg/ml)	2.43 ± 1.39	3.65 ± 1.41	1.5

* Data represents mean ± SD of at least five experiments

** Resistance index (RI) equals the ratio of IC_{50} values of resistant to sensitive cells

RI = (IC_{50} of A549RT-eto/ IC_{50} of A549)

Analysis of gene drug resistance gene

In order to analyze alterations in mRNA expression profiles associated with drug resistance in A549RT-eto cells, real-time PCR was used to compare the expression of drug resistant related genes (*mdr1, mrp1, mrp2, mrp3, bcrp, lrp, gst, topoisomerase IIa* and *topoisomerase IIb)* in the resistant cells with the parental A549 cells. Figure 3 showed that both cell lines expressed *mrp1, mrp2, mrp3, bcrp, lrp, gst, topoisomeraseIIa* and *topoisomeraseIIb* at comparable levels. In contrast, the expression level of *mdr1* in A549RT-eto was dramatically increased by 16-fold compared to the parental cell line. The ratios of gene expression in the resistant cell line as compared with parental cell were shown in Table 3. This result suggests that the major mechanism of acquired etoposide resistance in the cell line relates to the increase in the expression of *mdr1* gene.

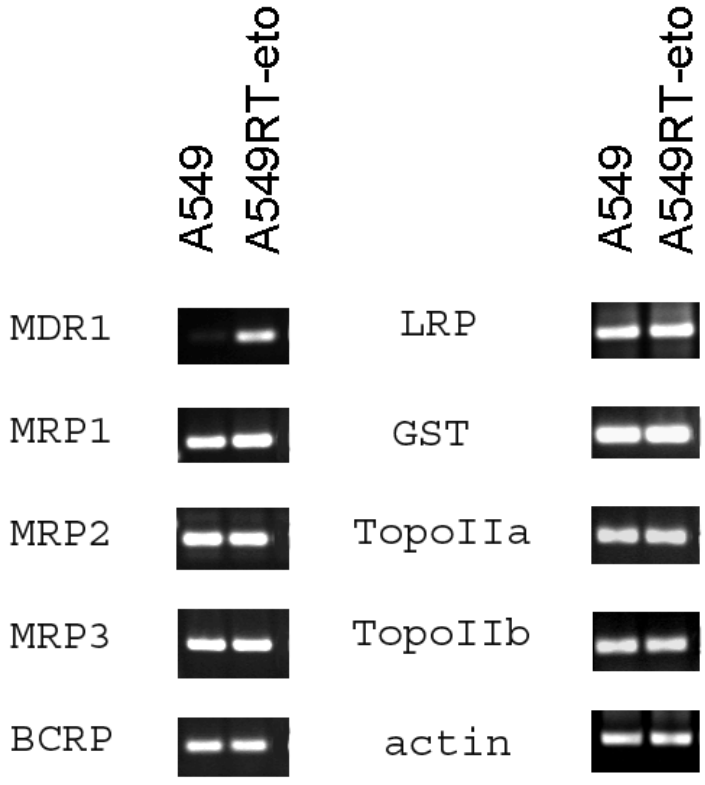


Figure 3 Expression of drug resistance related genes, mdr1, mrp1, mrp2, mrp3, bcrp, lrp, gst-pi, topoisomeraseII a and topoisomeraseIIb in A549 compared with resistant, A549RT-eto cells using real-time PCR.

Table 3 Ratios of target gene expression of the etoposide resistant compared with parental A549 cell lines under specific conditions

Gene	Ratio target gene expression
mdr1	16.00
mrp1	1.82
mrp2	0.52
mrp3	0.65
gst-π	1.13
bcrp	0.50
lrp	1.51
topoIIa	0.26
topoIIb	0.31

P-gp expression

The P-glycoprotein expression of the resistant cell line was compared with that of the parental cell line by western blot analysis using C219 antibody. No detectable levels of P-glycoprotein (170 kDa) were observed in A549 cells whereas this protein was highly expressed in A549RT-eto cells as shown in Figure 4. This result strongly suggests that the mechanism of drug resistance in A549RT-eto involves an increase in expression of P-glycoprotein.

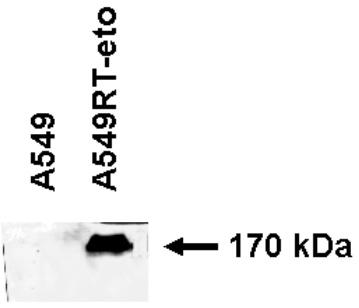


Figure 4 Western blot analysis of P-glycoprotein on membrane preparation

Drug accumulation

The nonfluorescent calceinAM passes through the cell membrane of viable cells. After penetrating into the cytoplasm, it is hydrolyzed by esterase to a fluorescent dye, calcein. CalceinAM is a substrate for P-glycoprotein and its transport out of cells has been demonstrated to reflect P-glycoprotein function**.** Calcein is not a P-gp substrate and cannot leave the cell *via* the plasma membrane, whereas calcein-AM is extruded from cells expressing P-glycoprotein. P-glycoprotein activity can therefore be assessed by measuring calcein accumulation in the cells. Accumulation of calcein in the parental and resistant cells was analyzed by FACS flow cytometry. Figure 5A showed a decrease in the accumulation of calcein in resistant cells. Moreover, the addition of 10 μM of P-glycoprotein inhibitor, verapamil, resulted in a marked increase in the accumulation of calcein in A549RT-eto (Figure 5B) suggesting that the acquired resistance in A549RT-eto is caused by the decrease in the intracellular drug accumulation.

(A) (B)

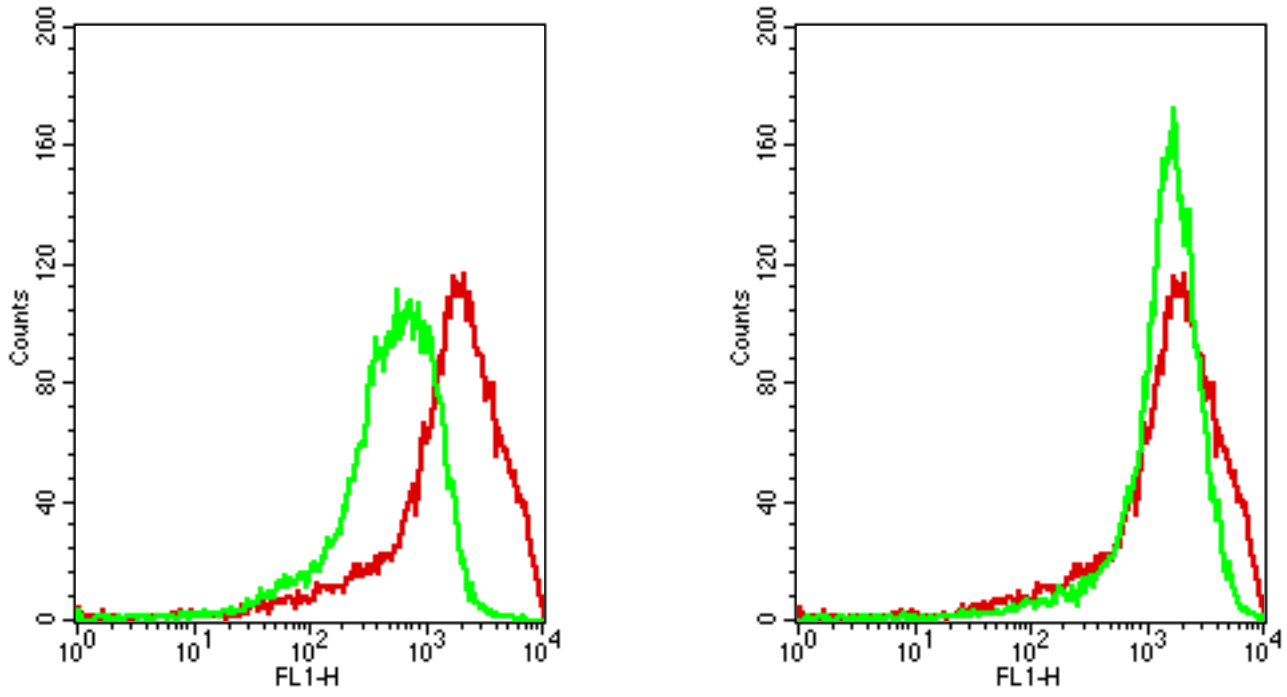


Figure 5 Accumulation of calcein-AM analyzed by flow cytometry. A) Accumulation of calcein-AM in A549 (red line) compared with resistant A549RT-eto (green line) cells. B) Accumulation of calcein-AM in A549RT-eto in the presence of P-gp inhibitor, verapamil.

Discussion

Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. Non-small cell lung cancer has been reported to be generally unresponsive to chemotherapy, even without previous drug treatment, which limits the chance of successful chemotherapy. Based upon gene expression analyses shown here, we have demonstrated that the parental lung carcinoma A549 cell line exhibits some intrinsic drug resistance properties as evidenced by the expression of drug resistant-related genes such as mrp1, mrp2, mrp3, bcrp, gst and lrp. In accordance with our data, MRP1 expression has been shown to be significantly increased in clinical samples and this was suggested to play a major role in the intrinsic resistance of non-small cell lung cancer (NSCLC) tissue.

In the present study, we have developed acquired resistance in the A549 cell line towards etoposide. Resistant cells (A549RT-eto) were developed from the A549 cell line by increasing the concentrations of etoposide to 1.5 μM. This resistant cell line, A549RT-eto, changed its morphology to a spindle-like shape from an epitheloid shape. However, the relationship between the morphology changes and alterations in gene expression in the A549RT-eto resistant cell line is still unclear. Morphology changes have been observed in resistant cell lines in several studies. Cisplatin-resistant neuroblastoma cell lines demonstrated alterations in their morphology without changes in cell growth. In an adriamycin-resistant leukemia cell line, morphological changes have been reported in association with the tumor cell environment rather than the abundance of P-glycoprotein in the plasma membrane. Alterations of actin cytoskeleton or mutations of the c-kit gene also resulted in morphological changes in cells. Thus, further studies will be required on the effect of the changes in the morphology in A549RT-eto cell line.

For MTT assays, A549RT-eto cells exhibited 28-fold greater resistance to etoposide and also showed cross-resistance to doxorubicin. The mechanism of acquired resistance appears to be associated with the over-expression of P-glycoprotein, as shown the western blot of Figure 4. The 170-kDa P-gp, encoded by the mdr1 gene, utilizes energy released from ATP hydrolysis to extrude a range of drugs such as vinblastine, vincristine, daunorubicin, doxorubicin, colchicine, paclitaxel and etoposide. An increase in P-gp activity can lower intracellular concentrations of drug, resulting in drug resistance. To test the P-glycoprotein activity, calceinAM was used. CalceinAM is

non-fluorescent that passes through the cell membrane of viable cells. After penetrating into the cytoplasm, it is hydrolysed by esterase to a fluorescent dye, calcein. CalceinAM is substrate for P-glycoprotein and its transport out cell has been demonstrated to reflect P-glycoprotein function. Calcein is not a P-glycoprotein substrate and can not leave the cell via plasma membrane. In our studies, correlations were observed between the results obtained by real time PCR, western blot analysis and flow cytometry. These data support previous studies indicating that the molecular mechanism of etoposide resistance is involved in the overexpression of P-glycoprotein.

It is also possible that other factors apart from P-glycoprotein may be involved, such as overexpression of MRP1, overexpression of lung resistance related protein (LRP) that alters nuclear/cytoplasmic drug distribution or alterations in topoisomerase II activity. Furthermore, up-regulation of bcl2 and other apoptosis pathway regulatory genes has been associated with etoposide resistance in lung cancer cell. Resistance mechanisms may vary depending on cell types, drugs and the manner in which the cells were treated. For example, decreased topoisomeraseII mRNA was observed in another A549 cell line showing acquired etoposide resistance, while other studies demonstrated the overexpression of P-glycoprotein or MRP1, also in the A549 cell line.

The mechanism involved in doxorubicin resistance has been suggested to be mediated by the multidrug resistance related protein (MRP1) or may be related to alternative mechanisms capable of altering drug efflux, such as P-glycoprotein. Our data showed that A549RT-eto is cross-resistant to doxorubicin, suggesting that doxorubicin can be pumped out from the cell via P-glycoprotein.

In conclusion, this work indicates that the mechanism of etoposide resistance in A549-eto cell line involves pumping out etoposide from the cell by P-glycoprotein. However, drug resistance in cancer cells may not be caused by a single factor, but rather by the coordinated effect of multiple factors involved in the drug response. Therefore, we are also investigating the roles of other factors in inducing MDR with acquired resistance to etoposide. This cell line also has potential for use as a model for screening P-glycoprotein inhibitors, as well as for characterizing MDR1 substrates and modulators in order to improve the efficacy of chemotherapy for lung cancer patients.

ผลงานวิจัยที่คาดว่าจะตีพิมพ์

บทความ

Yodsoi Kanintronkul, Kovit Pattanapanyasat, Rudee Surarit and Jisnuson Svasti Molecular mechanism of etoposide resistance in A549 lung cancer cells with acquired drug resistance ส่งไปตีพิมพ์วารสารนานาชาติ Biochemical and Biophysical Research Communications (ดังเอกสารแนบ)

กิจกรรมอื่น ๆที่เกี่ยวข้อง

Poster Presentation at Second Annual Symposium of Protein Society of Thailand, Sep 20-21, 2007, Bangkok, Thailand

Poster presentation at The Sixth Princess Chulabhorn International Science Congress, Nov 25-29 2007, Bangkok, Thailand

Poster presentation at National Cancer Conference, Dec 12-14 2007 Bangkok, Thailand

Poster Presentation at Third Annual Symposium of Protein Society of Thailand, Aug 28-29, 2008, Bangkok, Thailand

ภาคผนวก

Molecular mechanism of etoposide resistance in A549 lung cancer cells with acquired drug resistance

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This manuscript contains

25 pages including 3 Tables and 4 Figures

Abstract

The development of drug resistance is a major obstacle to effective cancer chemotherapy. In order to understand the mechanism of drug resistance, an etoposide-resistant lung cancer cell line A549RT-eto was established by continuous exposure of the A549 cell line to increasing concentrations of etoposide. This A549RT-eto cell line was approximately 28-fold more resistant to etoposide compared to parental A549 cell line. Moreover, A549RT-eto resistant cells showed cross-resistance to doxorubicin but not to taxol and cisplatin. To investigate the mechanism of drug resistance, the expression levels of drug resistance-related genes were determined using real-time PCR. Of all the genes investigated, only *mdr1* transcript levels were drastically increased in A549RT-eto cells compared to the parental cells. Since the *mdr1* gene encodes for P-glycoprotein (P-gp), a membrane protein involved in drug efflux pumps, the expression of P-gp and its transport activity were studied. Western blot analysis showed increased P-gp expression in the resistant cells compared to A549 cells. As determined by flow cytometry, the levels of calcein transported in the drug resistant cells were also enhanced, correlating with the increase in P-gp expression found in the Western blot. These studies therefore strongly suggest that expression of *mdr1* induced by etoposide plays a major role in the development of drug resistance in the A549RT-eto cell line.

Key words: Multidrug resistance, P-glycoprotein, Flow cytometry, Lung cancer cell

Lung cancer is currently one of the leading causes of cancer mortality for both males and females worldwide [1]. Non-small cell lung cancers (NSCLC) make up approximately 80% of all lung cancers and are generally treated with surgery before chemotherapy and radiotherapy. Small cell lung cancers (SCLC), on the other hand, are not surgically treated but are treated with chemotherapy and radiotherapy as a precautionary measure. The first line of chemotherapy regimens for lung cancer includes treatment with cisplatin, doxorubicin, vinblastin or etoposide [2]. However, chemotherapy for NSCLC is not as effective as for SCLC due to the development of multidrug resistance (MDR) in NSCLC cells. MDR in non-small cell lung cancer can be present at the time of diagnosis (intrinsic resistance) or may be acquired after cancer cells are exposed to chemotherapeutic drugs (acquired resistance) [3-4]. To overcome the problems related to drug resistance and to improve the clinical outcomes of patients with lung cancer, the molecular mechanisms of cancer chemoresistance must be more clearly elucidated.

Lung cancer patients are more commonly treated with etoposide [4], but drug resistance may decrease the efficacy of this drug. Various mechanisms have been proposed for etoposide resistance in cancer cells e.g., overexpression of efflux pumps in the cell membrane such as P-glycoprotein (P-gp) [5-7] and/or multidrug resistance associated protein (MRP) [8-9], intracellular redistribution of drug away from the target by lung related protein (LRP) [10-11], increased detoxification of compounds mediated by high levels of glutathione S-transferase (GST) [12-13] or reduction in the expression of topoisomerase IIa [14-15]. Alternative mechanisms for etoposide resistance may result from alterations in genes and proteins involved in apoptotic pathways [16-18].

Since the mechanism of etoposide resistance remains unclear, this study aims to investigate the molecular mechanism associated with the acquired resistance of a non-small cell lung carcinoma cell line, A549, to etoposide. Our findings demonstrate that development of drug resistance is associated with a decrease in the intracellular accumulation of etoposide mediated by the increased expression of *mdr1* gene and overexpression of P-gp.

Material and Methods

Materials

Etoposide, doxorubicin, cisplatin, 3-(4, 5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromine (MTT), calcein-acetoxymethylester (calcein-AM), verapamil and ProteoPrep Membrane Extraction kit were purchased from Sigma.. RNeasy kit, DNaseI and QuantiTect SYBR Green PCR master mix were purchased from Qiagen. Bradford reagent and taxol were purchased from Biorad and Bristol Myers Squibb, respectively. SuperscriptIII was obtained from Invitrogen. All other chemicals were analytical grade. Non-small cell lung adenocarcinoma cell line, A549 was obtained from American Type Culture Collection (ATCC). C219 antibody and rabbit anti-mouse immunoglobulin G (IgG) were obtained from Alexis and Dako Cytomation, respectively. Primer was ordered from Sigma-Proligo.

Development of drug resistant lung cancer cells

To develop etoposide resistant cells (A549RT-eto), the parental A549 cells were continuously exposed to increasing concentrations of etoposide up to 1.5 μM for 18 months. Cells were cultured in RPMI-1640 medium (Gibco) supplemented with 2.2 g/l $NaHCO_3$, 25 mM HEPES, 10% fetal bovine serum (FBS, Gibco) and 0.5% penicillin-streptomycin at 37°C in the presence of 5% CO_2. Resistant cells were maintained in the 1.5 μM of etoposide. Morphology of both etoposide resistant and the parental lung cancer cell lines was studied using an inverted microscope.

Cytotoxicity assay

The sensitivities of resistant and parental cells to various chemotherapeutic drugs were determined by MTT assay. Cells were cultured in drug-free medium for at least 1 week before the experiments were performed. Briefly, cells suspended in RPMI-1640 containing 10% FBS were seeded at 5 x 10^3 cells per well in a 96-well

plate, and incubated in 5% CO_2 at 37°C. After 24 hours, 100 μl media containing various concentrations of etoposide, doxorubicin, cisplatin and taxol were added and plates were incubated for 72 hours. Thereafter, the media were removed by aspiration, and 100 μl of fresh media with MTT was added to each well. The plates were incubated at 37°C for 2 hours. The dark blue formazan crystals formed by live cells were dissolved in 100 μl of dimethyl sulfoxide (DMSO) solution. Absorbances of individual samples were determined at 550 nm using spectrophotometer. The concentration required to inhibit growth by 50% (IC_{50} values) were calculated. Data were obtained from at least five independent experiments. Resistance index (RI) equals the ratio of IC_{50} values of resistant to sensitive cells.

Elucidation into the mechanism of multidrug resistance using real time PCR

The mRNA gene expression of *mdr1, mrp1, mrp2, mrp3, bcrp, lrp, gst-π, topoisomeraseIIa and topoisomeraseIIb* in the resistant cells, A549RT-eto, were compared with those of parental A549 cell line using real-time PCR.

Total RNA was isolated from cells using RNeasy kit. For cDNA synthesis, 1 μl of 10 pmol oligo dT primer was added to 2 μg RNA in 20 μl reaction and incubated for 10 minutes at 70°C. Then 200 units of SuperScript III reverse transcriptase was added to the mixture, followed by incubation first for 50 minutes at 42°C and then for an additional 15 minutes at 70°C. QuantiTect SYBR Green PCR master mix was used with 2 μl of cDNA and 10 pmol of specific primers. PCR was performed on a LightCycler (Roche Applied Science) in capillary glass tubes. Primer sequences were designed based on the reported sequences (Table 1) [19-22] to amplify portions of exons in the relevant genes related to drug resistance, using the β-actin gene as an internal control. An initial activation step at 95°C for 15 minutes was followed by 35

cycles comprising of denaturation at 94°C for 15 seconds, annealing at the respective T_a 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. The ratios of gene expression values were normalized using β-actin gene and calculated by the following formula:

$$\text{Ratio target gene expression} = \frac{\text{Fold change in target gene expression (A549RT-eto/A549)}}{\text{Fold change in reference gene expression (A549RT-eto/A549)}}$$

Western Blot Analysis

Membrane proteins were extracted using ProteoPrep Membrane Extraction Kit. Protein concentrations were determined by Bradford assay. Solubilized membrane proteins were separated by 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. After blocking with 10% non-fat dried milk in TBS/T buffer (20 mM Tris buffered saline pH 7.6 containing 0.1% Tween-20) for 1 hour at room temperature, the membrane was probed with 1:100 diluted specific monoclonal antibody C219 and incubated at 4°C overnight. After three washing steps in TBS/T buffer, the membrane was incubated in 1:5000 diluted rabbit anti-mouse immunoglobulin G for 1 hour. After washing, the reaction was developed using ECL (GE Healthcare).

Measurement of cellular accumulation of calcein-AM

Evaluation of P-gp activity was performed by the fluorescence measurement of intracellular accumulation of calcein produced by the 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 [23-24]. Briefly, cells were counted at $1x10^6$ cells and were incubated with 0.5 μM of calcein-AM at 37°C in the absence and presence of 10 μM P-gp inhibitor, verapamil for 1 hour. After incubation, cells

were washed twice with cold phosphate buffered saline (PBS), resuspended in ice-cold Hank's buffered salt solution and then calcein accumulation was measured using FACSCalibur™ flow cytometer (Becton Dickinson Biosciences) with excitation wavelength of 488 nm and emission of 530 nm.

Results

An etoposide resistant cell line (A549RT-eto) was established over a period of 18 months. There was no difference in doubling time between the A549 parental cells and the A549RT-eto resistant cells. However, the two cell lines showed differences in morphology, with A549 cells showing an epitheloid-like shape whereas A549RT-eto cells showed a spindle-like shape under an inverted microscope (Figure 1).

Cytotoxicity assay

Cytotoxic effects of etoposide on the two lung cancer cell lines were evaluated by measuring cell viability using the MTT assay. Dose response curves were obtained after 72 hours of exposure of both cell lines to increasing concentrations of etoposide. The concentration of etoposide inhibiting cell growth by 50% (IC_{50}) of A549RT-eto was 103.3 ± 5.6 μg/ml or 28-fold more resistant than A549, as shown in Table 2. Moreover, doxorubicin, cisplatin and taxol were also studied to investigate whether etoposide exposure causes cross-resistance to other MDR-related chemotherapeutic drugs. The resistant cells were cross-resistant to doxorubicin with 5-fold increase in IC_{50} to 0.91 ± 0.09 μg/ml, but did not show resistance to cisplatin and taxol (Table 2).

Analysis of gene drug resistance gene

In order to analyze changes in mRNA expression profiles associated with drug resistance in A549RT-eto cells, real-time PCR was used to compare the expression of drug resistant related genes in the resistant cells with the parental A549 cells. Figure 2 showed that both cell lines expressed *mrp1, mrp2, mrp3, bcrp, lrp, gst,*

topoisomeraseIIa and *topoisomeraseIIb* at comparable levels. In contrast, the expression level of *mdr1* in A549RT-eto was dramatically increased by 16-fold compared to the parental cell line. The ratios of gene expression in the resistant cell line are compared with those of the parental cell in Table 3. This result suggests that the major mechanism of acquired etoposide resistance in the cell line relates to the increased expression of the *mdr1* gene.

P-gp expression

The P-gp expression of the resistant cell line was compared with that of the parental cell line by Western blot analysis using C219 antibody. No P-gp (170 kDa) could be observed in A549 cells, whereas P-gp was highly expressed in A549RT-eto cells as shown in Figure 3. This result strongly suggests that the mechanism of drug resistance in A549RT-eto involves increased expression of P-gp.

Drug accumulation

Flow cytometric assessment of calcein accumulation which reflects P–gp function in both A549 and A549RT-eto cell lines showed a decrease in the accumulation of calcein in resistant cells (Figure 4A). Moreover, the addition of 10 μM of P-gp inhibitor, verapamil, resulted in a marked increase in the accumulation of calcein in A549RT-eto (Figure 4B) suggesting that the acquired resistance in A549RT-eto is caused by a decrease in the intracellular drug accumulation.

Discussion

Acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. NSCLC has been reported to be generally unresponsive to chemotherapy, even without previous drug treatment, which limits the chance of successful chemotherapy [1, 25]. Based upon gene expression analyses shown here, we have demonstrated that the parental lung carcinoma A549 cell line exhibits some

intrinsic drug resistance properties as evidenced by the expression of drug resistant-related genes such as *mrp1, mrp2, mrp3, bcrp, gst* and *lrp*. In accordance with our data, MRP1 expression has been shown to be significantly increased in clinical samples and this was suggested to play a major role in the intrinsic resistance of NSCLC tissue [26-28].

In the present study, we have developed acquired resistance in the A549 cells towards etoposide. Resistant cells (A549RT-eto) were developed from the A549 cell by increasing the concentrations of etoposide to 1.5 μM. A549RT-eto changed its morphology to a spindle-like shape from an epitheloid shape. However, the relationship between the morphology changes and alterations in gene expression in the A549RT-eto resistant cell is still unclear. Morphology changes have been observed in resistant cells in several studies [29-32]. Cisplatin-resistant neuroblastoma cell lines demonstrated alterations in their morphology without changes in cell growth [29]. In an adriamycin-resistant leukemia cell line, morphological changes have been reported in association with the tumor cell environment rather than the abundance of P-glycoprotein in the plasma membrane [30]. Alterations of actin cytoskeleton [31] or mutations of the c-kit gene also resulted in morphological changes in cells [32]. Thus, further studies will be required on the effect of the changes in the morphology in A549RT-eto cell line.

For MTT assays, A549RT-eto cells exhibited 28-fold greater resistance to etoposide and also showed cross-resistance to doxorubicin. The mechanism of acquired resistance appears to be associated with the over-expression of P-glycoprotein, as shown the western blot. The 170-kDa P-gp, encoded by the *mdr1* gene, utilizes energy released from ATP hydrolysis to extrude a range of drugs such as vincristine, doxorubicin, colchicine, and etoposide [5]. An increase in P-gp activity

can lower intracellular concentrations of drug, resulting in drug resistance. To test the P-gp activity, calceinAM was used. CalceinAM is non-fluorescent that passes through the cell membrane of viable cells. After penetrating into the cytoplasm, it is hydrolysed by esterase to a fluorescent dye, calcein. CalceinAM is substrate for P-glycoprotein and its transport out cell has been demonstrated to reflect P-glycoprotein function. Calcein is not a P-glycoprotein substrate and can not leave the cell via plasma membrane [24]. In our studies, correlations were observed between the results obtained by real-time PCR, Western blot analysis and flow cytometry. These data support previous studies indicating that the molecular mechanism of etoposide resistance is involved in the overexpression of P-glycoprotein [7-8]. However, resistance mechanisms may vary depending on cell types, drugs and the manner in which the cells were treated [33]. For example, decreased topoisomeraseII mRNA was observed in another A549 cell line showing acquired etoposide resistance [34-35], while other studies demonstrated the overexpression of P-glycoprotein [8] or MRP1 [11], also in the A549 cell line.

The mechanism involved in doxorubicin resistance has been suggested to be mediated by the multidrug resistance related protein (MRP1) [36] or may be related to alternative mechanisms capable of altering drug efflux, such as P-glycoprotein [5]. Our data showed that A549RT-eto is cross-resistant to doxorubicin, suggesting that doxorubicin can be pumped out from the cell *via* P-glycoprotein.

In conclusion, this work indicates that the mechanism of etoposide resistance in A549-eto cell line involves pumping out etoposide from the cell by P-glycoprotein. However, drug resistance in cancer cells may not be caused by a single factor, but rather by the coordinated effect of multiple factors involved in the drug response. Therefore, we are also investigating the roles of other factors in inducing MDR with

acquired resistance to etoposide. This cell line also has potential for use as a model for screening P-glycoprotein inhibitors, as well as for characterizing MDR1 substrates and modulators in order to improve the efficacy of chemotherapy for lung cancer patients.

Acknowledgements

This work was financially supported by the Thailand Research fund (Grant no. MRG5080092) and the Chulabhorn Research Institute. We are indebted to Dr. Pakorn Winayanuwattikun and Mr. Kriengsak Lirdprapamongkol for critical comments for the study and Dr. Nilubol Paricharttanakul for critical reading of the manuscript. We are also grateful to Mrs. Surada Lerdwana for technical assistance. K.P. is presently a Senior Research Scholar of the Thailand Research Fund.

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Figure Legend

Figure 1 Morphologies of parental (A549), and etoposide resistant (A549RT-eto) cell lines shown under 400X magnification by inverted microscope.

Figure 2 Expression of drug resistance related genes, mdr1, mrp1, mrp2, mrp3, bcrp, lrp, gst, topoisomeraseII a and topoisomeraseIIb in A549 compared with resistant, A549RT-eto cells using real-time PCR.

Figure 3 Western blot analysis of P-glycoprotein on membrane preparation from parental A549 and resistant A549RT-eto cell lines detected by using monoclonal antibody C219. The arrow indicates the position of P-glycoprotein (170 kDa).

Figure 4 Accumulation of calcein-AM analyzed by flow cytometry. A) Accumulation of calcein-AM in A549 (red line) compared with resistant A549RT-eto (green line) cells. B) Accumulation of calcein-AM in A549RT-eto in the presence of P-gp inhibitor, verapamil.

Table 1 Primers used in real-time PCR

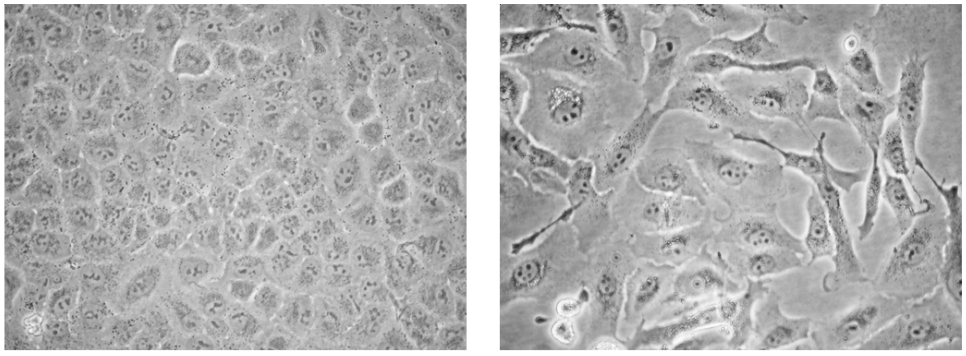
Gene	**Tm**	**Size of PCR (bp)**	**Forward primer**	**Reverse primer**
mdr1	62	206	GTCTTTGGTGCCATGGCCGT	ATGTCCGGTCGGGTGGGATA
mrp1	62	262	CTGACAAGCTAGACCATGAATGT	CCTTTGTCCAAGACGATCACCC
mrp2	62	202	GCCAGATTGGCCCAGCAAA	AATCTGACCACCGGCAGCCT
mrp3	56	452	GGGACCCTGCGCATGAACCTG	TAGGCAAGTCCAGCATCTCTGG
bcrp	62	205	TGGCTGTCATGGCTTCAGTA	GCCACGTGATTCTTCCACAA
lrp	64	342	GAGCAGTTCACAGTGTTGTCC	AAAGCCAAAGACAGCAGTGCG
grt-π	62	199	TACGGGCAGCTCCCCAAGTT	TGCCCGCCTCATAGTTGGTG
topoIIa	57	310	GGCTCGATTGTTATTTCCAC	ATGGTTGTAGAATTAAGAATAGC
topoIIb	57	310	GCTGTGGATGACAACCTC	CTGTGTTTCTGTCCACTAC
actin	62	574	GACCTGACTGACTACCTCATGA	AGCATTTGCGGTGGACGATGGAG

Table 2 **Cytotoxicity of anticancer drugs towards A549 and A549RT-eto cell lines**

Drugs	IC_{50}		Resistance Index
	A549	A549RT-eto	
Etoposide (μg/ml)	3.73 ± 0.86	103.33 ± 5.59	28
Doxorubicin (μg/ml)	0.19 ± 0.06	0.91 ± 0.09	5
Taxol (ng/ml)	3.02± 1.08	3.43 ± 1.10	1.1
Cisplatin (μg/ml)	2.43 ± 1.39	3.65 ± 1.41	1.5

Table 3 Ratios of target gene expression of the etoposide resistant compared with parental A549 cell lines under specific conditions

Gene	Ratio target gene expression
mdr1	16.00
mrp1	1.82
mrp2	0.52
mrp3	0.65
gst-π	1.13
bcrp	0.50
lrp	1.51
topoIIa	0.26
topoIIb	0.31



A549 A549RT-eto

Figure 1

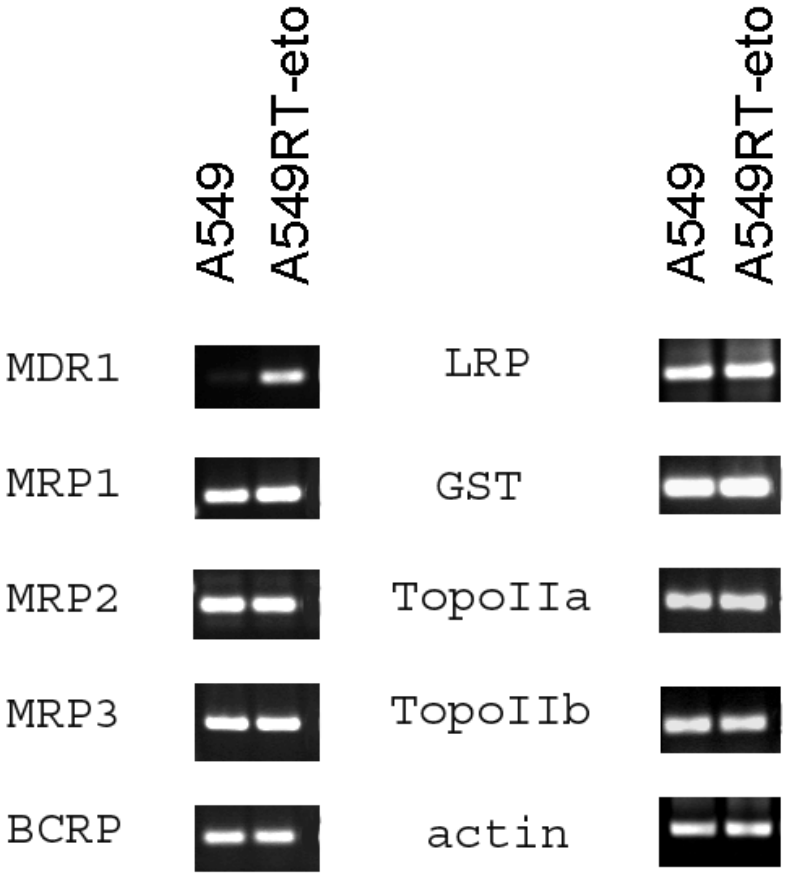

A549
A549RT-eto
A549
A549RT-eto
MDR1
MRP1
MRP2
MRP3
BCRP
LRP
GST
TopoIIa
TopoIIb
actin

Figure 2

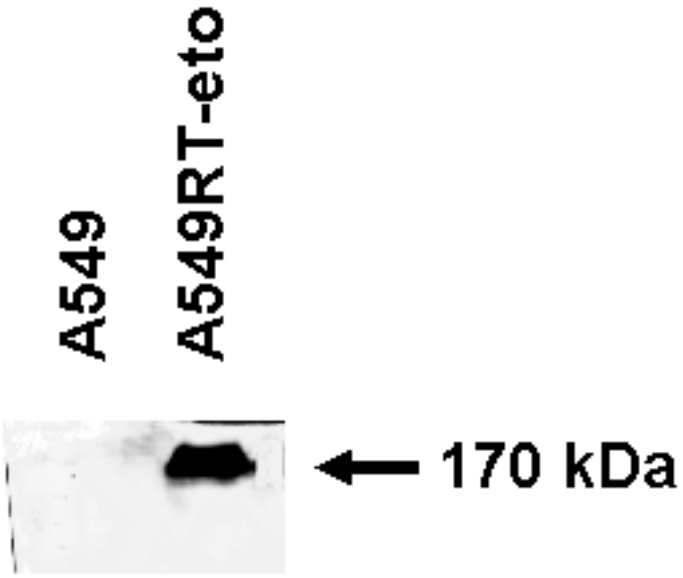
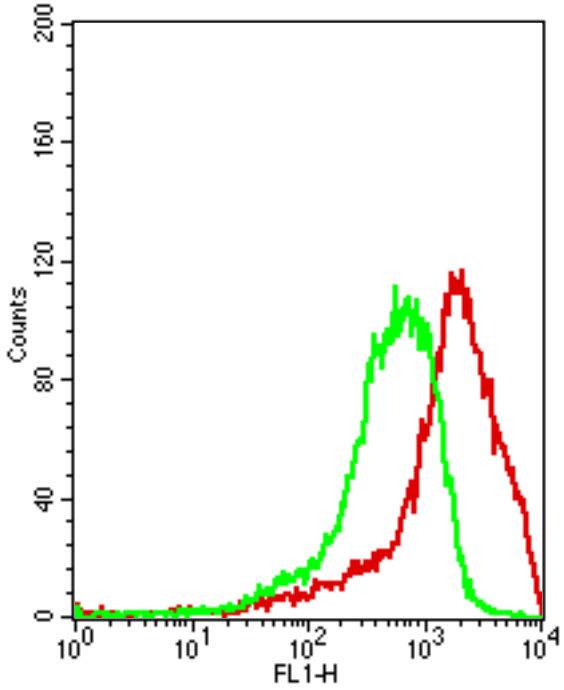


Figure 3

A)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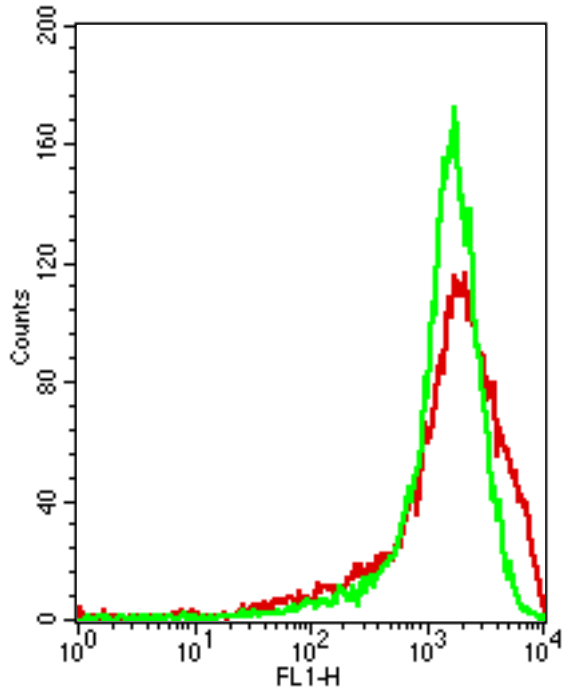
120
160
200
10^0
10^1
10^2
10^3
10^4
FL1-H

B)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10^1
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10^3
10^4
FL1-H

Figure 4