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Suppressive effect of tobacco smoke extracts on oral P-glycoprotein function and its impact in smoke-induced insult to oral epidermal cells

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ABSTRACT

P-glycoprotein (Pgp) participates in the export of numerous toxins, drugs, and physiological compounds. To examine the involvement of Pgp in smoke-induced oral cell insult, the effects of extracts of the mainstream tobacco smoke (TS) on Pgp were studied in an oral epidermal carcinoma cell line, OECM-1. TS was first extracted with cyclohexane (CTS) and the residues were further extracted with isopropanol (ITS). For comparison, cells were exposed to CTS and ITS at the concentrations according to their relative extraction yield. ITS but not CTS decreased the efflux of a Pgp substrate, rhodamine (Rh) 123, in a concentration- and time-dependent manner. The efflux was also decreased by co-exposure to CTS and ITS. However, immunoblot analysis revealed that the protein level of Pgp was not affected by ITS. Naphthalene, mainly detected in the ITS, decreased Rh 123 efflux. However, the efflux activity was not affected by benzo(a)pyrene and nicotine, which were present in the CTS and both extracts, respectively. Co-exposure to CTS in combination with ITS, naphthalene, or verapamil enhanced cell insult compared to single exposure. These results demonstrated that smoke and its constituent, naphthalene, diminished Pgp-mediated efflux. The reduction in Pgp function could be a stimulatory factor of TS-induced oral cell insult.

1. Introduction

The multidrug resistant (MDR) gene-encoded P-glycoprotein (Pgp) is responsible for the ATP-dependent export of numerous endogenous and exogenous compounds across the plasma membrane. Substrates of Pgp include physiological compounds, such as aldosterone, as well as environmental pollutants and toxins, such as benzo(a)pyrene and 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) (Walle and Walle, 1999; Yeh et al., 1992; Penny and Campell, 1994). The impairment in Pgp-mediated efflux can reduce the elimination of toxins and elevate their cytotoxicities. For example, the nephrotoxicity of cyclosporine A was enhanced by the Pgp inhibitor, sirolimus, and the neurotoxicity of loperamide was enhanced by the Pgp inhibitor, quinidine (Sadeque et al., 2000; Anglicheau et al., 2006).

Oral cancer has increased in both incidence and mortality over the past decade (Chung et al., 2005). Smoking represents the main stimulatory factor of the most common type of oral carcinoma, oral squamous cell carcinoma (OSCC) (Blot et al., 1988). Pgp has been identified in normal oral tissues, untreated primary oral tumors, and dysplastic lesions as analyzed by immunocytochemical staining (Jain et al., 1997; Muzio et al., 2000). Maternal exposure to tobacco smoke stimulates placental mdr1a messenger (m)RNA without affecting mdr1b mRNA in Wistar rats (Yan et al., 2006). However, a smoke extract decreased the function of a multidrug resistant-associated protein, MRP1, in bronchial epithelial cells (van der Deen et al., 2007). The constituent(s) responsible for this inhibition was not analyzed. Besides the respiratory tract, the oral cavity is also a tissue target directly exposed to tobacco and tobacco smoke. However, the effect of smoke on oral Pgp function has not been investigated. An oral epidermal carcinoma cell line, OECM-1 (or OEC-M1), derived from human gingiva has been used in the toxicological and cancer research (Lin et al., 2005; Yang and Meng, 1994; Yang et al., 2003). OECM-1 grew as adherent monolayer, had the morphology of epithelioid, and showed responsiveness to the modulators of cyclooxygenase and fibrotic toxins, such as areca nut (Yang and Meng, 1994; Yang et al., 2003).

Among the more than 4000 compounds present in tobacco smoke (TS), benzo(a)pyrene is one of the major constituents responsible for TS-induced mutagenicity and alterations in

Abbreviations: TS, tobacco smoke; Pgp, P-glycoprotein; OEC-M1, oral epidermal carcinoma cell line, Meng-1; CTS, the cyclohexane extract of tobacco smoke; ITS, the isopropanol extract of tobacco smoke; Rh 123, rhodamine 123.

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drug-metabolizing enzymes, such as CYP1A (Smith and Hansch, 2000). Pgp was able to pump out benzo(a)pyrene in MCF-7 breast cancer cells and human intestinal epithelium (Penny and Campell, 1994; Yeh et al., 1992). In a rat liver epithelial cell line, SDVI and a human intestinal cell line, Caco-2, benzo(a)pyrene caused Pgp induction (Fardel et al., 1996; Sugihara et al., 2006). However, benzo(a)pyrene did not affect the protein level of Pgp in *Xenopus laevis* (Colombo et al., 2003). There were heterogeneity in response to the inductive effect of benzo(a)pyrene on Pgp in different biological systems. Risner (1988) reported that cyclohexane is an efficient extraction solvent for determining the content of benzo(a)pyrene in TS. Therefore, in this report, TS was first extracted with cyclohexane to obtain the benzo(a)pyrene-enriched extract, and the residues were further extracted with isopropanol to prepare the isopropanol extract. To reveal the participation of Pgp in TS-induced oral cell insult, effects of cyclohexane and isopropanol extracts of TS on oral Pgp function and cell viability were investigated in this report. The contribution of benzo(a)pyrene and two other smoke constituents, nicotine and naphthalene, to TS-mediated Pgp functional changes were characterized.

2. Materials and methods

2.1. Chemicals and antibodies

Benzo(a)pyrene, 3-methylcholanthrene, naphthalene, nicotine, and rhodamine (Rh) 123 were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), and a mixture of antibiotics (10,000 units/ml penicillin, 10 mg/ml streptomycin, and 0.025 mg/ml amphotericin) were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Molecular weight markers for the immunoblot analyses were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The Pgp-immunoreacted monoclonal antibody, C219, was purchased from Merck-Calbiochem (Merck, Darmstadt, Germany).

2.2. Preparation of tobacco extracts

Cigarettes (LongLife, classic premium deluxe, 83 mm in length) were purchased from Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan). The mainstream smoke condensate was prepared as the TS from 100 cigarettes using an RM200 smoking machine (Borgwaldt KC, Hamburg, Germany) at an air flow of 18.8 cm/s under 98.8 kPa of air pressure and 60% humidity at 22 °C. The smoking machine utilized eight puffs per cigarette. The CO and CO_2 produced during cigarette smoking were 12.9–13.6 and 43.0–44.5 mg/cigarette, respectively. The smoke was collected on a glass-fiber filter and the filter was immersed in 400 ml cyclohexane twice for 24 h at room temperature and then filtered through filter paper (Whatman, Florham Park, NJ, USA). After filtration, the filtrate was dried using a rotary evaporator and yielded 0.130 g cyclohexane extracts from TS (CTS). The residues were further extracted by 400 ml isopropanol twice for 24 h at room temperature. After filtration, the filtrate was dried, and yielded 0.874 g isopropanol extract from TS (ITS).

2.3. Quantification of benzo(a)pyrene, nicotine, and naphthalene

TS was dissolved in acetonitrile. The contents of benzo(a)pyrene were determined using a high performance liquid chromatographic (HPLC) method modified from the method of the National Institute of Safety and Health (NIOSH) method 5506 (NIOSH, in press). Separation was performed using a 50-min gradient of 70% acetonitrile to 100% acetonitrile and then 100% acetonitrile for more 30 min at a flow rate of 0.5 ml/min. Benzo(a)pyrene was detected using an excitation wavelength of 290 nm and an emission wavelength of 425 nm. The quantities of nicotine and naphthalene were measured using gas chromatography (GC)–mass spectrometry (MS) following a method modified from Kim et al. (2005). A Network gas chromatography system (6890N, Agilent Technology, Santa Clara, CA, USA) equipped with a DB-5ms column (30 m × 0.25 mm × 0.25 μm) and a Network Mass Selective detector (Agilent Technology) was used. Helium was used as the carrier gas for the GC. Following sample injection, the GC column was held at 135 °C for 0.5 min, the temperature was programmed to 250 °C at 10 °C/min, held at 250 °C for 1 min, then programmed to 300 °C at 15 °C/min, and finally held at 300 °C for 3.5 min. The total run time was 20 min with a flow rate of 1 ml/min. The ion source was kept at 230 °C and the quadrupole at 150 °C. Scan and selected ion modes were used for the quantitative analyses of nicotine (m/z 84) and naphthalene (m/z 128), respectively. Data represent the mean of duplicate determinations.

2.4. Culture and treatments of oral cells

An oral epidermal carcinoma cell line, OECM-1, was generously provided by Prof. Kuo-Wei Chang (National Yang-Ming University, Taipei, Taiwan) and cultured in RPMI-1640 medium supplemented with a 10% (v/v) FBS and 1% (v/v) antibiotic mixture in a humidified atmosphere at 37 °C with 5% CO_2. Cell viability was analyzed by a cellular 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium (MTT) reduction assay (Alley et al., 1988) and trypan blue (0.4%) exclusion assay. Cell survival was expressed as the percentage of treated cells compared to vehicle control cells. Extracts of TS were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the medium was <0.5%. The pH value of the medium did not change with the addition of TS extracts. Cells (8–20 passages) were treated with various concentrations of tobacco extracts for 18 h at 60–70% confluence. After treatment, cultured cells approached about 80% confluence, and the activities and protein levels of Pgp were analyzed. Cells were treated with 1 μM 3-methylcholanthrene for 18 h to induce CYP1A1/2 activity. A human proximal tubular cell line, HK-2, was purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). HK-2 cells were cultured following the method of Romiti et al. (2002). The crude membrane fractions of untreated HK-2 cells were used as a marker for the immunoblot analysis of Pgp.

2.5. Rh 123 accumulation assay

OECM-1 cells were seeded onto a 6-well plate (1×10^5 cells/well), and 70–80% confluent cells were incubated with 5 μM Rh 123 in complete medium at 37 °C in a CO_2 incubator for 1 h. After two washes with an ice-cold potassium phosphate-buffered saline (PBS) solution, cells were lysed with 0.1% Triton X-100 for 30 min. The PBS solution contained 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.75 mM KH_2PO_4 (pH 7.2). The amount of Rh 123 was determined by measuring the fluorescence with 485 nm for excitation and 538 nm for emission (F-4500, Hitachi, Tokyo, Japan). The cellular Rh 123 content was calculated using a linear regression and was normalized with the protein content in each sample. The protein concentration was determined by the dye-binding assay following the instruction manual of the Bio-Rad Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Rh 123 efflux assay

The Pgp-mediated efflux was monitored by verapamil-inhibited Rh 123 efflux (Piquette-Miller et al., 1998; Lee and Piquette-Miller, 2003). Cells were seeded on a 6-well plate (1×10^5 cells/well), and 70–80% confluent cells were incubated with 5 μM Rh 123 in complete medium at 37 °C in a CO_2 incubator for 1 h. After two washes with an ice-cold PBS solution in the dark, cells were incubated with Rh 123-free medium in the absence and presence of 100 μM verapamil at 37 °C in a CO_2 incubator for 3 h. After three washes with an ice-cold PBS solution in the dark, the fluorescence of Rh 123 retained in the cell was measured using a microplate reader (Flex Station, Molecular Devices, Sunnyvale, CA, USA). The reduced intracellular fluorescence due to the efflux of Rh 123 was calculated as the percent of fluorescence measured at 0 h. The Pgp-mediated efflux was calculated as the difference in Rh 123 efflux in the absence and presence of the Pgp inhibitor, verapamil.

2.7. Immunoblot analysis of Pgp

The crude membrane fraction was prepared following the method of König et al. (1999) using a hypotonic buffer. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the discontinuous system of Laemmli (1970). Membrane fractional proteins (100 μg) were electrophoresed on a 6% (w/v) polyacrylamide gel for the analysis of Pgp. Markers with high molecular weight ranges (45–200 kDa, Bio-Rad Laboratories) were used for the molecular weight determination. Electrophoresis was carried out at 8 °C and 15 mA/gel during stacking and 30 mA/gel during separation. Following electrophoresis, proteins were transferred from the slab gel to a nitrocellulose membrane using the method of Towbin et al. (1979). The expressed Pgp protein was immunoreacted with the C219 monoclonal antibody (Kartner et al., 1985). Immunoreactive protein was detected by rabbit immunoglobulin Gs (IgGs) against mouse IgGs conjugated with horseradish peroxidase and immunostained using a chemiluminescence detection kit purchased from PerkinElmer LAS (Boston, MA, USA). The protein band density was analyzed by densitometry using ImageMaster (Pharmacia Biotech, Uppsala, Sweden).

2.8. Statistical analysis

The statistical significance of differences between two groups was evaluated by Student's t-test. The differences between >2 sets of data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons. A p value <0.05 was considered statistically significant.

3. Results

3.1. Effects of the tobacco smoke extracts and naphthalene on oral cell viability

The extracts of CTS and ITS were prepared as described in Section 2. Cell viability was monitored by measuring cellular reduction

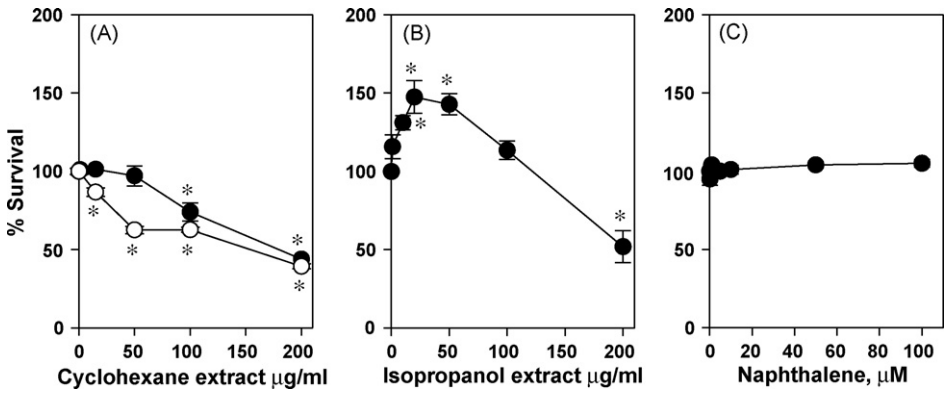

Fig. 1. (A) The viability of OECM-1 cells following the treatment of the cyclohexane extract of tobacco smoke in the absence (●) and presence (○) of 100 μM verapamil. Panels (B) and (C) shows the effects of isopropanol extract of tobacco smoke and naphthalene on the viability of OECM-1 cells, respectively. Cell viability was monitored by cellular MTT reduction activity. Data are presented as the mean ± S.E.M. of six determinations. An asterisk (*) indicates that the value significantly differed from the vehicle control value, $p < 0.05$. There was no significant difference in cell viability between vehicle control and verapamil-treated cells.

of MTT. Cell viability was not affected by the 18-h treatments with CTS at concentrations of up to 50 μg/ml. Exposure to CTS at 100 and 200 μg/ml decreased cell viability by 26% and 56%, respectively (Fig. 1A). The IC_{50} value for the decrease of cell viability in the treatment with CTS was 176 ± 17 μM. However, the IC_{50} value in the cotreatment with CTS and verapamil was 133 ± 31 μM. The presence of verapamil reduced the IC_{50} value for the decrease of cell viability. ITS at 1 μg/ml did not change the cellular MTT reduction. However, ITS caused a dose-dependent increase in viable cells at the concentrations of 10–50 μg/ml. ITS at 10, 20, and 50 μg/ml caused 31%, 47%, and 43% increases in MTT reduction, respectively (Fig. 1B). This increase in viable cells was diminished at higher ITS concentrations. At 100 μg/ml, ITS-treated cells showed a MTT reduction level similar to that of DMSO-treated cells. At 200 μg/ml, ITS decreased cell viability by 48%. The smoke constituent, naphthalene, at 0.2–100 μM did not affect cell viability (Fig. 1C).

3.2. Establishment of optimal assay condition for determining Pgp function in OECM-1 cells

To establish optimal assay condition for measuring Rh 123 accumulation in OECM-1 cells, the intracellular concentration of Rh 123 was determined after incubating cells with Rh 123 at initial concentrations of 1–10 μM in the culture media for 1 h. The accumulation of Rh 123 increased with an increasing Rh 123 concentration from 1 to 5 μM in the culture media. However, the cellular accumulation of Rh 123 at the initial 10 μM was not higher than that at 5 μM (Fig. 2A). Thus, 5 μM Rh 123 was used in the accumulation step of the assay. After Rh 123 accumulation, cells were re-incubated in a medium without Rh 123, and efflux began. After efflux for 1 and 3 h, 77% and 65% Rh 123 were retained in cells, respectively (Fig. 2B). A Pgp inhibitor, verapamil, at 100 and 150 μM decreased the Rh 123 efflux to 43% and 37% of the control, respectively (Fig. 2C). Thus, Pgp-mediated Rh 123 efflux was determined using a 3-h efflux period in the absence and presence of 100 μM verapamil.

3.3. Dose–response and time-course effects of tobacco extracts on Rh 123 efflux activity

According to the extraction yield, 1–20 μg/ml CTS and 50–200 μg/ml ITS were studied. The exposure of cells to CTS for 18 h had no effect on Pgp-mediated Rh 123 efflux activity (Fig. 3A). The Rh 123 efflux was not affected by 50 μg/ml ITS. However, 100 and 200 μg/ml ITS significantly decreased the efflux by 55% and 53%, respectively. Thus, cells were treated with 100 μg/ml ITS in the time-course study. Treatment of cells with 100 μg/ml ITS for 3 and 6 h did not affect the Rh 123 efflux activity (Fig. 3B). However,

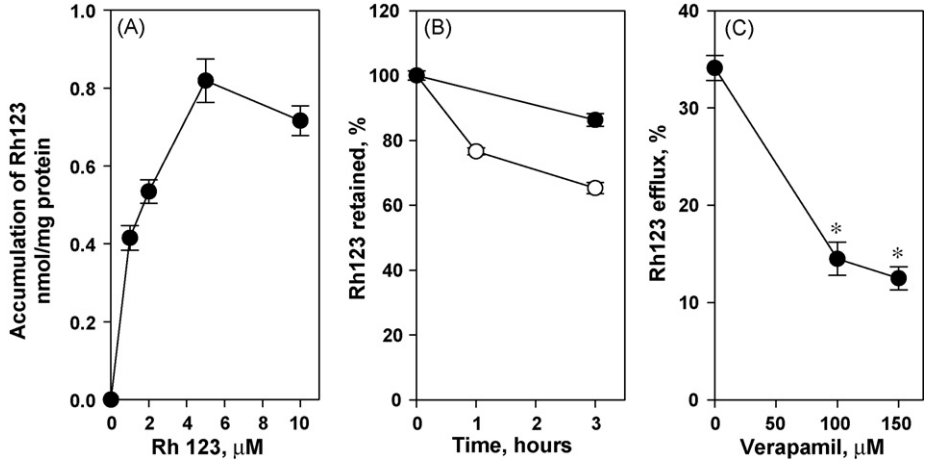

Fig. 2. (A) Rhodamine (Rh) 123 accumulation in OECM-1 cells at various initial concentrations of Rh 123. Cells were incubated with increasing concentrations of Rh 123 for 1 h, and cellular fluorescence was determined after two washes with PBS solution. Data represent the mean ± S.E.M. of three individual experiments with three to six replicates. (B) Rh 123 retained in OECM-1 cells after efflux for different time periods. Results are presented as the mean ± S.E.M. of three to six determinations in the absence (○) and presence (●) of 100 μM verapamil. (C) Effect of verapamil on Rh 123 efflux in OECM-1 cells. Results are presented as the mean ± S.E.M. of three individual experiments with three to six replicates. An asterisk (*) indicates that the value significantly differed from the control value, $p < 0.05$.

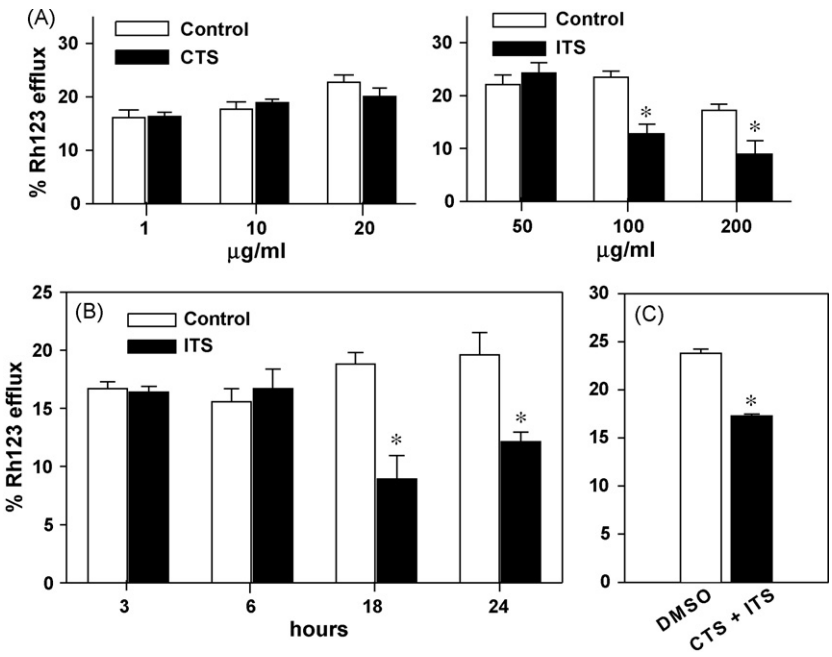

Fig. 3. Effects of tobacco smoke (A) extracts on Rh 123 efflux. OECM-1 cells were treated with increasing concentrations of the cyclohexane (CTS) and isopropanol (ITS) extracts of tobacco smoke condensate. (B) Time-course effects of ITS on Rh 123 efflux. Cells were exposed to 100 μg/ml ITS for the time period as indicated. (C) Effects of the co-exposure to 15 μg/ml CTS and 100 μg/ml ITS on Rh 123 efflux. Data are presented as the mean ± S.E.M. of six determinations. Experiments were repeated at least twice. An asterisk (*) indicates that the value significantly differed from the control value, $p < 0.05$.

ITS caused 55% and 38% decreases in Rh 123 efflux after treatment for 18 and 24 h, respectively. Based on the extraction yield, the exposure to 1 cigarette/100 ml resulted in the exposure to 15 μg/ml CTS and 100 μg/ml ITS. Thus, cells were exposed to a medium containing CTS (15 μg/ml) and ITS (100 μg/ml) for 18 h. This co-treatment significantly decreased the Rh 123 efflux activity by 21% (Fig. 3C).

3.4. Immunoblot analyses of Pgp in control and ITS-treated cells

Functional Pgp was reported to be expressed in HK-2 cells, and the Pgp member, MDR-1, was identified in HK-2 cells (Romiti et al., 2002). Thus, the crude membrane fraction of HK-2 cell lysate was loaded as a marker of Pgp. Immunoblot analyses of the crude membrane proteins of OECM-1 cells showed that one protein with an apparent molecular mass of 159 kDa immunoreacted with the C219 monoclonal antibody (Fig. 4). This protein had a gel-mobility equal to the Pgp protein in HK-2 cells. The relative band densities of Pgp in control and ITS-treated cells were 100 ± 8% and 95 ± 2% of three separate experiments, respectively. ITS treatment did not significantly reduce the protein level of Pgp in OECM-1 cells.

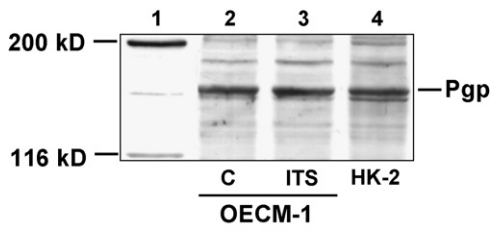

Fig. 4. Immunoblot analyses of P-glycoprotein (Pgp) in OECM-1 cells. Cells were treated with 100 μg/ml of the isopropanol extract of tobacco smoke (ITS) for 18 h. Control cells were treated with the same concentration of DMSO as ITS-treated cells. Electrophoresis and immunodetection were carried out as described in Section 2. Membrane fractional proteins (100 μg) were loaded for immunoreaction with C219. Lane 1 contains the molecular weight markers. Lanes 2 and 3 contain membrane proteins from control and ITS-treated OECM-1 cells, respectively. Lane 4 contains membrane proteins from untreated HK-2 cells.

3.5. Contents of benzo(a)pyrene, nicotine, and naphthalene in the TS extracts

HPLC analyses revealed that there was 35 μg benzo(a)pyrene/g extract in CTS, whereas the contents of benzo(a)pyrene in ITS was under the detection limit (Table 1). Fig. 5A shows the full-scan GC–MS chromatograms of CTS and ITS. Results of the GC–MS analyses showed that nicotine was the main constituent of all extracts and its contents in CTS and ITS were 53 and 86 mg/g extract, respectively. The chromatogram showed that CTS apparently had more-hydrophobic constituents than ITS, since the main peaks of CTS appeared at retention times of longer than 15 min. In contrast, the main constituents of ITS appeared at retention times of shorter than 6 min, and almost no peaks were detected at retention times of longer than 10 min. Naphthalene, which appeared at 2.4 min, could only be quantitatively detected in ITS (Fig. 5B). There was 0.15 mg naphthalene in 1 g ITS.

3.6. Effects of benzo(a)pyrene, nicotine, and naphthalene on Rh 123 efflux

The concentration of nicotine in a mixture containing 15 μg/ml CTS and 100 μg/ml ITS was 72 μM. Treatment with 50 and 100 μM nicotine for 18 h had no effects on Pgp-mediated Rh 123 efflux activity (data not shown). Benzo(a)pyrene was mainly collected in CTS, and its concentration in 15 μg/ml CTS was 2 nM. Treatment with benzo(a)pyrene at higher concentrations (1 and 10 μM) for 18 h, had no effect on Pgp function (data not shown). The concentration of

Table 1
Contents of benzo(a)pyrene, nicotine, and naphthalene in the extracts.

Extract	Benzo(a)pyrene (μg/g extract)	Nicotine (mg/g extract)	Naphthalene (mg/g extract)
CTS	35 ± 4	53	n.d.
ITS	n.d.	86	0.15

Data represent the mean and mean ± S.D. of duplicate and triplicate determinations, respectively. n.d.: not detectable.

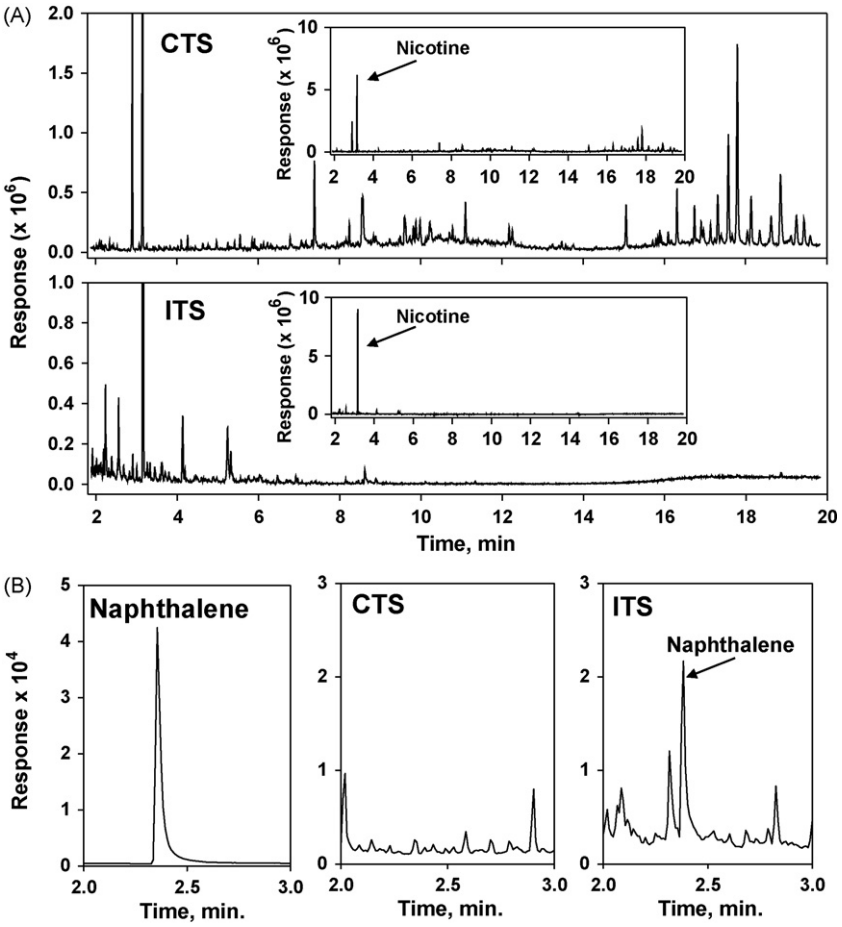

Fig. 5. The gas chromatograms of GC–MS analyses of the cyclohexane (CTS) and isopropanol (ITS) extracts of tobacco smoke. Panel (A) shows the separation profile of the full-scan modes. Nicotine appeared at a retention time of 3.2 min (the insert). Panel (B) shows the representative gas chromatograms of select-ion mode ($m/z = 128$) of naphthalene, CTS, and ITS. Naphthalene (3 μg/ml) and tobacco extracts (10 mg/ml) were dissolved in methanol and ethylacetate, respectively. One μl was injected into GC and the response in mass spectrometer was measured.

naphthalene in 100 μg/ml ITS was 0.2 μM. Naphthalene at 0.2 μM was not sufficient to inhibit Rh 123 efflux activity (Fig. 6). However, 18 h treatment with naphthalene at concentrations of 1–100 μM significantly decreased cellular Rh 123 efflux activity.

3.7. *Effects of the exposure to CTS in combination with ITS or naphthalene on cell viability*

Consistent with the decreased Rh 123 efflux by ITS, 18 h exposure to 100 μg/ml ITS caused a significant increase in Rh 123 accumulation (Fig. 7A). Single exposure of OECM-1 to 15 μg/ml CTS, 100 μg/ml ITS, or 0.2 μM naphthalene did not affect cell viability as monitored by the MTT reduction assay (Figs. 1 and 7B). However, co-exposure to 15 μg/ml CTS and 100 μg/ml ITS significantly decreased cell viability to 57% that of DMSO-treated cells (Fig. 7B). Co-exposure to 15 μg/ml CTS and 0.2 μM naphthalene also significantly decreased cell viability to 69% that of DMSO-treated cells. CTS at 50 μg/ml showed 89% cell viability. Co-incubation of cells with 50 μg/ml CTS and 0.2 μM naphthalene showed cell viability similar to that of CTS or naphthalene alone. However, co-exposure of cells to 50 μg/ml CTS and 10 μM naphthalene significantly decreased cell viability compared to cells treated with CTS alone. To further examine the contribution of CYP1A to the decreased cell viability, cells were concomitantly exposed to a CYP1A inducer, 3-methylcholanthrene (1 μM), and naphthalene (0.2 μM) for 18 h. This co-exposure did not decrease cell viability (Fig. 7C). Due to the interference of verapamil on MTT assay (Vellonen et al., 2004), the

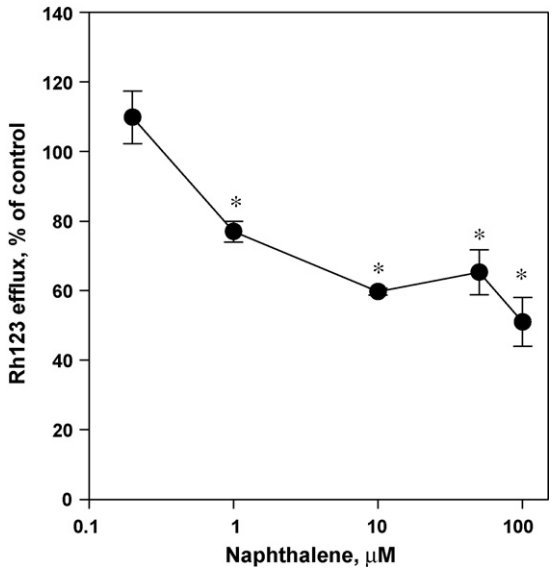

Fig. 6. Effect of naphthalene on Rh 123 efflux in OECM-1 cells. Cells were treated with increasing concentrations of naphthalene for 18 h. Control cells were treated with DMSO. Experiments were repeated at least twice. Results are presented as the mean ± S.E.M. of six determinations. An asterisk (*) indicates that the value significantly differed from the control value, $p < 0.05$.

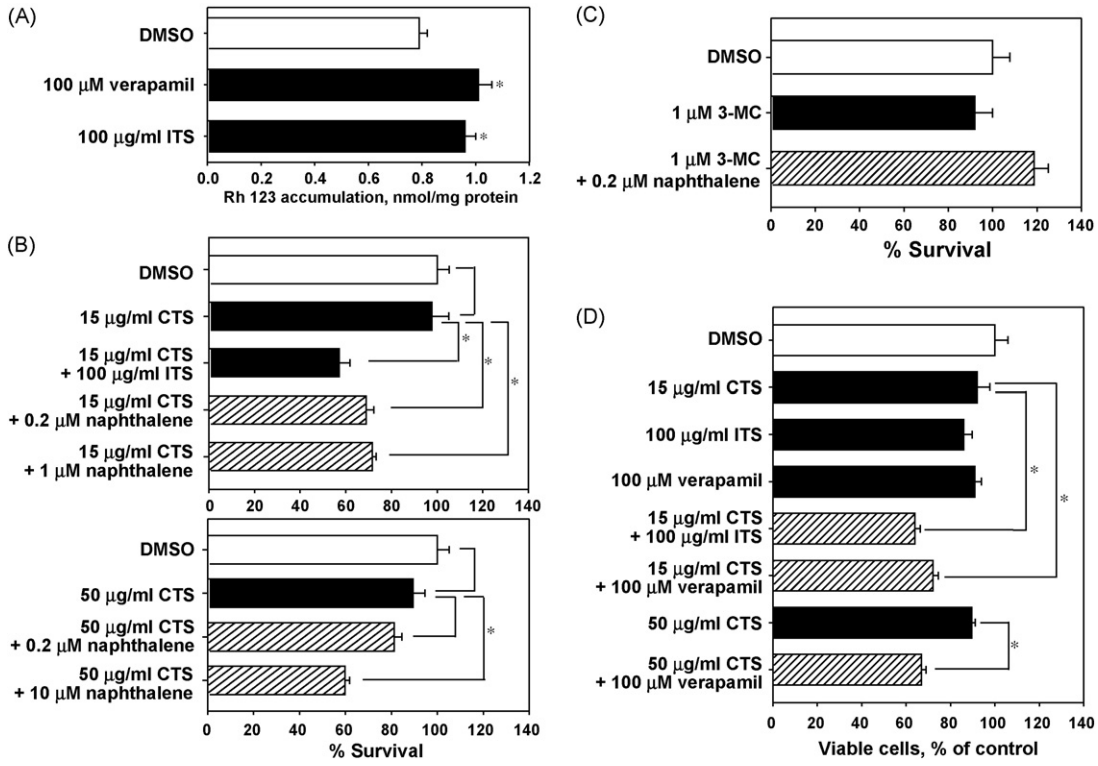

Fig. 7. (A) Effect of verapamil and the isopropanol extract of tobacco smoke (ITS) on the accumulation of rhodamine (Rh) 123 in OECM-1 oral cancer cells. (B) Oral cell viability after single or co-exposure to the cyclohexane extract of tobacco smoke (CTS) in combination with the ITS or naphthalene. Cell viability was monitored by cellular MTT reduction activity. Control cells were treated with DMSO. Data represent the mean ± S.E.M. of six determinations. (C) Effect of single or co-exposure to naphthalene and 3-methylcholanthrene (3-MC) on cell viability. Data represent the mean ± S.E.M. of six determinations. (D) Oral cell viability after single or co-exposure to the CTS in combination with the ITS or verapamil. The percentage of viable cells was determined by trypan blue exclusion 18 h after the extract and verapamil treatments. Data represent the mean ± S.E.M. of three determinations. An asterisk (*) indicates a significant difference between the two groups, $p < 0.05$.

number of living cells was further examined by a trypan blue exclusion assay. Consistent with the results of MTT assay, co-treatment with 15 μg/ml CTS and 100 μg/ml ITS decreased cell viability compared to single treatments (Fig. 7B and D). In the presence of a Pgp inhibitor, verapamil (100 μM), treatments with 15 and 50 μg/ml CTS significantly decreased cell viability to 72% and 65% that of DMSO-treated cells, respectively (Fig. 7D).

4. Discussion

Smoking is known to be an important risk factor for OSCC (Blot et al., 1988). Our study of TS showed that ITS but not CTS at 10–50 μg/ml increased the MTT reduction, suggesting the stimulation of cell proliferation. Our BrdU incorporation analysis demonstrated this stimulatory effect of ITS (results not shown). Another GC–MS study we performed indicated that a proliferation-stimulating agent, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), was mainly present in the ITS (unpublished results). In the exposure to the smoke from 1 cigarette in 100 ml medium, cells were exposed to 15 μg/ml CTS and 100 μg/ml ITS according to their respective extraction yield from TS. Under these exposures, cell viability was not affected. ITS but not CTS decreased the Rh 123 efflux activity. Thus, the TS-induced Pgp dysfunction might be attributed mainly to its isopropanol extractable portion after removing CTS. Increasing the exposure concentration of ITS to 200 μg/ml, cell viability and Pgp function were decreased. The Pgp dysfunction might be associated with reduced cell viability. Co-treatment of oral cells with ITS and CTS caused a decrease of Rh 123 efflux less potent than ITS alone. The CTS may interfere with the inhibitory mechanism of ITS on the export of Rh 123 and reduced the inhibitory potency of ITS. Although the ITS-mediated decrease of Rh 123 efflux activity was reduced by co-treatment with CTS, the cell insult was enhanced by this co-treatment. From the study of digoxin and cyclosporin A, Okamura et al. (1993) suggested that there could be more than one substrate binding sites in Pgp. Since CTS alone did not affect the export of Rh 123, CTS and/or its metabolites could be pumped out by Pgp though a binding site different from Rh 123 pathway. ITS-mediated Pgp inhibition could increase the accumulation of CTS and/or its metabolites and enhanced cell insult. Besides of the ITS-mediated Pgp inhibition, the cell insult may also be attributed to other factors, such as the synergistic cytotoxicity and cross activation of ITS and CTS ingredients. Therefore, the cell insult was highly increased.

Competition with substrates for export was the most common mechanism of Pgp inhibition. Thus, cells were treated with ITS during the accumulation or efflux periods instead of with 18 h of treatment. ITS could not affect Rh 123 accumulation and efflux, whereas verapamil elevated Rh 123 accumulation and decreased Rh 123 efflux. These results suggested that ITS did not directly compete with Rh123 for being pumped out. Results of our time-course study showed that ITS decreased Pgp function after treatment for 18 and 24 h, whereas 3 h and 6 h treatment did not affect Pgp function. These results suggest that a time period of more than 6 h was required to cause Pgp functional loss. However, our immunoblot analysis of membrane proteins showed that the level of Pgp was not affected by ITS. Other factors including ATP loss, nitration and phosphorylation of transporter protein, biophysical changes in the plasma membrane, and induction of metabolizing enzymes responsible for the formation of a metabolite which is the Pgp substrate, have been proposed or proven to be factors causing functional inhi-

bition of transporters (Fenyvesi et al., 2008; Idriss et al., 2000; Riganti et al., 2005). All these processes may require a certain period of exposure. However, the formation of nitric oxide and nitrated proteins were too low to be detected using Greiss reagent and immunoblot analysis, respectively (data not shown). Thus, further investigations on MS analysis of Pgp modification, enzyme induction, plasma membrane environment, and metabolomic profiles, may help clarify the contributions of these pathways.

Our determination using GC–MS showed the most abundant constituent in the TS extracts was nicotine, which was not a Pgp substrate (Wang et al., 2005). CTS had the highest benzo(a)pyrene content, but it did not affect Rh 123 efflux in OECM-1 cells. Nicotine and benzo(a)pyrene did not affect Rh 123 efflux, either. Activation of p53 was reported to be necessary for the induction of Pgp by benzo(a)pyrene in hepatoma cells (Mathieu et al., 2001). However, OECM-1 cells exhibit restriction fragment length polymorphism in p53 gene and no p53 protein was detected using immunoblot analysis (Kim et al., 1993). On the other hand, the benzo(a)pyrene concentrations and exposure time periods (10–50 μM for 24 h) for Pgp induction in Caco2 and SDVI cells was greater than those used in our study (Fardel et al., 1996; Sugihara et al., 2006). The differences in treatment regimen and cell types may cause the lack of induction in our study. In smokers with abnormal p53 expression, our results suggested that smoking may increase the risk of oral cell insult through inhibiting Pgp function. Naphthalene was not a Pgp substrate (Chang et al., 2006) and could be detected only in the ITS. However, it decreased Pgp efflux activity after 18 h treatment. Although we could not exclude the contribution of the other smoke constituents to this Pgp inhibition, naphthalene contributed at least partially to Pgp inhibition by ITS. To clarify the Pgp-inhibitory mechanism of naphthalene, it would be of interest to investigate naphthalene-triggered signaling and effects of naphthalene oxidation metabolites on Pgp function in oral cells in the future.

Different from the unchanged MTT reduction in cells with single treatments, co-treatment of cells with 15 μg/ml CTS in combination with 100 μg/ml ITS or 0.2 μM naphthalene decreased cell viability. Although 0.2 μM naphthalene was insufficient to cause a significant decrease of Rh 123 efflux, it was sufficient to enhance the cell insult under lower CTS exposure concentration. This might be due to the differences in IC_{50} values for inhibiting the efflux of different Pgp substrates. When cells were exposed to 50 μg/ml CTS, cell viability was decreased by co-treatment with 10 μM naphthalene but not with 0.2 μM naphthalene. Higher concentration of naphthalene was required to enhance cell insult when cells were exposed to higher concentration of CTS. Based on this dependence on the concentration ratio of naphthalene to CTS, naphthalene metabolites formed during 18 h exposure may competitively inhibit the export of CTS and/or its metabolites and caused cell insult. On the other hand, cytotoxicity of naphthalene could be elevated by over-expression of CYP1A1 in ovarian cells (Greene et al., 2000), suggesting the activation of naphthalene cytotoxicity by CYP1A1. 3-Methylcholanthrene is a prototype CYP1A inducer. Pre-treating cells with 3-methylcholanthrene, CTS and ITS increased the 7-ethoxyresorufin O-deethylation activity from 1.0 ± 0.5 pmol/min/mg protein of DMSO-treated cells to 160.5 ± 20.3, 48.1 ± 3.6 and 21.8 ± 3.7 pmol/min/mg protein, respectively. However, co-treatment with 3-methylcholanthrene and naphthalene (0.2 μM) did not decrease the MTT reduction level. These results suggest that the decreased cell viability following co-exposure to naphthalene (0.2 μM) and CTS (15 μg/ml) was not due to the activation of naphthalene by CTS-induced CYP1A. In addition, co-treatment with CTS and verapamil decreased the cell viability, suggesting that Pgp may play a protective role against the cell insult induced by CTS. Although we could not exclude the other causes for enhanced cell damage, results of co-treatment indicated that the reduction in Pgp function might be involved in the smoke-induced oral toxic event through diminishing the export of smoke toxins out of cells.

In summary, our results demonstrate that Pgp-mediated Rh 123 efflux was inhibited by ITS in OECM-1. Benzo(a)pyrene-enriched CTS did not affect Rh 123 efflux. Consistent with these results, the ITS constituent, naphthalene, but not the CTS constituent, benzo(a)pyrene, decreased Rh 123 efflux. Concurrent exposure to CTS in combination with naphthalene, ITS, or verapamil appeared to be more hazardous than individual agents. In the future, further experiments including small interfering RNA-mediated knockdown and over-expression of Pgp may provide further evidence to show the protective role of Pgp against smoke-induced cell insult.

Conflicts of interest

There were no competing interests.

Acknowledgments

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References

Alley, M.C., Scudeiro, D.A., Monks, A., Hursey, M.L., Czerwinsk, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., Boyd, M.R., 1988. Feasibility of drug screening with panels of human tumor cell lines using microculture tetrazolium assay. Cancer Res. 48, 589–601.

Anglicheau, D., Pallet, N., Rabant, M., Marquet, P., Cassinat, B., Meria, P., Beaune, P., Legendre, C., Thervet, E., 2006. Role of P-glycoprotein in cyclosporine cytotoxicity in the cyclosporine–sirolimus interaction. Kidney Int. 70, 1019–1025.

Blot, W.J., McLaughlin, J.K., Winn, D.M., Austin, D.F., Greenberg, R.S., Preston-Martin, S., Bernstein, L., Schoenberg, J.B., Stemhagen, A., Fraumeni Jr., J.F., 1988. Smoking and drinking in relation to oral and pharyngeal cancer. Cancer Res. 48, 3282–3287.

Chang, J.H., Kochansky, C.J., Shou, M., 2006. The role of P-glycoprotein in the bioactivation of raloxifene. Drug Metab. Dispos. 34, 2073–2078.

Chung, C.H., Yang, Y.H., Wang, T.Y., Shieh, T.Y., Warnakulasuriya, S., 2005. Oral precancerous disorder associated with areca quid chewing, smoking, and alcohol drinking in southern Taiwan. J. Oral Pathol. Med. 34, 460–466.

Colombo, A., Bonfanti, P., Orsi, F., Camatini, M., 2003. Differential modulation of cytochrome P-450 1A and P-glycoprotein expression by aryl hydrocarbon receptor agonists and thyroid hormone in *Xenopus laevis* liver and intestine. Aquat. Toxicol. 63, 173–186.

Fardel, O., Lecureur, V., Corlu, A., Guillouzo, A., 1996. P-glycoprotein induction in rat liver epithelial cells in response to acute 3-methylcholanthrene treatment. Biochem. Pharmacol. 51, 1427–1436.

Fenyvesi, F., Fenyvesi, É., Szente, L., Goda, K., Bacsó, Z., Bácskay, I., Váradi, J., Kiss, T., Molnár, É., Janáky, T., Szabó Jr., G., Vecsernyés, M., 2008. P-glycoprotein inhibition by membrane cholesterol modulation. Eur. J. Pharm. Sci. 34, 236–242.

Greene, J.F., Zheng, J., Grant, D.F., Hammock, B.D., 2000. Cytotoxicity of 1,2-epoxynaphthalene is correlated with protein binding and in situ glutathione depletion in cytochrome P4501A1 expressing Sf-21 cells. Toxicol. Sci. 53, 352–360.

Idriss, H.T., Hannun, Y.A., Boulpaep, E., Basavappa, S., 2000. Regulation of volume-activated chloride channels by P-glycoprotein: phosphorylation has the final say! J. Physiol. 524, 629–636.

Jain, V., Das, S.N., Luthra, K., Shukla, N.K., Ralhan, R., 1997. Differential expression of multidrug resistance gene product, P-glycoprotein, in normal, dysplastic and malignant oral mucosa in India. Int. J. Cancer 74, 128–133.

Kartner, N., Evernden-Porelle, D., Bradley, G., Ling, V., 1985. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature (London) 316, 820–823.

Kim, M.S., Li, S.L., Bertolami, C.N., Cherrick, H.M., Park, N.H., 1993. State of p53,Rb and DCC tumor suppressor genes in human oral cancer cell lines. Anticancer Res. 13, 1405–1414.

Kim, I., Darwin, W.D., Huestis, M.A., 2005. Simultaneous determination of nicotine, cotinine, norcotinine, and trans-3′-hydroxycotinine in human oral fluid using

solid phase extraction and gas chromatography–mass spectrometry. J. Chromatogr. B 814, 233–240.
König, J., Rost, D., Cui, Y., Keppler, D., 1999. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology 29, 1156–1163.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) 227, 680–685.
Lee, G., Piquette-Miller, M., 2003. Cytokines alter the expression and activity of multidrug resistance transporters in human hepatoma cell lines; analysis using RT-PCR and cDNA microarrays. J. Pharm. Sci. 92, 2152–2163.
Lin, S.C., Lu, S.Y., Lee, S.Y., Lin, C.Y., Chen, C.H., Chang, K.W., 2005. Areca (betel) nut extract activates mitogen-activated protein kinases and NF-κB in oral keratinocytes. Int. J. Cancer 116, 526–535.
Mathieu, M.C., Lapierre, I., Brault, K., Raymond, M., 2001. Aromatic hydrocarbon receptor (AhR)·AhR nuclear translocator- and p53-mediated induction of the murine multidrug resistance mdr1 gene by 3-methylcholanthrene and benzo(a)pyrene in hepatoma cells. J. Biol. Chem. 276, 4819–4827.
Muzio, L., Staibano, S., Pannone, G., Mignogna, M., Serpico, R., Rubini, C., Fioroni, M., Fanali, S., Piattelli, A., 2000. The human multidrug resistance gene (MDR-1): immunocytochemical detection of its expression in oral SCC. Anticancer Res. 20, 2891–2898.
NIOSH, in press. Manual of Analytical Methods. National Institute of Occupational Safety and Health, Washington, DC. Available at http://www.cdc.gov/niosh/nmam.
Okamura, N., Hirai, M., Tanigawara, Y., Tanaka, K., Yasuhara, M., Ueda, K., Komano, T., Hori, R., 1993. Digoxin–cyclosporin A interaction: modulation of the multidrug transporter P-glycoprotein in the kidney. J. Pharmacol. Exp. Ther. 266, 1614–1619.
Penny, J.I., Campell, C.F., 1994. Active transport of benzo(a)pyrene in apical membrane vesicles from normal human intestinal epithelium. Biochim. Biophys. Acta 1226, 232–236.
Piquette-Miller, M., Pak, A., Kim, H., Anari, R., Shahzamani, A., 1998. Decreased expression and activity of P-glycoprotein in rat liver during acute inflammation. Pharm. Res. 15, 706–711.
Riganti, C., Miraglia, E., Viarisio, D., Costamagna, C., Pescarmona, G., Ghigo, D., Bosia, A., 2005. Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. Cancer Res. 65, 516–525.
Risner, C.H., 1988. The determination of benzo(a)pyrene in the total particulate matter of cigarette smoke. J. Chromatogr. Sci. 26, 113–120.
Romiti, N., Tramonti, G., Chieli, E., 2002. Influence of different chemicals on MDR-1 P-glycoprotein expression and activity in the HK-2 proximal tubular cell line. Toxicol. Appl. Pharmacol. 183, 83–91.
Sadeque, A.J., Wandel, E., He, H., Shah, S., Wood, A.J.J., 2000. Increased drug delivery to the brain by P-glycoprotein inhibition. Clin. Pharmacol. Ther. 68, 231–237.
Smith, C.J., Hansch, C., 2000. The relative toxicity of compounds in main cigarette smoke condensate. Food Chem. Toxicol. 38, 637–646.
Sugihara, N., Toyama, K., Michihara, A., Akasaki, K., Tsuji, H., Furuno, K., 2006. Effects of benzo(a)pyrene on P-glycoprotein-mediated transport in Caco-2 cell monolayer. Toxicology 223, 156–165.
Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.
van der Deen, M., de Vries, G.E., Visserman, H., Zandbergen, W., Postma, D.S., Timens, W., Timmer-Bosscha, H., 2007. Cigarette smoke extract affects functional activity of MRP1 in bronchial epithelial cells. J. Biochem. Mol. Toxicol. 21, 243–251.
Vellonen, K.S., Honkakoski, P., Urtti, A., 2004. Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity. Eur. J. Pharm. Sci. 23, 181–188.
Walle, U.K., Walle, T., 1999. Transport of the cooked-food mutagen 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) across the human intestinal Caco-2 cell monolayer: role of efflux pump. Carcinogenesis 20, 2153–2157.
Wang, J.S., Markowitz, J., Donovan, J., 2005. P-glycoprotein does not actively transport nicotine and cotinine. Addict. Biol. 10, 127–129.
Yan, Y.E., Wang, H., Wang, T., Zeng, H.G., 2006. Indole-3-carbinol alters placental cytochrome P450 1A1 and P-glycoprotein levels in rats: AQ potential role in intensifying fetal intrauterine growth-retardation produced by tobacco smoke. Exp. Toxicol. Pathol. 58, 39–47.
Yang, S.C., Lin, S.C., Chiang, W.F., Yen, C.Y., Lin, C.H., Liu, S.Y., 2003. Areca nut extract treatment elicits the fibroblastoid morphological changes, actin re-organization and signaling activation in oral keratinocytes. J. Oral Pathol. Med. 32, 600–605.
Yang, C.S., Meng, C.L., 1994. Regulation of PG synthase by EGF and PDGF in human oral breast, stomach, and fibrosarcoma cancer cell lines. J. Dent. Res. 73, 1407–1415.
Yeh, G.C., Lopaczynska, J., Poore, L.M., Phang, J.M., 1992. A new functional role for P-glycoprotein: efflux pump for benzo(a)pyrene in human breast cancer CMF-7 cells. Cancer Res. 52, 6692–6695.