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Budesonide reduces multidrug resistance-associated protein 1 expression in an airway epithelial cell line (Calu-1)

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Abstract

The objective of this study was to determine the expression and activity of multidrug resistance-associated protein (MRP1) in a human airway epithelial cell line (Calu-1) and to further assess whether budesonide, a potent antiasthma corticosteroid, alters the expression and activity of MRP1 in these cells. Reverse transcriptase polymerase chain reaction (RT-PCR) and the Western blot analysis demonstrated the MRP1 mRNA and MRP1 protein in Calu-1 cells. Indomethacin, probenecid, and verapamil significantly enhanced the fluorescein accumulation and reduced the fluorescein efflux, consistent with the MRP1 activity in the Calu-1 cells. Following 14-day budesonide treatment, fluorescein accumulation increased and fluorescein efflux decreased, consistent with the inhibition of MRP1 activity by budesonide. At a concentration (10 μM) devoid of cytotoxicity, budesonide treatment decreased MRP1 mRNA and MRP1 protein expression in Calu-1 cells by 38% and 42%, respectively. In addition, budesonide (10 μM) enhanced the sensitivity of the MRP1 overexpressing COR-L23R cells to vincristine, suggesting the chemosensitizing effect of budesonide. Thus, budesonide inhibits MRP1 expression and may be useful as a chemosensitizer in tumor chemotherapy.

Keywords: Budesonide; Calu-1 cell; MRP (Multidrug resistance-associated protein)

1. Introduction

Multidrug resistance to cytotoxic drugs leads to treatment failure in lung cancer, the leading cause of cancer death in the United States (Nishio et al., 1999). Overexpression of drug efflux pumps, such as multidrug resistance-associated protein (MRP) transporters, is one reason for the development of drug resistance in cancer patients (Scagliotti et al., 1999). MRPs belong to the ATP-binding cassette (ABC) family of transporters, which can reduce the intracellular accumulation of some anticancer drugs by an energy-dependent efflux mechanism (Borst et al., 2000). MRPs are abundantly expressed in the normal tracheal and bronchial epithelial cells of the respiratory tract and this expression is elevated in patients with lung cancer (Flens et al., 1996; Wright et al., 1998). MRP overexpression in lung cancer subjects decreases the intracellular accumulation of anticancer drugs, thereby reducing the chemosensitivity (Nakano et al., 1998). This is a principal mechanism by which lung cancer cells acquire resistance to drugs, such as daunorubicin, etoposide, and vincristine (Scagliotti et al., 1999). Sensitivity to chemotherapy can be improved by co-administering MRP inhibitors (Berger et al., 1997). For instance, the sensitivity of the human lung carcinoma cells (COR L23) to doxorubicin was increased eight times, with the use of dipyridamole, an inhibitor of MRP (Curtin and Turner, 1999). Thus, inhibition of MRP is of potential value in the treatment of lung cancer.

The human MRP family consists of at least seven members (MRP1–MRP7) (Kool et al., 1997, 1999). Within the normal lung, MRP1, MRP4, and MRP5 have been identified (Borst et al., 2000). On the other hand, MRP1, MRP2, and MRP3 have been identified in the tumor lung (Young et al., 2001). The objective of this study was to determine whether budesonide, a potent antiasthma corticosteroid, modulates MRP1 expression in the airway epithelial cells. Budesonide has high glucocorticoid receptor affinity, airway selectivity, and prolonged tissue retention (Szefler, 1999). The effect of budesonide on activity and/or expression of MRPs has not been previously tested in any biological system. In this study, we are specifically interested in the modulation of MRP1 expression, because MRP1 expression in the normal lung and tumor lung is higher compared

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to other various tissues including the intestine, liver, brain, and kidney (Barrand et al., 1997; Pascolo et al., 2000). In addition, the function of MRP1 in normal cells and its role in conferring multidrug resistance in tumor cells is well characterized.

Other corticosteroids have been previously shown to alter the activity and/or expression of drug efflux pumps. For instance, dexamethasone can either down-regulate or up-regulate the expression of drug efflux pumps (Fardel et al., 1993; Demeule et al., 1999; Courtois et al., 1999). A reduction in the MRP1 expression and/or activity is likely to improve the chemosensitivity in lung cancer patients. However, the modulation of MRP1 activity and/or expression by any corticosteroid in the respiratory epithelial cells has not been reported. In the present study, we determined the effects of budesonide on MRP1 expression in the airway epithelial cells. The findings of this study, besides being valuable in the treatment of lung cancer, are useful in understanding the effects of antiasthma drugs on the activity and expression of MRP1 in airway epithelial cells.

Besides some anticancer drugs, MRP1 in normal tissues can export a variety of solutes, such as steroid glucuronides, steroid hormones, organic anions and glucuronide, glutathione, and sulfate conjugated compounds by an energy-dependent efflux mechanism (Jedlitschky et al., 1996; Keppler and Konig, 2000; Borst et al., 2000). However, it should be noted that the physiological role of MRP1 in the normal lung is not established. Because budesonide therapy for asthma is mostly long-term, the airway epithelium is exposed to this drug for prolonged periods. Under these conditions, if the inhaled budesonide alters the MRP1 expression and/or activity, the intracellular retention of the endogenous MRP1 substrates such as leukotriene C_4 and conjugated estrogen may be affected (Loe et al., 1996; Wijnholds et al., 1997). Since leukotriene C_4 is a potent chemical mediator of human bronchial asthma, the findings of this study may also be of relevance to the effects of budesonide in asthma therapy (Weltman, 1999).

Thus, the airway epithelial cells express MRP1, which can reduce the cellular retention of drugs and metabolites in normal and tumor cells (Wright et al., 1998; Borst et al., 2000). Although the expression of MRPs can likely be regulated by corticosteroids, there are no previous reports on the interaction of corticosteroids with MRP1. In addition, there are no reports on the interaction of budesonide with MRPs in any cell type. For the first time, we determined these possibilities by assessing the interaction of budesonide with MRP1 in cells representative of the airway epithelia (Calu-1). Calu-1 is a human airway epithelial cell line, derived from human non-small-cell lung carcinomas. These cells secrete mucus, possess cilia, and have extensive endopeptidase activity, similar to the normal airway epithelium (Botti et al., 2000; Lang and Murlas, 1992, 1993). For these reasons, this cell line has been widely used as a model for studying the molecular events associated with the human airway and/or bronchial epithelial cells (Lee et al., 1992; Lang and Murlas, 1992, 1993). This is the first report demonstrating the modulation of MRP1 expression and activity by any corticosteroid. In addition, this is the first study to provide evidence for the expression and activity of MRP1 in Calu-1 cells.

2. Materials and methods

2.1. Chemicals

All cell culture supplies were obtained from Gibco (Grand Island, NY) and Becton Dickinson Labware (Franklin Lakes, NJ). Verapamil hydrochloride, probenecid, indomethacin, Triton-X, budesonide, fluorescein, vincristine, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, triazolyl blue) were purchased from Sigma (St. Louis, MO). All agents were of analytical grade and used without further purification.

2.2. Preparation of solutions

Accumulation and efflux studies of fluorescein were conducted using an assay buffer (pH 7.4), which contained 1.14 mM $CaCl_2$, 1.2 mM $MgSO_4$, 3 mM KCl, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 122 mM NaCl, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). Prior to each experiment, the assay buffer was pre-equilibrated to 37 °C for 30 min.

2.3. Cell culture

Calu-1 cells obtained from the American Type Culture Collection (ATCC; Rockville, MD) were grown in McCoy's 5a medium with 10% fetal bovine serum and supplemented with 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin sulfate. The cells were plated in 75 cm^2 cell culture flasks and were subcultured after attaining confluence on day 6 or 7. The cells were split 1:10 during each passage. The passages used for the experiments ranged from 28 to 35. All the accumulation, efflux, Western blot, and reverse transcriptase polymerase chain reaction (RT-PCR) studies were done using day 6 cells grown either in 48-well tissue culture plates or cell culture flasks. For the accumulation studies performed in 48-well culture plates, Calu-1 cells were seeded at 15000 cells/cm^2. A549 cells used as the MRP1 positive control cells in the RT-PCR studies were obtained from ATCC. A549 cells were grown in Dulbecco's modified eagle medium (DMEM/F-12) medium with 10% fetal calf serum and supplemented with 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin sulfate. The doxorubicin-resistant COR-L23R cells used as the MRP1 positive controls in the Western blot and vincristine sensitivity studies were

obtained from Dr. Donald W. Miller (Nebraska Medical Center, Omaha, NE).

2.4. RT-PCR study

2.4.1. Expression of MRP1 mRNA in Calu-1 cells

Using the Access RT-PCR System (Promega, Madison, WI), MRP1 mRNA expression was identified in Calu-1 cells. To do this, total RNA was extracted from the confluent Calu-1 and A549 cells using the RNA STAT-60™ RNA isolation kit (TEL-TEST, Friendswood, TX) as per manufacturer's recommendations. The RT-PCR for MRP1 was performed as described by Pascolo et al. (2000). The amplification products were separated on a 3% agarose gel and visualized by staining with ethidium bromide. PCR product of 287 bp encoding MRP1 was obtained. The sense and antisense primer sequences for the MRP1 expression were CTGTTTTGTTTTCGGGTTCC and GATGGTGGACTGGATGAGGT, respectively. On the other hand, the sense and antisense primer sequences for GAPDH, used as an internal control were CGATGCTGGCGCTGAGTAC and CGTTCAGCTCAGGGATGACC, respectively.

2.5. Western blot study

2.5.1. Expression of MRP1 protein in Calu-1 cells

Cell monolayers of Calu-1 were solubilized in phosphate-buffered saline (pH 7.4) containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN) and 1% sodium dodecyl sulfate (SDS) and the total protein content of the cell lysates was estimated. For the Calu-1 cells, six lysate samples (5, 10, 15, 20, 25, and 30 μg) were loaded onto the preformed 7.5% polyacrylamide gels (Bio-Rad, Hercules, CA). Cell lysates of the human lung carcinoma cells, COR-L23R, used as MRP1 positive controls (Thomas et al., 1994) in the Western blot analysis, were loaded at 10-μg protein. Molecular weight markers ranging from 220 to 14.3 kDa (Amersham Life Science, Arlington Heights, IL) were used to identify the corresponding bands of MRP1. The proteins on the gels were separated using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were run at 40 V for 30 min and then continued at 70 V for another hour. The proteins were then transferred onto the polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) at 4 °C using a current of 480 mA. The immunoblots were done using the MRP1 specific antibody, MRPm6 (Kamiya, Seattle, WA). The transferred proteins were treated with the blocking buffer which contained 0.3% Tween-20 and 1% BSA and incubated with the specific antibodies overnight at 4 °C. The antibody was used at 1:100 dilutions. Following a series of washes with the washing buffer, a secondary horseradish peroxidase conjugated mouse immunoglobulin (Ig) antibody (Amersham Life Science) was added (1:1500) and incubated at 4 °C for 1 h. MRP1 bands were visualized using a chemiluminescence kit (Amersham Life Science).

2.6. MRP1 functional studies

Functional assay for MRP1 in the Calu-1 cells was performed using the fluorescein accumulation and fluorescein efflux studies with and without indomethacin (10 μM), probenecid (100 μM), and verapamil (100 μM). Bakos et al. (2000) have provided evidence that indomethacin decreases the ATP-ase activity of MRP1 in *Spodoptera frugiperda* (Sf9) ovarian cells expressed with MRP1. Probenecid, a well-known organic anion transport inhibitor, also inhibits the MRP1 activity (Gollapudi et al., 1997; Sun et al., 2001). Verapamil, a well-known chemosensitizer known to inhibit the P-glycoprotein (P-gp)-mediated transport at 10 μM, has been shown to be effective in inhibiting MRP activity at higher concentrations (Barrand et al., 1993). Fluorescein, as well as MRP inhibitors used in this study, interact with other MRPs besides MRP1. Therefore, the measured activity while consistent with MRP1 activity, does not rule out the contribution of other MRPs.

2.6.1. Fluorescein accumulation studies

Cell monolayers grown in 48-well plates were pre-equilibrated with assay buffer at 37 °C for 45 min. Subsequently, the monolayers were exposed to 100 μM fluorescein with or without MRP inhibitors. The accumulation of fluorescein was terminated after either 3 or 12 h by removing the marker solution and washing the monolayers three times with ice-cold phosphate-buffered saline (PBS). The monolayers were solubilized for 3 h using 1.0% Triton-X and the intracellular fluorescein was estimated using a spectrofluorometer (RF 5000 U, Shimadzu, Kyoto, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 510 nm. The solubilized cellular aliquots were also used to estimate the cellular protein content using a Pierce protein assay kit (Rockford, IL) and a microkinetics reader (Fischer Scientific, Pennsylvania). The cellular accumulation of fluorescein was normalized to the cellular protein content.

2.6.2. Fluorescein efflux studies

Following 3-h fluorescein (100 μM) accumulation in assay buffer at 37 °C, the monolayers were washed three times with ice-cold PBS and exposed to solutions with or without MRP inhibitors with the same concentrations as in the accumulation studies. The cumulative efflux of fluorescein into the assay buffer was assessed at the end of 30 min. At the end of the efflux study, the cell monolayers were solubilized with 1% Triton-X solution and the protein content was determined. Fluorescein in the efflux medium was analyzed using a spectrofluorometer. The cellular efflux of fluorescein was normalized to the protein content.

2.7. Effect of budesonide treatment on the MRP1 expression

The effect of 14-day budesonide (10 μM) treatment on MRP1 mRNA expression was determined using RT-PCR. In addition, the effect of this treatment on the MRP1 protein

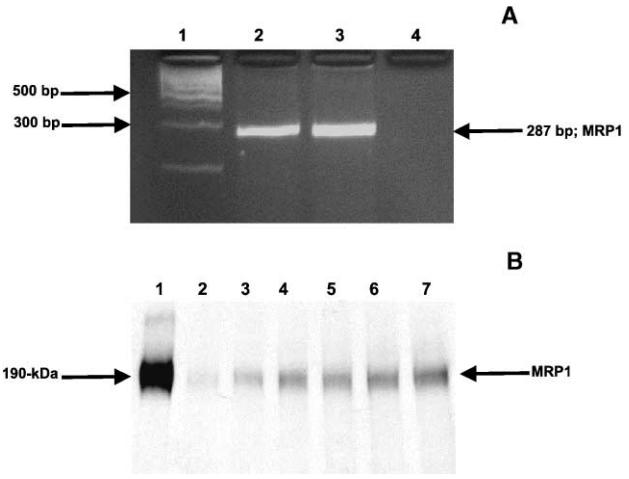

Fig. 1. MRP1 expression in the Calu-1 cell monolayers. (A) MRP1 mRNA expression in Calu-1 cells. Aliquots (3 μg) of total RNA were subjected to RT-PCR and the PCR products were separated on a 3% agarose gel. Key-lane 1: marker, lane 2: Calu-1 cells; lane 3: A549 cells (MRP positive cells); and lane 4: negative control (no RNA template). (B) MRP1 protein expression in the Calu-1 cells. Western blot for the MRP1 protein expression was performed with MRPm6, a specific antiMRP1 antibody. COR-L23 (MRP positive cells) cell lysates were loaded at 10 μg in lane 1 and the Calu-1 cell lysates were loaded at 5, 10, 15, 20, 25, and 30 μg in lanes 2, 3, 4, 5, and 6, respectively.

expression was determined using Western blot analysis. For MRP1 mRNA studies, following the budesonide treatments, total RNA (3 μg) of untreated and budesonide-treated cells was subjected to MRP1 mRNA RT-PCR, as described above. In addition, RT-PCR was performed for GAPDH, used as an internal control. For the MRP1 protein expression studies, following the budesonide treatment, lysates of untreated and budesonide-treated cells (25 μg) were loaded onto preformed 7.5% polyacrylamide gels (Bio-Rad) and the Western blot for MRP1 expression was performed, as described above. The densitometric analysis for MRP1 mRNA and MRP1 protein expressions was performed using the Nucleovision™ Imaging System (Nucleotech, San Mateo, CA).

Time-course of budesonide (10 μM) effect on MRP1 mRNA expression was also determined in Calu-1 cells. Calu-1 cells cultured in T-25 flasks were incubated with budesonide (10 μM) for 1,4, and 7 days. At the end of these incubation periods, total RNA was extracted and the RT-PCR was performed for MRP1 and GAPDH mRNA expressions.

2.8. Effect of the budesonide treatment on MRP1 activity

Calu-1 cells were subjected to subchronic (14 days) treatment with budesonide. The treatment involved a 14-day exposure of the Calu-1 cells to budesonide (1, 10, 100 μM). The medium was changed every 2 days. In all the experiments, control cells were maintained in a culture medium devoid of budesonide. Following the subchronic budesonide treatment, budesonide-containing medium was removed, cell monolayers were washed three times with the assay buffer and fluorescein accumulation was performed for 3 h. In addition, fluorescein efflux was assessed at the end of 30 min, following an initial fluorescein loading for 3 h.

2.9. Effect of budesonide on the cytotoxicity of vincristine

The cytotoxicity of anticancer drugs such as vincristine, an MRP1 substrate, can be increased by drugs inhibiting the MRP1 expression. Cytotoxicity can be determined using the cell viability indicating tetrazolium dye assay (Narasaki et al., 1997; Nakano et al., 1998). In this study, MRP1 over-expressing COR-L23R cells seeded at densities of 5000 cells/well in 96-well plates were treated with various concentrations of vincristine (0.1–5 nM) with or without budesonide (10 μM). Following this treatment for 4 days at 37 °C, 20 μl of MTT (5 mg/ml) was added to each well and the contents were incubated for 4 h at 37 °C. Subsequently, the formed formazan crystals were dissolved by adding 100 μl of 2-propanol-0.1 N HCl to each well.

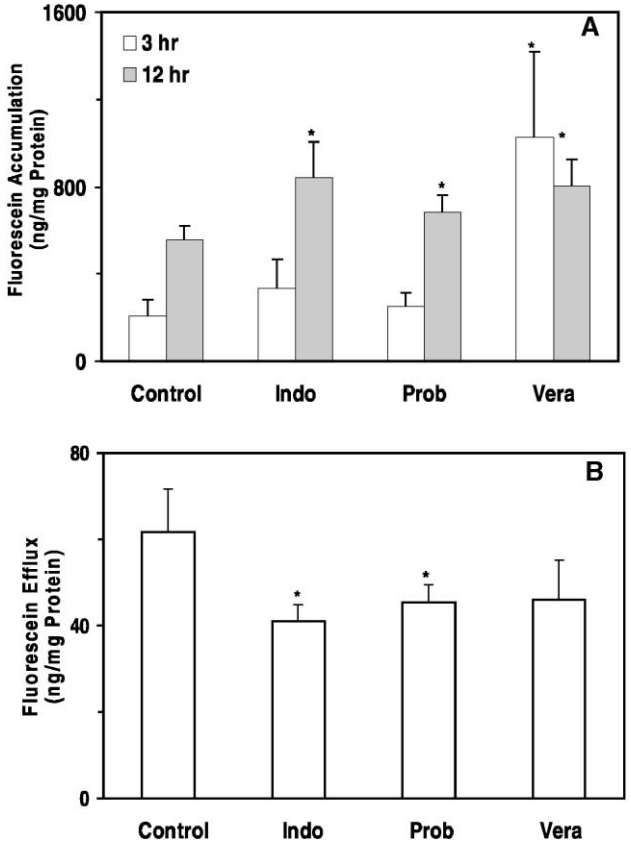

Fig. 2. MRP functional activity in the Calu-1 cell monolayers. (A) Fluorescein accumulation and (B) fluorescein efflux in the absence and presence of the MRP inhibitors, 10 μM indomethacin (Indo), 100 μM probenecid (Prob), and 100 μM verapamil (Vera). Fluorescein efflux at the end of 30 min was measured after an initial fluorescein loading for 3 h. Data are expressed as the mean ± S.D. for $n=4$. * Indicates the significant difference from the controls in the accumulation and efflux studies.

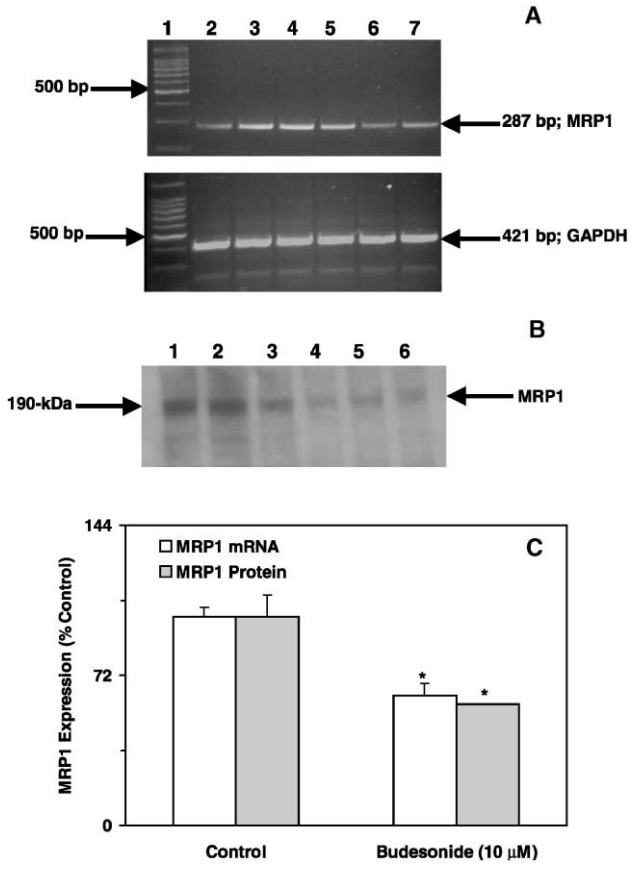

Fig. 3. Influence of the 14-day budesonide (10 μM) treatment on the MRP1 mRNA and MRP1 protein expression in the Calu-1 cell monolayers. (A) MRP1 mRNA expression in the Calu-1 cell monolayers. Key-lane 1: molecular weight markers (100 bp); lanes 2, 3, and 4: controls; and lanes 5, 6, and 7: budesonide (10 μM) treatment. (B) MRP1 protein expression in the Calu-1 cell monolayers. Lanes 1, 2, and 3: control lysates loaded at 25 μg. Lanes 4, 5, and 6: budesonide treated cell lysates loaded at 25 μg. (C) Densitometric analysis for the MRP1 expression. For MRP1 mRNA studies, data is expressed as the mean ratio of MRP1/GAPDH mRNA band intensity. Data for MRP1 mRNA and MRP1 protein are expressed as the mean ± S.D. for three independent experiments ($n = 3$). * Indicates significant difference from controls in the expression studies.

Absorbances were read on a microtiter plate (Fischer Scientific) at 550 nm. The cell viability was determined as the relative formation of formazan following the vincristine treatments when compared with the controls treated with vincristine-free medium.

2.10. Cytotoxic effect of budesonide and MRP inhibitors

Cytotoxic effect of budesonide and the MRP inhibitors was assessed by performing a colorimetric MTT assay (Bandi and Kompella, 2001). For the budesonide effects, Calu-1 cells plated at a density of 1×10^4 cells/well in a 96-well microtiter plate were cultured for 14 days with budesonide (1, 10, 100 μM). The medium was changed every 2 days. In all the experiments, control cells were maintained in a culture medium devoid of budesonide. For the MRP inhibitor effects, the viability was determined following 12-h treatment with indomethacin (10 μM), probenecid (10 μM), and verapamil (10 μM). In both the experiments, the percentage of viable cells with all tested budesonide and/or MRP inhibitor concentrations were calculated relative to the untreated cells.

2.11. Statistical analysis

All data are expressed as the mean ± S.D. or S.E.M. and the comparison of the mean values was performed using a paired Student's *t*-test. Statistical significance was accepted at $p < 0.05$ levels.

3. Results

3.1. MRP1 expression

MRP1 mRNA expression was determined using a RT-PCR method (Fig. 1A). In the Calu-1 cells, a 287-bp band corresponding to MRP1 mRNA (lane 2) was observed. A similar band was observed in the MRP1 overexpressing A549 cells (lane 3) (Trussadi et al., 1998). Lane 4, with no visible bands is the negative control, which contained no RNA template.

MRP1 protein expression in the Calu-1 cells was examined by performing Western blot analysis. In Calu-1 cells (Fig. 1B), a band at 190 kDa corresponding to MRP1 was

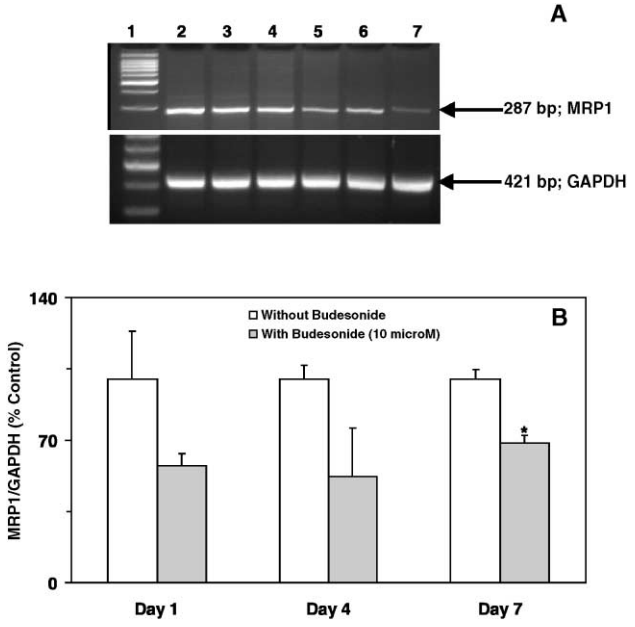

Fig. 4. Time-dependent effect of budesonide (10 μM) on the MRP1 mRNA expression in the Calu-1 cells. (A) MRP1 and GAPDH expression in the Calu-1 cells. Key-Lane 1: molecular weight markers (100 bp); Lanes 2, 4, and 6: controls from days 1, 4, and 7, respectively; Lanes 3, 5, and 7: budesonide (10 μM) treatment for 1, 4, and 7 days, respectively. (B) Densitometric analysis of the MRP1 and GAPDH mRNA expression in the Calu-1 cells. Data is expressed as the mean ratio of MRP1/GAPDH mRNA band intensity for three independent experiments. * Indicates significant difference from controls in the expression studies.

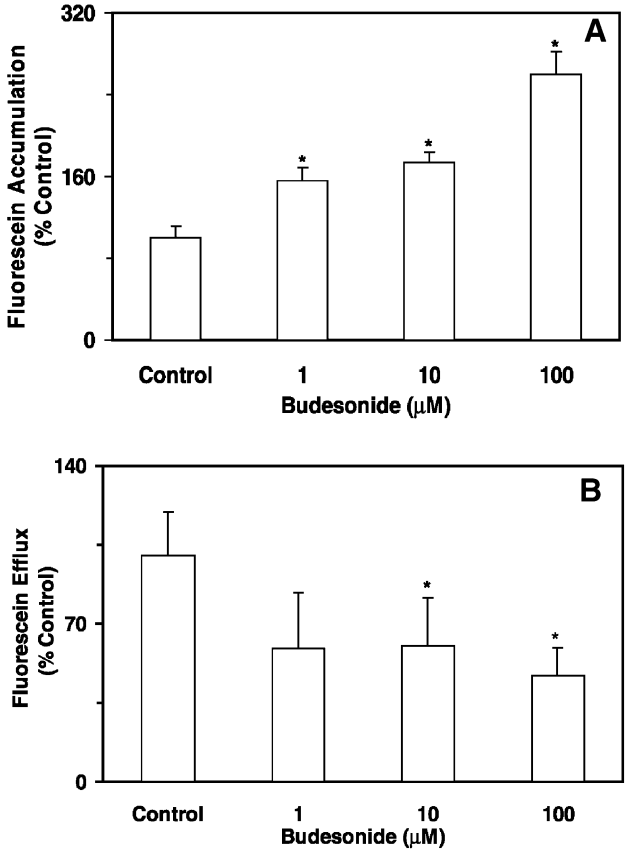

Fig. 5. Influence of the budesonide treatment on the MRP activity in the Calu-1 cell monolayers. At the end of the treatment period, budesonide was removed from the medium and fluorescein accumulation and efflux studies were conducted. (A) 3-h fluorescein accumulation in the Calu-1 cells. (B) Fluorescein efflux from the Calu-1 cells at the end of 30 min following an initial fluorescein loading for 3 h. Data are expressed as the mean ± S.D. or S.E.M. for $n=4$. * Indicates significant difference from controls in the accumulation and efflux studies.

observed. A similar band was detected in immunoblots of the MRP1 overexpressing COR-L23 cells (Fig. 1B).

3.2. MRP1 functional activity

In controls, the fluorescein accumulation increased with time (Fig. 2A). Compared to the controls at 12 h, indomethacin, probenecid, and verapamil significantly increased the fluorescein accumulation by 52%, 24%, and 45%, respectively.

The cumulative fluorescein efflux at the end of 30 min was reduced by 33%, 27%, and 25% in the presence of indomethacin, probenecid, and verapamil, respectively (Fig. 2B), with the effects of indomethacin and probenecid being statistically significant.

3.3. Effect of budesonide treatment on MRP1 expression

MRP1 and GAPDH mRNA expression in the Calu-1 cells indicated bands at 287 and 421 bp, corresponding to MRP1 and GAPDH, respectively (Fig. 3A). With the 14-day budesonide (10 μM) treatment, the MRP1 mRNA expression was decreased by 38% (Fig. 3C). Such a reduction was also feasible with a 7-day budesonide treatment but not with a 1- or 4-day treatment (Fig. 4).

Following 10-μM budesonide treatment for 14 days, the intensity of the MRP1 protein band at 190 kDa in the immunoblots was significantly reduced by 42% (Fig. 3B and C).

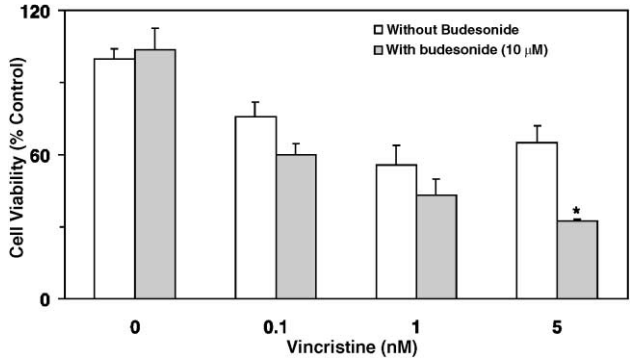

Fig. 6. Effect of the budesonide on the vincristine-mediated cytotoxicity in the COR-L23R cells. The sensitivity of the COR-L23R cells to the vincristine was determined in the presence and absence of budesonide (10 μM) using an MTT assay. Data are expressed as the mean ± S.E.M. for $n=5$. * Indicates significant difference from controls.

3.4. Effect of budesonide treatment on MRP1 activity

Compared to the controls, the fluorescein accumulation significantly increased by 56%, 73%, and 160% with 1, 10, and 100 μM budesonide treatments for 14 days (Fig. 5A).

Compared to the controls, the fluorescein efflux decreased by 41%, 40%, and 51% with 1, 10, and 100 μM budesonide treatments (Fig. 5B), with the effects being significant at 10 and 100 μM, consistent with the reduction of the MRP1 activity with the budesonide treatment.

3.5. Chemosensitizing effect of budesonide

Vincristine reduced the cell viability in a concentration-dependent manner (Fig. 6). Compared to the 64% cell via-

Table 1

Treatment	%Control absorbance[a]
(a) Effect of a 14-day budesonide treatment on the cell viability	
Controls	100 ± 12.1
Budesonide (1 μM)	118 ± 7.4
Budesonide (10 μM)	85 ± 5
Budesonide (100 μM)	51 ± 5 *
(b) Effect of a 12-h MRP inhibitor treatment on the cell viability	
Controls	100 ± 7.34
Indomethacin (1 μM)	95.6 ± 13.1
Probenecid (100 μM)	96.1 ± 8.8
Verapamil (100 μM)	85.4 ± 18.6

[a] In the MTT assay, the absorbances were measured at a test wavelength of 550 nm and a reference wavelength of 630 nm. Data is expressed as the mean ± S.D. or S.E.M. for $n=4$ or 5.

* Indicates significant difference from controls.

bility in the presence of 5 nM vincristine, co-treatment with 10-μM budesonide for 4 days reduced the cell viability to 32%.

3.6. Cytotoxic effect of budesonide and MRP inhibitors

The results of the cytotoxic effect of the budesonide treatment are shown in Table 1. Budesonide treatment induced cytotoxicity in the Calu-1 cells at 100 μM (Table 1a). The effects observed at 1 and 10 μM budesonide were not statistically significant. MRP inhibitors did not alter the cell viability significantly (Table 1b).

4. Discussion

In the present study, we have investigated the expression and activity of MRP1 in airway (Calu-1) epithelial cells and further assessed the effects of budesonide on MRP1 expression and activity in these cells. RT-PCR analysis using MRP1 specific primers (Pascolo et al., 2000) indicated that MRP1 mRNA in Calu-1 cells, similar to A549 cells (Fig. 1A), a positive control for the MRP1 expression (Trussardi et al., 1998). Western blot analysis indicated a 190-kDa protein corresponding to MRP1 in the lysates of Calu-1 cells (Fig. 1B), similar to COR-L23 cells, another positive control for MRP1 expression. MRPm6 antibody used in the Western blot studies was previously used to indicate MRP1 expression in bovine brain microvessel endothelial cells (BBMEC) and a human pancreatic adenocarcinoma cell line (PANC1) (Miller et al., 1996; Huai-Yun et al., 1998; Aukunuru et al., 2001). In addition, MRPm6 was reported not to cross-react with other members of the MRP family including MRP2–MRP6 (Scheffer et al., 2000).

Cellular accumulation and the efflux of fluorescein provided a functional evidence for the MRP activity in the Calu-1 cells. Fluorescein, an anionic molecule at physiologic pH, is a likely substrate for the MRP-like anionic efflux pumps (Huai-Yun et al., 1998; Miller et al., 1999; Sun et al., 2001; Aukunuru et al., 2001). We observed an increase in the fluorescein accumulation (Fig. 2A) and a decrease in the fluorescein efflux (Fig. 2B) in the presence of the MRP inhibitors, consistent with a role for MRP in restricting fluorescein accumulation. Because fluorescein accumulation was reduced following the transfection of MRP1 in the MDCKII cells (Sun et al., 2001), our results are consistent with the presence of an MRP1-like activity in Calu-1 cells. However, because of the possible export of fluorescein by MRPs other than MRP1, the involvement of other MRPs in the observed activity cannot be ruled out.

Since corticosteroids are used on a long-term basis, we exposed budesonide to Calu-1 cells for 14 days in order to understand the effect of prolonged corticosteroid exposure on MRP1 expression and activity. It is evident from Fig. 3 that this subchronic budesonide treatment decreased the MRP1 mRNA and MRP1 protein expression in Calu-1 cells. This effect occurred as early as 1 week (Fig. 4). Consistent with this reduced MRP1 expression, budesonide increased the intracellular accumulation of fluorescein and reduced fluorescein efflux (Fig. 5). Thus, this study demonstrated for the first time that budesonide inhibits the expression and activity of MRP1. Previous studies have demonstrated that the inhibition of the MRP-mediated efflux may involve a depletion of cellular glutathione (GSH) content, direct interaction of the drug and/or drug-metabolites with the efflux pump, possibly via competition or direct interaction for its drug-binding sites, and/or any alteration in the expression of the transporter (Lautier et al., 1996). Our results are consistent with Fardel et al. (1993), who reported that treatment with dexamethasone (10 μM) decreased the expression of another drug efflux pump, P-glycoprotein (P-gp), thereby reducing the efflux of doxorubicin, a putative P-gp substrate. While previous reports suggested the modulation of MRP2 expression by dexamethasone (Demeule et al., 1999; Courtois et al., 1999), this is the first report on the effect of any corticosteroid on MRP1 expression. Thus, budesonide treatment reduced MRP1 expression in Calu-1 cells.

The results of our study are relevant to the chemotherapy of lung tumors since the overexpression of the MRP gene is linked to the prognosis of non-small-cell lung cancer and to the loss of chemosensitivity in these subjects (Oshika et al., 1999). In order to determine whether budesonide enhances the chemosensitivity of anticancer drugs, we determined the cytotoxic effects of vincristine with and without budesonide in COR-L23R cells. Vincristine is a putative MRP1 substrate, which has been widely utilized in performing chemosensitizing assays (Mao et al., 2000; Narasaki et al., 1997; Nakano et al., 1998). The results of our study indicated that budesonide, at concentrations devoid of cytotoxicity (Table 1a), enhanced the sensitivity of COR-L23R to vincristine (Fig. 6), possibly by inhibiting MRP1 expression. Because vincristine interacts with both MRP1 and MRP2, we cannot rule out a role for MRP2 in the observed results (Mao et al., 2000; Keppler et al., 1999). However, as the vincristine studies were performed in MRP1 overexpressing COR-L23R cells, we believe that our results are consistent with the inhibition of the MRP1-mediated efflux of vincristine.

The ability of budesonide to reduce MRP1 expression, as well as its ability to reduce the expression and secretion of the vascular endothelial growth factor (VEGF) (Bandi and Kompella, 2001), an angiogenic factor implicated in the cancer progression, are likely explanations for the usefulness of budesonide in cancer treatment (Wattenberg et al., 1997). As the current studies were done in a tumor cell line (Calu-1), further studies in whole animal models are required to establish the relevance of these results under in vivo conditions.

Previous reports indicated that the leukotriene C_4 excretion was reduced in an MRP-knock out mice (Wijnholds et al., 1997) and that MRP1 can export leukotriene C_4, suggest-

ing that MRP1 is likely to play an important role in the inflammatory disorders. Because the inhibitory effect of budesonide on leukotriene C_4 is well documented (Wang et al., 1997), we speculate that the inhibition of MRP1 activity is one possible explanation for the anti-inflammatory effect of budesonide. As the respiratory epithelium has high MRP1 expression (Flens et al., 1996; Wright et al., 1998) and because budesonide is widely used in the treatment of the respiratory inflammatory disorders such as asthma and nasal polyps (Szefler, 2001; Jankowski et al., 2001), it remains to be investigated whether the effects of budesonide contribute to its clinical effects.

In conclusion, our results suggest that MRP1 is functionally and biochemically present in the airway (Calu-1) epithelial cells. In addition, budesonide treatment inhibits the MRP activity, MRP1 mRNA, and MRP1 protein expression in the Calu-1 cells. Thus, budesonide may likely be a useful adjuvant to overcome the multidrug resistance in chemotherapy. This, in conjunction with its ability to inhibit VEGF expression and secretion, makes budesonide a potential therapeutic agent for cancer treatment.

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