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Research Paper

Formulation and Evaluation of Aerosolized Celecoxib for the Treatment of Lung Cancer

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Purpose. We examined the effect of aerosolized celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, on the *in vitro* cytotoxicity and apoptotic response of docetaxel against the human lung carcinoma cell lines A549 and H460.

Methods. A metered dose inhaler (MDI) formulation of celecoxib was prepared and evaluated for its medication delivery and aerodynamic properties. The *in vitro* cytotoxicity of the aerosolized celecoxib-MDI alone or in combination with docetaxel was assessed using a six-stage viable impactor by a previously established method. The induction of apoptosis was evaluated by morphologic examination (acridine orange and Hoechst staining) and DNA fragmentation. Furthermore, in an attempt to identify molecular targets involved in the anticancer mechanisms of celecoxib and docetaxel, we examined their effect on the expression of an array of markers involved in the COX-2 dependent and independent pathways.

Results. The celecoxib-MDI had a medication delivery of 231.3 μg/shot, mass median aerodynamic diameter (MMAD) of 1.4 μm (GSD = 1.9), and respirable fraction of 50.7%. The celecoxib-MDI (2 shots) in combination with docetaxel had cell kills as high as 81.3% and 67.7% in A549 and H460 cells, respectively. Hoechst and acridine orange staining showed an enhanced induction of apoptosis in A549 and H460 cells exposed to aerosolized celecoxib with docetaxel, which was further confirmed by DNA fragmentation. Western blot analysis showed a significant reduction in $cPLA_2$ expression in both A549 and H460 cells treated with the combination of celecoxib with docetaxel. In the COX-2 independent pathway, there was a significant increase in the expression of PPAR-γ and p53, whereas pro-caspase-3 expression was significantly decreased, which may contribute to the enhanced apoptotic response observed with the combination treatment.

Conclusions. Our results suggest that aerosolized celecoxib significantly enhances the *in vitro* cytotoxicity and apoptotic response of docetaxel against A549 and H460 cells, and this enhanced activity is mediated via alterations in expression of various molecular targets involved in apoptosis.

KEY WORDS: apoptosis; celecoxib; cytotoxicity; docetaxel; inhalation.

INTRODUCTION

During the past several years, the outcome for the treatment of primary and metastatic lung cancer in humans has not improved dramatically even with the availability of new chemotherapeutic agents. The 5-year survival rate is less than 15%, and lung cancer still has the highest mortality rate among cancer-related deaths (1,2). Non-small cell lung cancer (NSCLC) exhibiting adenocarcinoma histology represents the majority of patient lung cancers (3). As conventional treatments for lung cancer have had limited success, alternative strategies are being evaluated. It is central to the treatment of lung cancer that the chemotherapeutic agent be administered locoregionally and continuously in high enough concentrations in order to eradicate tumors more effectively with minimal systemic side effects. Therefore, inhalation drug delivery represents a potential drug delivery route for treatment of lung cancer. Most recently, the nebulized liposomal formulation of 9-nitrocamptothecin has been evaluated clinically in patients with advanced pulmonary malignancies showing feasibility and safety (4). However, many drawbacks may be expected with pulmonary delivery of anticancer agents. The efficiencies of most inhalation drug delivery systems are very low, and it is generally accepted that less than 20% of an emitted aerosolized dose reaches the lower respiratory tract (5,6). Although many strides have been made in improving the respiratory delivery of drugs, it is unlikely that a system will ever deliver 100% of the emitted dose to the target site in the lungs. This creates a dilemma in the delivery of anticancer agents with narrow therapeutic indexes, as there is nonspecific deposition. Therefore, another aspect to consider in

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ABBREVIATIONS: COX-2, cyclooxygenase-2; $cPLA_2$, cytosolic phospholipase A_2; GSD, geometric standard deviation; HFA, hydrofluoroalkane; 5-LOX, 5-lipoxygenase; MDD, medication delivery device; MDI, metered dose inhaler; MMAD, mass median aerodynamic diameter; NSAID, nonsteroidal anti-inflammatory drug; NSCLC, non-small cell lung cancer; PGE_2, prostaglandin E_2; PPAR, peroxisome proliferator-activated receptor; p53, tumor suppressor protein.

evaluating inhalation drug delivery for the treatment of lung cancer is the delivery of drugs with a higher therapeutic index. The increasing knowledge of cancer biology has led to new potentially targeted therapies that interfere with essential pathways in carcinogenesis. Cyclooxygenase (COX)-2 has emerged as a novel therapeutic target for the prevention and treatment of various types of cancers.

Cyclooxygenases are the rate-limiting enzymes involved in the conversion of arachidonic acid to prostaglandins (PGs). COX exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues, whereas COX-2 is an inducible form that is regulated by a variety of factors, including cytokines, growth factors, and tumor promoters. COX-2 and its associated PGs have been shown to play a role in the modulation of cell growth and development of cancer (7). COX-2 is expressed constitutively in many human carcinomas, including colon, lung, breast, gastric, esophagus, prostate, head/neck, and bladder. In addition, high levels of COX-2 mRNA and protein expression have been correlated with poor prognosis in lung cancer patients (8).

COX-2 inhibitors have been found to reduce the tumor growth rate by themselves and exert potent synergistic cytotoxic effects with anticancer drugs in experimental lung cancer models. In addition, COX-2 inhibitors have been shown to exert antiproliferative and proapoptotic effects in a variety of cancer cell lines, including NSCLC cell lines (9,10). The mechanism by which these NSAIDs affect tumor growth and proliferation are still a subject of intense debate. Despite a lot of studies reporting COX-2 dependent and independent actions of COX-2 inhibitors, its mechanism of action has not yet been completely understood. In addition, the underlying mechanism for the synergistic cytotoxic effects of COX-2 inhibitors with anticancer drugs *in vitro* has not been clearly defined.

Previous investigations in our lab have shown the potential of inhalation delivery of COX-2 inhibitors for the enhancement of the activity of cytotoxic drugs used in the treatment of lung cancer. For this purpose, aerosolized nimesulide in combination with doxorubicin was used as a model for proof-of-principal of this concept (11). These preliminary findings were very promising and prompted additional studies with a more selective drug for the treatment of NSCLC. Celecoxib is a highly selective inhibitor of COX-2 and is FDA approved for the treatment of adenomatous polyps in familial adenomatous polyposis (FAP), a genetic predisposition to colon cancer. In addition, docetaxel is a drug of choice for the treatment of NSCLC, as it is indicated for the treatment of patients with locally advanced or metastatic NSCLC. Therefore in this study, we examined the effect of celecoxib on the *in vitro* cytotoxicity of docetaxel against NSCLC cell lines A549 and H460. In addition, we evaluated the *in vitro* potentiation of the cytotoxicity of docetaxel by aerosolized celecoxib against these NSCLC cell lines. Further, we attempted to determine the underlying mechanism responsible for the enhanced activity of the combination therapy of celecoxib with docetaxel.

MATERIALS AND METHODS

Materials

Celecoxib and docetaxel were provided as generous gifts from Pfizer (Skokie, IL, USA) and Aventis (Collegeville, PA, USA), respectively. The human NSCLC cell lines, A549 and H460, were obtained from American Type Culture Collection (Rockville, MD, USA). A549 cells were grown in F12K medium supplemented with 10% fetal bovine serum. H460 cells were grown in RPMI medium supplemented with 10% fetal bovine serum. All tissue culture media contained penicillin (5000 U/ml), streptomycin (0.1 mg/ml), and neomycin (0.2 mg/ml). These cells were grown in standard tissue culture conditions, passaged at 80–90% confluence, and cytotoxicity experiments were performed between 2 and 20 passages. All tissue culture chemicals and anti-β-actin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Acridine Orange Stain Dropper solution and bisBenzimide Hoechst 33258 Trihydrochloride stain were obtained from Becton Dickinson and Company (Sparks, MD, USA) and Sigma, respectively. HFA 134a and 227 were obtained from Du Pont (Ingleside, TX, USA) and Solvay Fluorides Inc. (Hanover, Germany), respectively. The six-stage viable impactor and eight-stage Andersen cascade impactor, Mark II, were obtained from Graseby Andersen (Smyrna, GA, USA). Continuous and noncontinuous valves were kindly provided by 3M Pharmaceuticals (St. Paul, MN, USA). A PGE_2 high sensitivity colorimetric enzyme immunoassay kit was purchased from Assay Designs (Ann Arbor, MI, USA). Antibodies against COX-2, mPGE synthase-1, 5-LOX, and PPAR-γ were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The antibodies $cPLA_2$, pro-caspase-3, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, and bovine anti-goat IgG-HRP were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-poly-ADP-ribose polymerase (PARP) was purchased from ABR Bioreagents (Golden, CO, USA).

Solubility of Celecoxib in HFA 134a and 227

The solubility of celecoxib, at various concentrations of ethanol, was determined in the HFA propellants (134a and 227) after equilibration for 48 h. Excess celecoxib was added to a clean 15 ml glass vial containing 0, 0.4, 0.8, or 1.2 g of ethyl alcohol (200 proof) and crimped with a continuous valve. HFA 134a or 227 was then added from a pressure burette attached to a filling machine to bring the final weight in each vial up to 10 g. The vials were then placed on a platform shaker (Innova 2000, New Brunswick Scientific, Edison, NJ, USA) at 150 rpm and allowed to shake for 48 h. The solubility was determined by transferring the contents to a chilled receiving vial via an assembly consisting of two transfer buttons connected to a 0.45 μm Acrodisc filter. The weight of the transferred portion in the receiving vial was recorded. Then the receiving vial was placed in dry ice (for about 30 min), the valve was decrimped, and the contents were poured into a clean, pre-chilled, volumetric flask through a glass funnel. After allowing time for the propellant to evaporate, the vial and the valve were rinsed and the appropriate amount of methanol was added to the volumetric flask. The amount of celecoxib in methanol was then determined by using a spectrophotometer (Beckman DU 640) at a wavelength of 250 nm, and the solubility of celecoxib in the propellant system was calculated as percent weight.

Formulation of Celecoxib-MDI

Forty-five milligrams of celecoxib was placed in a clean 15 ml glass vial containing 1.0 g of ethyl alcohol (200 proof).

The vial was immediately crimped with a continuous valve followed by the addition of HFA-134a as described above. The vial was then placed on a platform shaker at 150 rpm and allowed to shake overnight. The continuous valve was then replaced with a 50 μl noncontinuous valve.

Medication Delivery (Ex-actuator Dose) of Celecoxib-MDI

The MDI formulation was primed by firing five shots into waste. The formulation was then fired once into the medication delivery device (MDD) with a glass wool filter under a flow rate of 30 L/min. The MDD chamber was then diluted with 25 ml of methanol and assayed with a spectrophotometer as previously described. The formulation was tested at least three times.

Aerodynamic Particle Size Distribution

An eight-stage Andersen cascade impactor, Mark II, was used to assess the aerodynamic size distribution of the celecoxib-MDI. The formulation was primed by firing five shots into waste. Then five shots (at 10 s intervals) were fired into the cascade impactor under a flow rate of 28.3 L/min. The deposited celecoxib was determined from the actuator, throat, jet stage, impactor stages 0–7 and filter by transferring each component to individual polyethylene bags and rinsing with appropriate volume of methanol. Each individual plate was coated with 10% Pluronic L10 (BASF Corporation, Parsippany, NJ, USA) in ethanol solution (to prevent particle bounce). The samples were analyzed on a spectrophotometer at a wavelength of 250 nm. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were obtained, based on impaction data, using established software in our laboratory. Other parameters, such as percent throat deposition, respirable mass, and respirable fraction were calculated based on the known amount of drug deposited on the various components. A cut off diameter of less than 4.7 μm was used to assess the respirable mass and fraction. Impaction experiments were conducted at least three times.

In Vitro Cytotoxicity of Celecoxib Alone and in Combination with Docetaxel Against Various Lung Cancer Cell Lines

The NSCLC cell lines (A549 and H460) and a small cell lung cancer (SCLC) cell line (SHP 77) were seeded at a density of 10,000 and 20,000 cells per well, respectively, in a 96-well plate. The cells were allowed to incubate overnight (12 h) and subsequently treated with various concentrations of celecoxib alone (2.5–80 μg/ml), docetaxel alone (0.01–1 μg/ml), and docetaxel (0.0001–0.1 μg/ml) combined with a fixed nontoxic concentration of celecoxib. For the combination treatments, celecoxib was used at 10 μg/ml in the NSCLC cells and at 5 μg/ml in the SCLC cell line. The cells were incubated for 72 h after treatment, and the cytotoxicity was assayed by crystal violet dye uptake assay (by measuring the absorbance at 540 nm) for the NSCLC cells and CellTiter 96 AQ_{ueous}One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) for the SCLC cell line.

In Vitro Cytotoxicity of Aerosolized Celecoxib-MDI Alone and Its Combination with Docetaxel Against A549 and H460 Cells

A six-stage viable impactor was used to assess the *in vitro* cytotoxicity of aerosolized celecoxib, alone or in combination with a known concentration of docetaxel. A549 or H460 cells (1 million in 20 ml of medium per Petri plate) were plated in Petri plates and placed on stages 3, 4, 5, and 6 of the viable impactor. The cells were then exposed to the celecoxib-MDI for two shots. After the exposure, the Petri plates were taken from the impactor, covered with sterile aluminum lids provided with the Petri plate (Graseby Andersen, Smyrna, GA, USA) and incubated at 37°C for 72 h. These operations (plating of cells in glass Petri plates, assembly of Petri plates on the various stages of viable impactor, exposure of cells to the aerosolized celecoxib, removal of Petri plates from the impactor and covering with the lids) were performed in a biological safety cabinet (class II, type A/B3, NuAire, Inc., Plymouth, MN, USA). The Petri plate along with its lid assembly is similar to that of a standard tissue culture well assembly, and as all the operations were performed under biological safety cabinet using established tissue culture precautions, sterility can be maintained throughout the incubation period. At the end of incubation, the medium in the Petri plate was discarded. The cells were rinsed three times with sterile PBS and detached by adding trypsin. The cells were spun down with a centrifuge and resuspended in 5 ml of medium for control where there were more viable cells and 1 ml for the treatments with less viable cells. The viable cells were then counted with a hemocytometer using trypan blue solution (0.4%). Untreated cells were used as control. The same procedure described above was used to assess the effect of combination therapy of celecoxib-MDI in conjunction with docetaxel, except a fixed concentration of docetaxel (0.001 or 0.0005 μg/ml) was added to cells in the Petri plate. To determine the aerosolized dose of the celecoxib-MDI in the six-stage viable impactor, 20 ml of 50% PEG 400 solution was used as the collection medium in Petri plates in place of the tissue culture medium, and 5 shots were fired (at 10 s intervals) under a flow rate of 28.3 L/min. PEG 400 was used as the collection medium, as celecoxib was freely soluble in this medium. The concentration of celecoxib was determined by spectrophotometer, and the corresponding dose was calculated.

Apoptosis Determination

DNA Fragmentation

One million A549 or H460 cells were plated in 10 ml of medium in 25 cm^2 flasks and incubated overnight. Subsequently, the cells were treated with celecoxib (10 μg/ml), docetaxel (0.01 μg/ml), and celecoxib (10 μg/ml) with docetaxel (0.01 μg/ml) for 24 h. Untreated cells were used as control. About 2×10^6 cells (both viable and nonviable) were used for the isolation of DNA. Soluble DNA was extracted from the cells using a phenol/chloroform extraction method. Briefly, cells were pelleted by centrifugation and resuspended in 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 25 mM EDTA; 0.5% SDS; 0.1 mg/ml Proteinase K) and allowed to incubate at 37°C for 4 h. Genomic DNA was ex-

tracted with phenol-choroform-isoamyl alocohol (25:24:1 at pH 8.0) and precipitated by adding sodium acetate (3M, pH 5.2) and ethanol. The precipitate was reconstituted and treated with RNase A (50 μg/ml). Aliquots of DNA (5 μg) were electrophoresed on a 1.6% agarose gel impregnated with ethidium bromide (0.1 μg/ml). Gels were then photographed under UV illumination using Polaroid 667 film.

Determination of Apoptosis Induced by the Aerosolized Celecoxib in Combination with Docetaxel by Acridine Orange and Hoechst Staining

A549 or H460 cells (1 million in 20 ml of medium with or without 0.005 μg/ml docetaxel) were exposed to aerosolized celecoxib on the fifth stage of the viable impactor, in the same way as described above for the *in vitro* cytotoxicity of aerosolized celecoxib. After exposure of the cells to the aerosolized celecoxib, the Petri plate was incubated at 37°C for 15 min to provide sufficient time for the aerosolized drug to uniformly mix with the medium. Subsequently, the cell suspension was aspirated from the Petri plate, and 1 ml of the cell suspension was then plated into Nunc Lab-Tek chamber slide, which was subsequently incubated for 72 h. The cells were then fixed with 0.25% glutaraldehyde, washed twice with PBS, stained with acridine orange or Hoechst, and finally observed with a fluorescent microscope (Olympus BX40, Olympus Optical Co., Ltd. Tokyo, Japan). The morphologic criteria used to detect apoptotic cells were (a) cytoplasmic and nuclear shrinkage, (b) chromatin condensation, (c) cytoplasmic blebbing and the presence of apoptotic bodies.

Western Blot Analysis

A549 and H460 cells were treated with celecoxib (10 μg/ml) alone, docetaxel (0.01 μg/ml) alone, and their combination. The control cells were untreated. The cells were then lysed in RIPA B lysis buffer and centrifuged to obtain the cell lysate. Equal protein concentrations of the lysates from control and treatment were then added to SDS-PAGE loading buffer with 5% 2-mercaptoethanol, heated for 5 min at 100°C, and loaded on 8%, 10%, or 15% gels. Separated proteins were electroblotted to nitrocellulose membranes (Osmonics Inc., Westborough, MA, USA) at 100 V for 1 h. After that the membranes were incubated for 2 h in a blocking solution containing 5% nonfat skimmed milk in TBS-T and then incubated with COX-2, $cPLA_2$, mPGE synthase-1, 5-LOX, PPAR γ, PARP, pro-caspase-3, p53 or β-actin antibodies. The blot was then washed and incubated with the respective secondary antibodies. Signals were detected by chemiluminescent Substrate (Pierce, Rockford, IL, USA) on Kodak Biomax ML imaging film.

Prostanglandin E_2 Measurement

One million A549 or H460 cells were plated in 6-well plates and incubated overnight. Subsequently, cells were treated with celecoxib (10.0 μg/ml) alone, docetaxel (0.01 μg/ml) alone, and their combination for 24 h. One hour prior to harvesting, cells were treated with 50 μM of arachidonic acid (Cayman Chemicals). The culture medium was removed, and samples were centrifuged to remove any cellular debris. PGE_2 production was measured by an enzyme immunoassay using PGE_2 High Sensitivity Correlate-EIA Kit (Assay Design, Inc., Ann Arbor, MI, USA) as per manufacture's instructions.

Statistical Analysis

One-way ANOVA followed by Tukey's multiple comparison test was performed to determine the significance of difference in the *in vitro* cytotoxicity of different treatments. The statistical analysis was performed using GraphPad PRISM version 2.0 software (San Diego, CA, USA).

RESULTS

Solubility of Celecoxib in HFA 134a and 227

Figure 1 shows the structures of celecoxib as well as docetaxel, while Fig. 2 shows the solubility profiles of celecoxib in HFA propellants containing various concentrations (0–12% w/w) of ethyl alcohol. Celecoxib had a solubility of 0.008 ± 0.0004 (%w/w) and 0.002 ± 0.0003 (%w/w) in HFA 134a and 227, respectively. The addition of ethanol increased the solubility of celecoxib at all of the concentrations evaluated in both HFA propellants. The highest solubility of celecoxib (0.70% w/w) was observed in HFA 134a with 12% ethyl alcohol.

Characterization of Various Celecoxib-MDI Formulations

Based on the solubility profiles, various celecoxib-MDI formulations were prepared and characterized for their aerodynamic properties. Table I summarizes the aerodynamic characteristics of the celecoxib-MDI formulations using com-

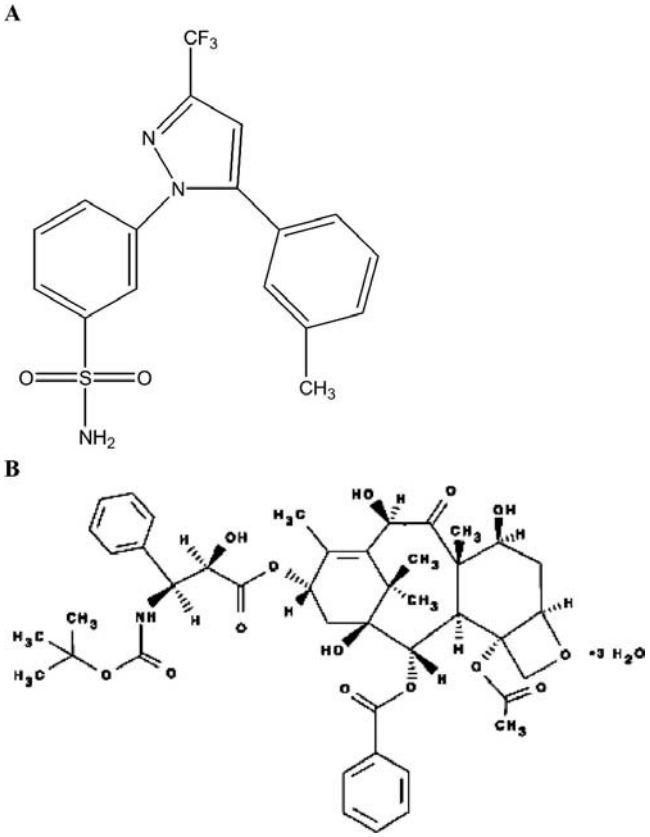

Fig. 1. Chemical structures of (A) celecoxib and (B) docetaxel.

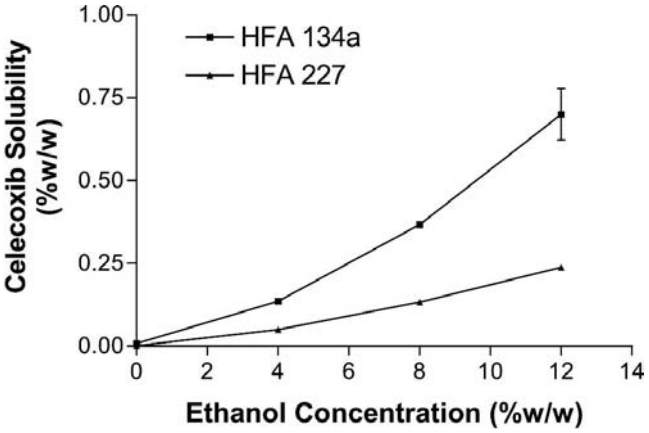

Fig. 2. Solubility of celecoxib in HFA propellants as a function of ethyl alcohol concentration. Each point represents mean ± SD (n = 3).

mercially available Qvar80 actuators. Formulations 1–3 were prepared using HFA 134a, 10% w/w ethanol, and varying concentrations of celecoxib (0.25–0.45% w/w). Increasing the drug concentration resulted in an increasing respirable mass, ranging from 71.6 to 117.3 μg/shot. Celecoxib showed a limited solubility in HFA 227 as a function of ethanol concentration. Therefore, formulation 4 was prepared in HFA 227 with 15% ethanol. This formulation had the highest MMAD (1.65 ± 0.5 μm) and the lowest respirable mass (44.8 ± 2.2 μg/shot). Based on this data, formulation 3 (0.45% w/w celecoxib, 10% w/w ethanol in HFA 134a) was used as a model MDI formulation for further studies. The deposition pattern of this celecoxib-MDI obtained from the Andersen cascade impactor is illustrated in Fig. 3.

In Vitro Cytotoxicity of Celecoxib Alone and in Combination with Docetaxel Against Various Lung Cancer Cell Lines

Table II shows the *in vitro* cytotoxicity data for celecoxib, docetaxel, and the combination of celecoxib with docetaxel against the various lung cancer cell lines. The combination of celecoxib with docetaxel reduced the IC_{50} values for docetaxel by 45–85% in the various lung cancer cell lines.

In Vitro Cytotoxicity of Aerosolized Celecoxib-MDI Formulation Alone and as a Combination Therapy with Docetaxel Against A549 and H460 Cells

In vitro cytotoxicity of the aerosolized celecoxib formulation alone and in combination with docetaxel against (A) A549 and (B) H460 cells kept on stages 3–6 of the viable impactor was performed (Fig. 4). In A549 cells, the celecoxib-MDI (2 shots) had a cell kill of 42.0% on stage 5. Docetaxel, when used alone at a concentration of 0.001 μg/ml, had a cell kill of 52.5%. However, when A549 cells were treated with 0.001 μg/ml docetaxel and exposed to 2 shots of the celecoxib-MDI, a cell kill as high of 81.3% was observed on the fifth stage of the viable impactor. In H460 cells, the celecoxib-MDI (2 shots) had a cell kill of 25.9% on stage 5. Docetaxel, when used alone at a concentration of 0.0005 μg/ml, had a cell kill of 46.0%. However, when A549 cells were treated with 0.0005 μg/ml docetaxel and exposed to 2 shots of the celecoxib-MDI, a cell kill as high of 67.7% was observed on the fifth stage of the viable impactor. Figure 5 shows the amount of aerosolized celecoxib deposited on each stage was quantified following 5 shots of the celecoxib-MDI formulation using 20 ml of 50% PEG 400 solution in place of tissue culture medium. The experimentally determined dose of celecoxib deposited on stages 5 and 6 of the viable impactor was 41.9 and 23.8 μg/shot, respectively. This corresponds to concentrations of 4.2 and 2.4 μg/ml, after administering 2 shots for stages 5 and 6, respectively, in 20 ml of collection medium used in the Petri plates.

Apoptosis Determination

The induction of apoptosis in the combination treatment of aerosolized celecoxib with docetaxel in A549 and H460 cells was evaluated by acridine orange and Hoechst staining. In A549 cells, acridine orange staining showed that there was no appreciable apoptosis in control and aerosolized celecoxib (2 shots) treated cells, and 19% apoptotic cells in the docetaxel (0.005 μg/ml) treated cells (Fig. 6 A, B, and C). However, the cells treated with combination of aerosolized celecoxib (2 shots) and docetaxel (0.005 μg/ml) showed an enhanced apoptotic response (61%) (Fig. 6D). The Hoechst staining confirmed this enhanced induction of apoptosis in the combination treated cells (Fig. 6D). The same trends were found with the H460 cells, as the combination treated cells showed the highest percentage of apoptotic cells (Fig. 7).

The enhanced apoptotic response of celecoxib with docetaxel was also confirmed by DNA degradation as shown in Fig. 8 in (A) A549 and (B) H460 cells. The combination of celecoxib (10 μg/ml) with docetaxel (0.005 μg/ml) had the most intense smear patterns indicating an enhanced apoptotic response, as compared to the control, celecoxib (10 μg/ml), and docetaxel (0.005 μg/ml) treatments alone in both A549 and H460 cells.

Table I. Aerodynamic Characteristics of Various Celecoxib-MDI Formulations

Number	Formulations	MMAD (μm)	GSD	Medication delivery (μg/shot)	Respirable mass (μg/shot)	Respirable fraction (%)	Throat deposition (%)
1	CXB 0.25%, 10% EtOH/134a	1.26 (0.05)	1.9 (0.13)	134.4 (1.7)	71.6 (0.1)	53.3 (0.6)	41.4 (1.4)
2	CXB 0.35%, 10% EtOH/134a	1.27 (0.02)	1.9 (0.08)	180.2 (5.4)	91.3 (3.4)	50.7 (1.3)	45.1 (1.2)
3	CXB 0.45%, 10% EtOH/134a	1.43 (0.08)	1.9 (0.03)	231.3 (9.5)	117.3 (6.6)	50.7 (2.4)	44.9 (2.3)
4	CXB 0.25%, 15% EtOH/227	1.67 (0.05)	2.4 (0.2)	129.5 (3.5)	44.8 (2.2)	34.6 (2.6)	56.6 (2.4)

Celecoxib impaction data on various stages of cascade impaction was used to calculate mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), throat deposition, respirable mass, and respirable fraction values as described in the "Materials and Methods" section. Data represented as mean ± SD (n = 3).

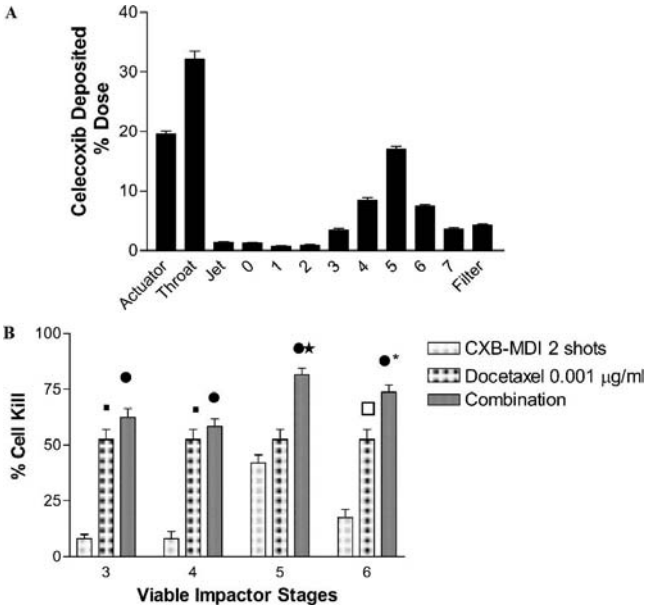

Fig. 3. Andersen cascade impactor (A) deposition profile of the model celecoxib-MDI (0.45% celecoxib, 10% w/w ethanol in HFA 134a). (B) Percentage of cell kill at different stages of the impactor. Data expressed as the percentage of the total drug deposited on all stages of the impactor including actuator and throat and represented as mean ± SD (n = 3).

Prostaglandin E_2 Measurements

The production of PGE_2 after various treatments was determined (Fig. 9 A and B). PGE_2 production, in cells treated with the combination of celecoxib (10 μg/ml) and docetaxel (0.01 μg/ml), was significantly reduced by 95.3% and 95.9% in A549 and H460 cell, respectively ($p < 0.001$ as compared to control cells). This same trend was also noted in the cells treated with celecoxib (10 μg/ml) alone, indicating that the celecoxib dose was sufficient in inhibiting the COX-2 mediated synthesis of PGE_2 from the exogenous arachidonic acid. Docetaxel (0.01 μg/ml) alone had no effect in reducing the PGE_2 production.

Mechanisms of Celecoxib and Docetaxel Action on the Inhibition of Cellular Proliferation

Western blot analysis was used to assess the expression of various proteins that will aid in elucidating mechanisms involved in the increased cytotoxic and apoptotic response seen in the combination treatment of celecoxib with docetaxel. Figure 10 shows the effect of various treatments (celecoxib alone, docetaxel alone, and celecoxib with docetaxel combination) on molecular targets involved in the arachidonic acid pathway. In A549 cells, celecoxib alone or celecoxib with docetaxel combination has no significant effect on COX-2 expression as compared to the control cells. On the other hand, celecoxib alone has significantly reduced COX-2 protein, while the combination of celecoxib with docetaxel treatment resulted in a significant increase in COX-2 expression in H460 cells. However, docetaxel alone produced an enhanced COX-2 protein expression in both A549 and H460 cells. There was a significant decrease in $cPLA_2$ and 5-LOX expression ($p < 0.001$ and $p < 0.05$, respectively) by the combination treatment in A549 cells, as compared to control cells. Similarly, the combination of celecoxib with docetaxel resulted in a significant decrease in the expression of $cPLA_2$ and 5-LOX ($p < 0.001$ and $p < 0.05$, respectively) in H460 as compared to the untreated control cells. We observed no significant difference in the expression of mPGE synthase-1 among the different treatments in A549 cells, where as its expression was significantly increased in both docetaxel alone and celecoxib with docetaxel treatments in H460 cells. The differences in the expression levels of various enzymes in the arachidonic acid pathway in A549 and H460 cells may be differences in COX-2 expression levels in A549 and H460 cells, as A549 cells are known to express significant COX-2 protein, whereas its expression in H460 cells is moderate. However, further studies are needed to support these findings.

Figure 11 shows Western blot analysis of alternative molecular targets involved in apoptosis and cell death. In A549 cells, protein expression of PPAR-γ, PARP, and p53 were significantly increased in the celecoxib and docetaxel treatment ($p < 0.001$) as compared to the controls. The significant increase in PARP expression (indicating PARP cleavage) directly correlates with a significant decrease in the pro-caspase protein expression in the combination treatment ($p < 0.001$) indicating an enhanced induction of apoptosis through the caspase pathway. Similar protein expression patterns were also noted in H460 cells.

DISCUSSION

Aerosol drug delivery in lung cancer patients holds promise as a means to obtain an enhanced therapeutic effect and to minimize the systemic toxicity of cytotoxic drugs. In the recent years, this approach has been pursued actively to

Table II. Effect of Celecoxib in the *in Vitro* Cytotoxicity of Docetaxel Against Various Lung Cancer Cell Lines[a]

Cell line	IC_{50} with celecoxib alone (μg/ml)[b]	IC_{50} with docetaxel alone (μg/ml)[c]	IC_{50} with celecoxib and docetaxel (μg/ml)[d]	% Reduction in IC_{50} of docetaxel
A549	19.10 ± 1.32	0.022 ± 0.003	0.0013 ± 0.002	45
H460	18.19 ± 0.72	0.014 ± 0.005	0.002 ± 0.002	85
SHP 77	10.20 ± 1.66	0.033 ± 0.002	0.0083 ± 0.001	75

[a] The human lung cancer cell lines were A549 (adenocarcinoma), large-cell carcinoma type H460 (large-cell carcinoma), and SHP 77 (undifferentiated large cell variant of small cell lung carcinoma).
[b] Celecoxib was employed in the concentration range of 2.5–80 μg/ml to determine the IC_{50} values.
[c] Docetaxel was employed in the concentration range of 0.01–1 μg/ml to determine the IC_{50} values.
[d] Docetaxel was employed in the concentration range of 0.0001–0.1 μg/ml against a fixed concentration of celecoxib 10 μg/ml. Data represented as +SD (n = 4).

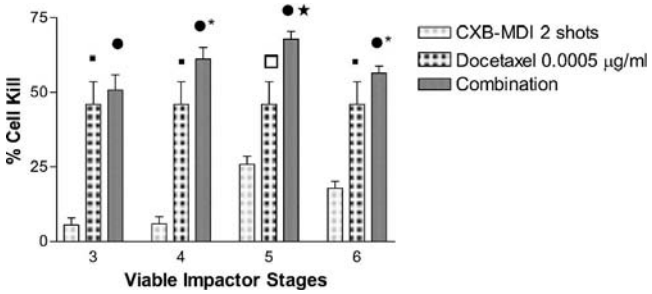

Fig. 4. *In vitro* cytotoxicity profiles of aerosolized celecoxib (MDI-2 shots) alone and in combination with docetaxel (0.001 or 0.0005 μg/ml) against (A) A549 and (B) H460 cells on the third, fourth, fifth, and sixth stages of the viable impactor. CXB: celecoxib. Data represented as mean ± SD (n = 4). Statistical significance of the difference in cytotoxicity: □, aerosolized celecoxib vs. docetaxel, $p < 0.01$; ■, aerosolized celecoxib vs. docetaxel, $p < 0.001$; ●, combination vs. aerosolized celecoxib, $p < 0.001$; ★, combination vs. docetaxel, $p < 0.01$; *, combination vs. docetaxel, $p < 0.005$.

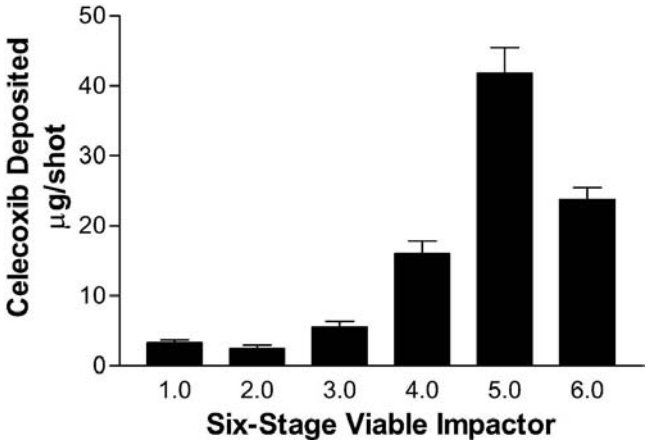

Fig. 5. Celecoxib deposited (μg/shot) on the different stages of the viable impactor. Data represented as mean ± SD (n = 3).

deliver various types of drugs to treat lung cancer in both preclinical and clinical studies. These include doxorubicin (D. White *et al., Proc Am Soc Clin Oncol.* Abstract 2739, 2001), paclitaxel (12), antitumor genes (13), camptothecins (14), vitamin E analogues (15), retinyl palmitate (16), and budesonide (17). We have previously demonstrated the feasibility and *in vitro* antitumor activity of aerosolized methotrexate (18) and docetaxel (Shaik *et al.,* AAPSPharm Sci Vol. 3, No.3, 2001). It has been found that the overexpression of COX-2 in lung tumors contributes to tumor progression and is associated with reduced patient survival. Thus COX-2 emerged as an important pharmacological target in the treatment and/or prevention of lung cancer. Further, nonselective COX inhibitor, sulindac sulfide has been shown to have synergistic cytotoxic effect in lung cancer cell lines (19). More recently, Hida *et al.* (10,20) have shown the potential of selective COX-2 inhibitors such as nimesulide and JTE-522 in enhancing the cytotoxicity of docetaxel and vinorelbine in lung cancer cell lines. Therefore, we hypothesized that inhaled delivery of selective COX-2 inhibitors may provide high local pulmonary concentrations and thus can be used to enhance the activity of cytotoxic drugs used in lung cancer. As inhaled nonselective COX inhibitor, indomethacin has been used in lung cancer patients to treat bronchorrhea, we reasoned that such an approach could be extended to more selective COX-2 inhibitors along with conventional administration of anticancer drugs, representing a more practical and viable approach, which could lead to an enhanced therapeutic effect in lung cancer patients. In this context, we have recently demonstrated the potential of an aerosolized selective COX-2 inhibitor, nimesulide in increasing the doxorubicin activity in A549 lung tumor cells. This paper describes our findings on the use of a highly selective COX-2 inhibitor, celecoxib delivered as an aerosol in combination with docetaxel in lung cancer cell lines. Further, the important molecular mechanisms mediating the enhanced *in vitro* antitumor effect have also been described.

Celecoxib, 4-[5-(4-methylphenyl)-3-trifluoro-methyl-1H-pyrazoyl-1-yl] benzene sulfonamide, is a 1,5-diaryl-substituted pyrazole with a pKa of 11.1 (Fig. 1). Celecoxib was the first specific inhibitor of COX-2 to be approved by the U.S. Food and Drug Administration (FDA) on December 31, 1998. Celecoxib is indicated (a) for relief of the signs and symptoms of osteoarthritits, (b) for relief of the signs and symptoms of rheumatoid arthritis, and (c) to reduce the number of adenomatous colorectal polyps in familial adenomatous polyposis (FAP) as an adjunct to usual care (400 mg twice a day). Celecoxib is available for human use as oral capsules (100 mg and 200 mg celecoxib). The mechanism of action of celecoxib is believed to be due to the inhibition of prostaglandin synthesis, primarily via inhibition of COX-2. It possesses a selectivity ratio for COX-1:COX-2 activity of >375. Peak plasma levels of celecoxib occur approximately 3 h after an oral dose. Celecoxib is highly protein bound within the clinical dose range with an apparent volume of distribution at steady state being 400 L suggesting extensive distribution to the tissues. This larger value when compared with NSAID may relate to the lipophilic nature of celecoxib or be reflective of a low bioavailability. Celecoxib metabolism is primarily mediated via cytochrome P450 2C9 with three metabolites, a primary alcohol, the corresponding carboxylic acid and its glucuronic acid conjugate, have been identified in human plasma. The elimination half-life ($t_{1/2}\beta$) is between 11.2 and 15.6 h.

We used the MDI approach to aerosolize celecoxib, as MDIs are the most widely used and have been found to be suitable for *in vitro* testing of aerosolized drugs as has been demonstrated in our previous studies (11,18). MDIs have been used to deliver all-*trans*-retinoic acid, which has shown promise in both chemoprevention and treatment of emphysema (21). We developed solution-based MDI formulations of celecoxib using HFA propellants (134a and 227). Celecoxib was found to have a higher solubility in the presence of 12% ethanol in HFA 134a (0.7% w/w) as compared to 0.2% solubility observed in HFA 227. As compared to our previous data on nimesulide (11), celecoxib showed more than a 5-fold increase in solubility in HFA 134a with ethanol. Thus, the differences in the solubility of different COX-2 inhibitors in HFA propellants may be attributed to the differences in the physiochemical properties of the drugs and their interactions with the propellants. Drug solubility in HFA 134a does not follow correlations similar to aqueous systems (22). The increased solubility of celecoxib in HFA 134a with ethanol enabled us to choose a model formulation (0.45% w/w celecoxib with 10% ethanol), which could deliver 230 μg of drug per

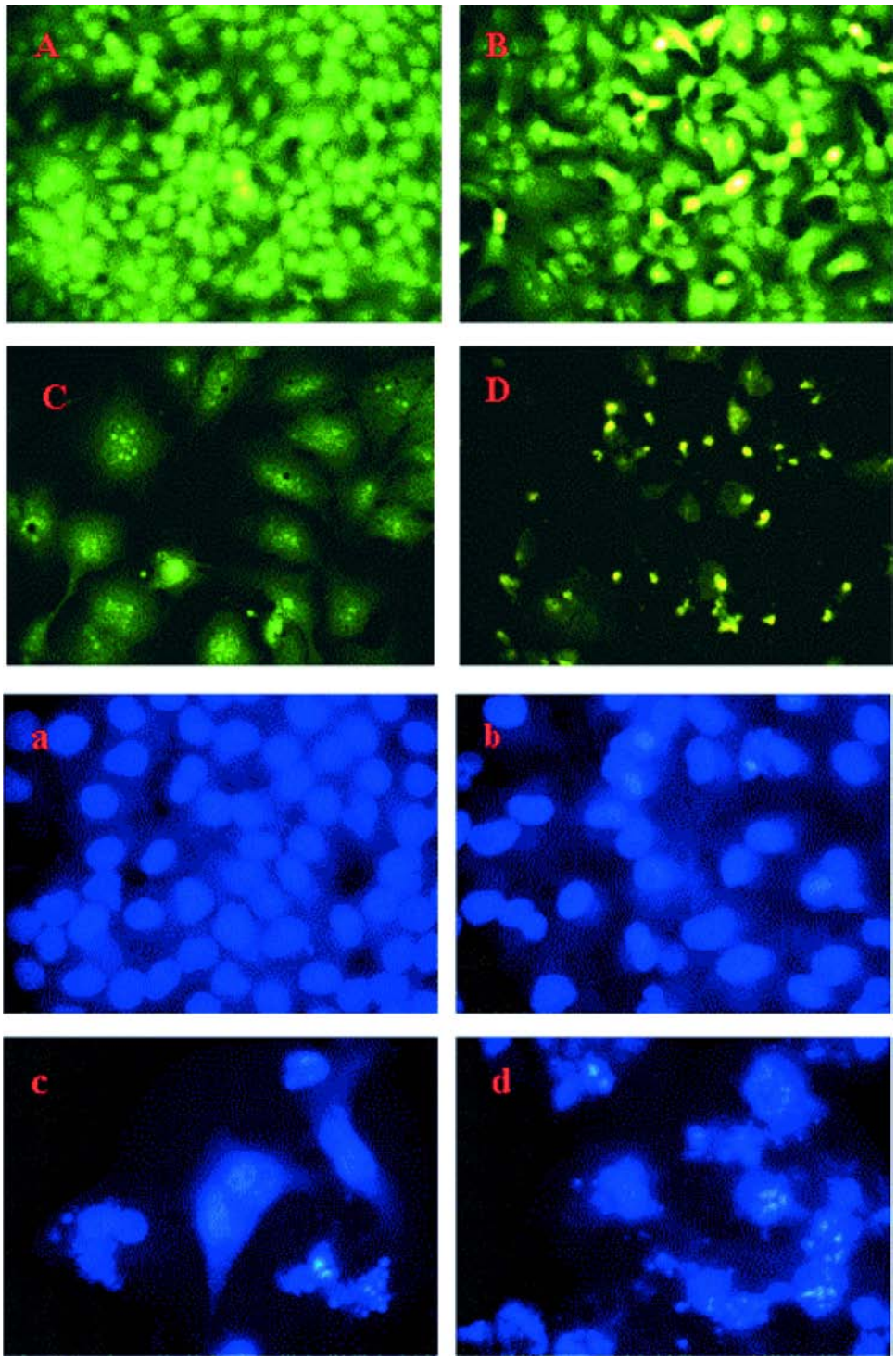

Fig. 6. Acridine orange (A, B, C, D) and Hoechst (a, b, c, d) staining of A549 cells: (A & a) untreated control cells; (B & b) cells exposed to aerosolized celecoxib (2 shots); (C & c) cells treated with docetaxel (0.005 μg/ml); (D & d) cells treated with aerosolized celecoxib (2 shots) + docetaxel (0.005 μg/ml). Original magnification ×40. The cells were exposed to aerosolized celecoxib on the fifth stage of the viable impactor, incubated for 15 min, and 1 ml of cell suspension was transferred to chamber slide. The slide was incubated for 72 h and stained with acridine orange or Hoechst as described in "Materials and Methods."

actuation with a high respirable fraction of 50.7% (Table I). Despite some differences in the drug and ethanol contents, between the model celecoxib-MDI formulation used in this study to our previous study using nimesulide, we observed similar aerodynamic characteristics for MMAD, throat deposition and respirable fraction values. These results indicate that consistently high respirable fraction (approximately 50%), low MMAD (<1.5 μm) and GSD (<3.0) values could

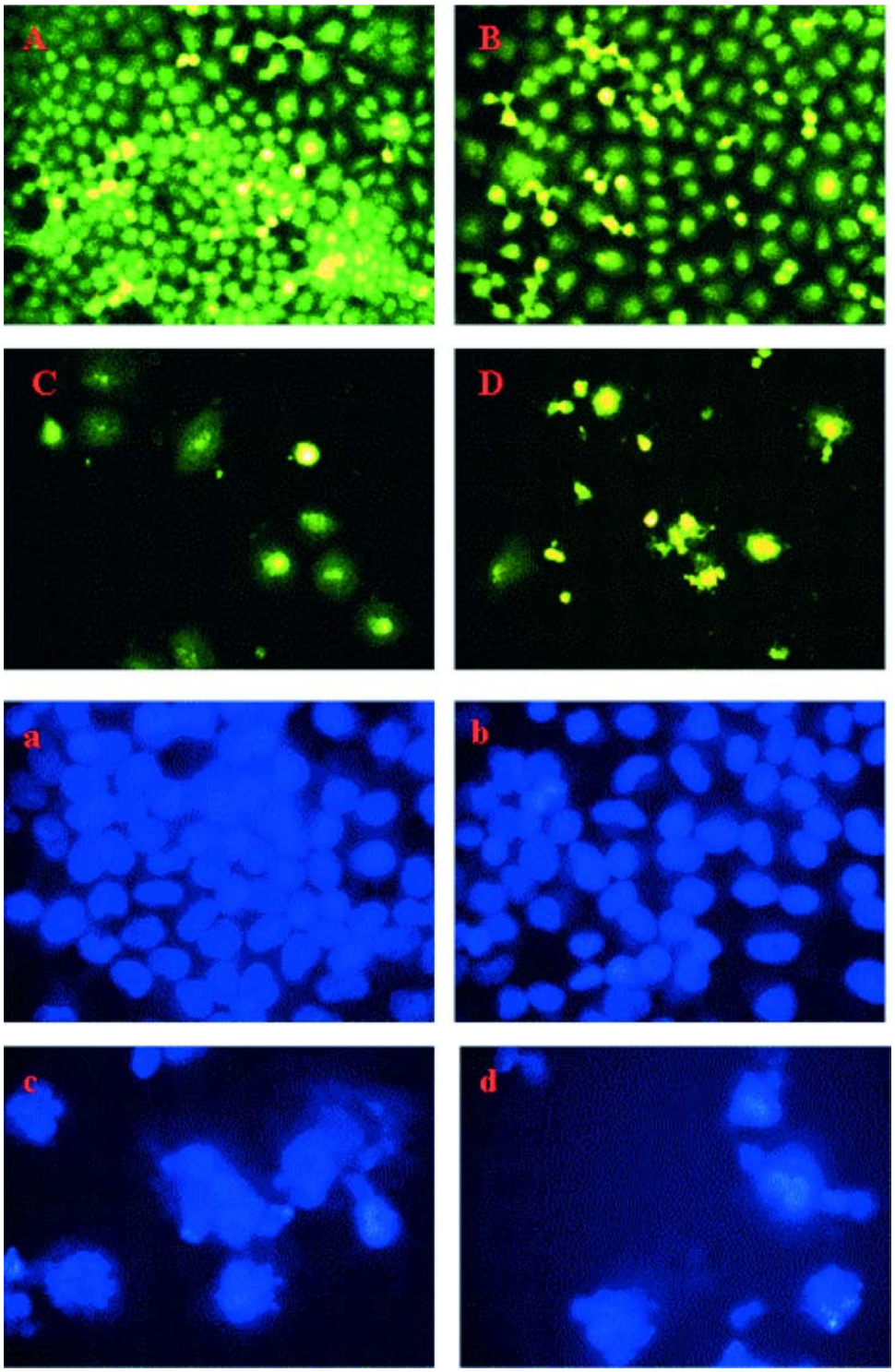

Fig. 7. Acridine orange (A, B, C, D) and Hoechst staining (a, b, c, d) of H460 cells: (A & a) untreated control cells; (B & b) cells exposed to aerosolized celecoxib (2 shots); (C & c) cells treated with docetaxel (0.005 μg/ml); (D & d) cells treated with aerosolized celecoxib (2 shots) + docetaxel (0.005 μg/ml). Original magnification ×40. The experimental conditions were similar to those described for Fig. 6.

be obtained with solution based HFA 134a formulations for COX-2 inhibitors, suggesting that approximately half of the actuated dose may be deposited in the bronchioalveolar regions of the lungs (23). The celecoxib-MDI formulation used in the current study has been found to be stable physically and chemically for one month at room temperature. Further, in this period, there was no significant alterations in the aerodynamic properties as mentioned in Table I.

Our studies showed that celecoxib significantly enhanced the *in vitro* cytotoxic activity of docetaxel in a panel of lung cancer cell lines (Table 2). Celecoxib, a more selective inhibitor of COX-2 than nimesulide, has a COX-1/COX-2 ratio of

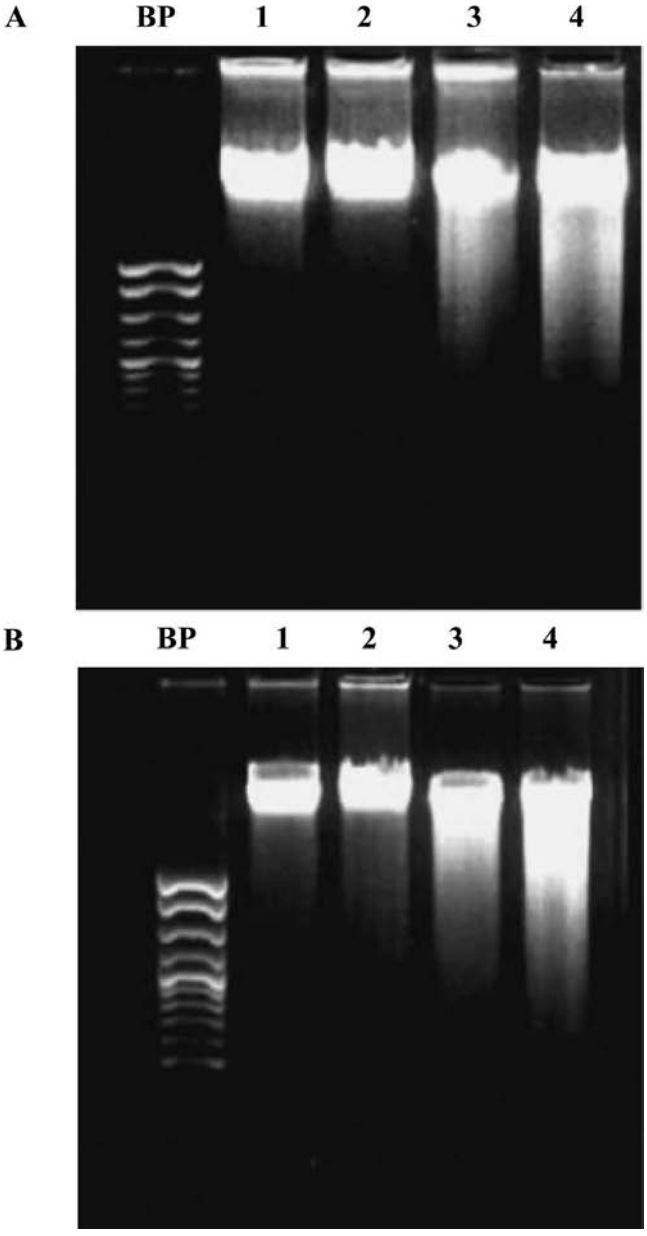

Fig. 8. Electrophesis of DNA from (A) A549 and (B) H460 cells after 48 h of exposure. Lane BP, 100 bp DNA marker; lane 1, untreated control cells; lane 2, celecoxib 10 μg/ml; lane 3, docetaxel 0.005 μg/ml; lane 4, celecoxib 10 μg/ml + docetaxel 0.005 μg/ml. Each well was loaded with 5 μg of DNA.

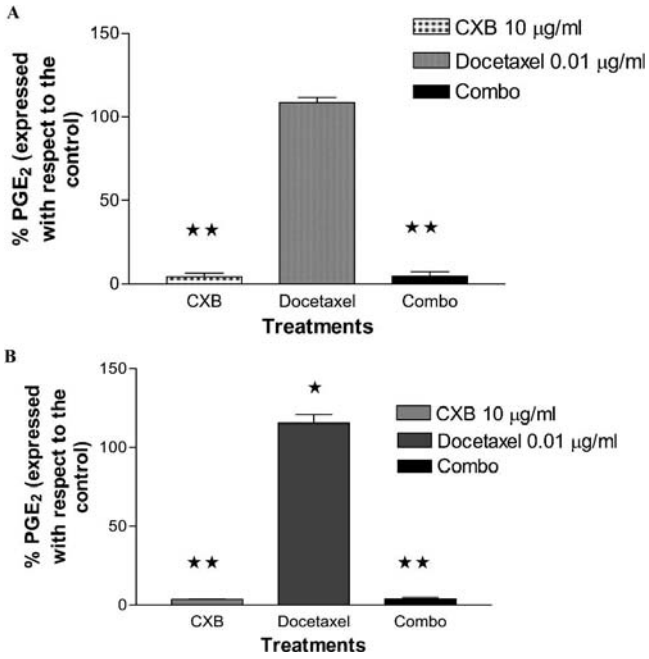

Fig. 9. Effect of celecoxib 10 μg/ml, docetaxel 0.01 μg/ml, and the combination (celecoxib 10 μg/ml + docetaxel 0.01 μg/ml) on PGE_2 production in (A) A549 and (B) H460 cells after 24 h of exposure. Data expressed as mean percent ± SD of PGE_2 production with respect to control (no treatment) (n = 3). Statistical significance of the difference in PGE_2 with respect to control cells: ★, $p < 0.01$; ★★, $p < 0.001$.

375 compared to nimesulide, which only has a ratio of 17.69. This directly correlates in the observed lower IC_{50} values for celecoxib (19.1 and 18.2 μg/ml) compared to nimesulide (74.6 and 54.9 μg/ml) in A549 and H460 lung cancer cells, respectively (11). The role of celecoxib in enhancing the cytotoxic activity of docetaxel was not restricted to human nonsmall cell lung carcinomas (A549 and H460), but was also observed in a small cell lung carcinoma (SHP77) as reported in Table II. The *in vitro* cytotoxicity of aerosolized celecoxib in combination with docetaxel was determined using a previously established method (11). Our findings showed that the celecoxib-MDI (2 shots) could enhance the *in vitro* cytotoxicity of docetaxel on the lower stages of the viable impactor in A549 and H460 cells (Fig. 4). In addition, the enhanced cytoxicity was mediated by an increased induction of apoptosis, as shown by increased apoptotic staining and DNA degradation (Figs. 6–8). These results indicate that a sufficient *in vitro* dose could be delivered by only 2 shots from a solution MDI. As with solution MDI formulations, dose is limited based upon the solubility of the drug in the formulation. This has proven to be a major concern in previous work evaluating the nimesulide-MDI, as 40 shots were needed to reach a sufficient *in vitro* dose for activity, due to its limited solubility in the ethanol/HFA 134a formulation. Clinical achievable concentrations of celecoxib can be obtained with the celecoxib-MDI, as concentrations in human plasma have been reported to range from 0.5 to 5 μM. In the *in vitro* apoptosis studies using acridine orange and Hoechst staining, docetaxel was used at a dose of 0.005 μg/ml which was higher than the dose (0.001–0.0005 μg/ml) used for cytotoxicity study using the viable impactor. To visualize the cellular morphologic changes associated with apoptosis, a higher dose of docetaxel was used than the antiproliferative dose of docetaxel used for the *in vitro* cytotoxicity studies using viable impactor. This is supported by our earlier study using aerosolized nimesulide and doxorubicine on A549 cell line where higher dose of doxorubicine was used for apoptosis staining studies than used in the viable impactor (11).

The *in vitro* antiproliferative effects of COX-2 inhibitors are thought to be mediated through both COX dependent and independent mechanisms of action. The growth inhibitory and apoptotic response of COX-2 inhibitors in lung cancers has been shown to be dependent upon the expression of COX-2 (10,24). Recently, Waskewich *et al.* (25) have shown

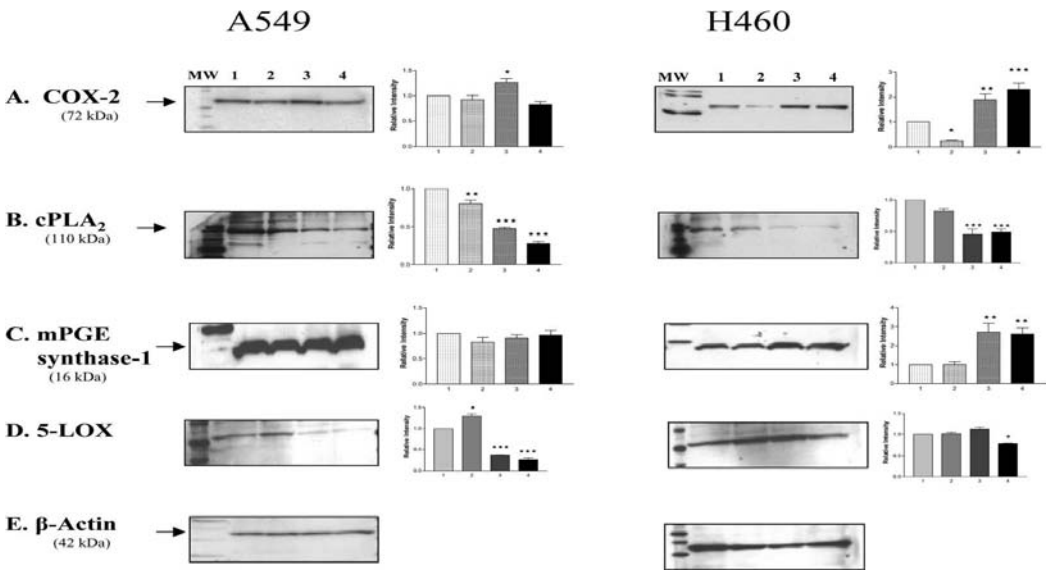

Fig. 10. Western blotting of (A) COX-2, (B) $cPLA_2$, (C) mPGE synthase-1, (D) COX-1, and (E) β-actin in A549 and H460 cells in response to celecoxib and docetaxel treatments. Band intensities expressed as relative intensity and were represented as mean ± SD (n = 3). Lane 1: Untreated control cells; lane 2: celecoxib (10 μg/ml); lane 3: docetaxel (0.01 μg/ml); lane 4, celecoxib (10 μg/ml) + docetaxel (0.01 μg/ml). Lane MW: Magic Mark Western molecular weight markers (Invitrogen, Carlsbad, CA, USA). Statistical significance of the difference in relative intensity of the band in the different treatments with respect to control cells: ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$.

that celecoxib exhibits antiproliferative effect in both COX-2 positive and negative epithelial cell lines. Further, exogenous addition of PGE_2 failed to reverse the cytotoxicity of the combined use of COX inhibitors with anticancer drugs in lung cancer cell lines (11,26). These results suggest the contribution of other COX-2 independent mechanisms in the growth inhibitory activities of COX-2 inhibitors.

In this study, we attempted to elucidate the molecular mechanisms involved in the enhanced *in vitro* cytotoxic response of celecoxib with docetaxel in A549 and H460 cells. Figure 12 summarizes the overall findings of the COX-2 independent molecular mechanisms involved in the enhanced apoptotic response of this combination in both the NSCLC cell lines A549 and H460. Within the arachidonic acid pathway, $cPLA_2$ expression was significantly reduced in the combination therapy, as compared to the control, and the indi-

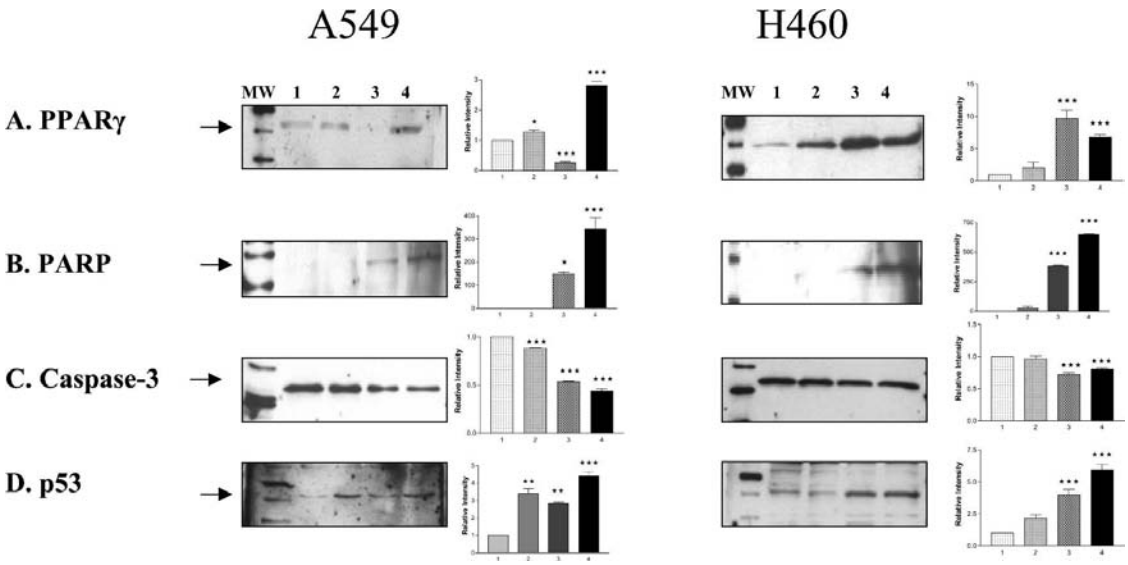

Fig. 11. Western blotting of (A) PPARγ, (B) PARP, (C) caspase-3, and (D) p53 in A549 and H460 cells in response to celecoxib and docetaxel treatments. Band intensities were expressed as relative intensity and are represented as mean ± SD (n = 3). Lane 1: Untreated control cells; lane 2: celecoxib (10 μg/ml); lane 3: docetaxel (0.01 μg/ml); lane 4, celecoxib (10 μg/ml) + docetaxel (0.01 μg/ml). Lane MW: Magic Mark Western molecular weight markers (Invitrogen). Statistical significance of the difference in relative intensity of the band in the different treatments with respect to control cells: ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.001$.

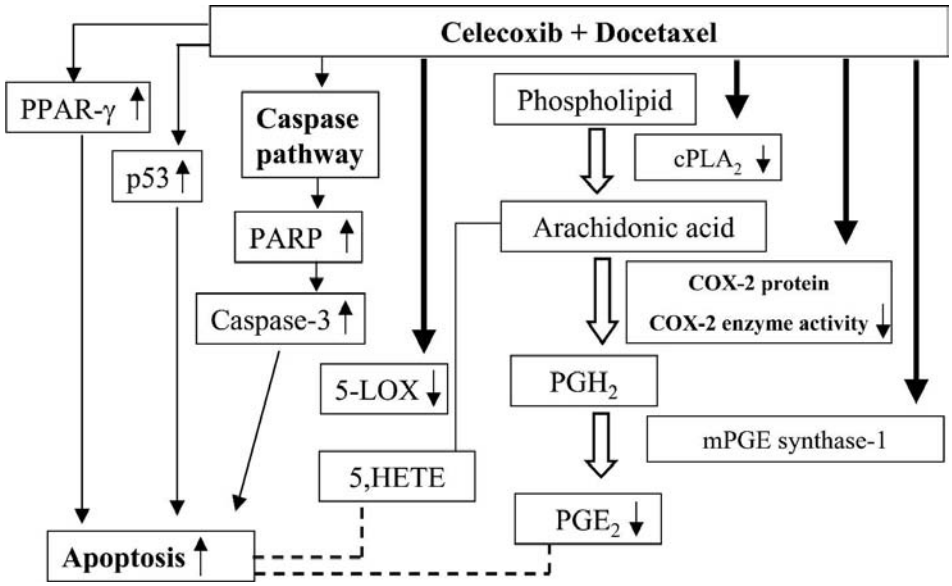

Fig. 12. This is a hypothetical model describing the possible mechanism(s) involved in the antiproliferative and apoptotic effects of the combination of celecoxib with docetaxel in lung tumor cell lines. The combination of celecoxib with docetaxel may alter the biosynthesis of various prostaglandins from arachidonic acid. The combination treatment has an inhibitory effect on $cPLA_2$, which may inhibit the release of arachidonic acid from membrane lipids. The COX-2 enzyme inhibitory activity of celecoxib may inhibit the biosynthesis of PGE_2 from arachidonic acid via COX-2. However, the combination treatment showed a cell-line specific effect on COX-2 and mPGE synthase protein expression. It is interesting to note that the combination of celecoxib with docetaxel has not significantly reduced the COX-2 protein in A549 cells, whereas an induced COX-2 protein expression has been observed in H460 cells. We also observed that celecoxib with docetaxel inhibits 5-LOX, an enzyme involved in the metabolism of arachidonic acid via lipoxygenase pathway. 5-LOX inhibitors have been shown to have chemopreventive effects in animal lung carcinogenesis models. These mechanisms may have a direct growth inhibitory and apoptotic effect (dotted line) or indirectly contribute to apoptosis. One possible mechanism is that cellular accumulation of arachidonic acid (possibly via decreased PGE_2 biosynthesis and decreased metabolism of arachidonic acid via inhibition of 5-LOX) may stimulate the conversion of sphingomyelin to ceramide, (32) a known mediator of apoptosis. The combination of celecoxib with docetaxel treatment resulted in an increase in the expression of PPAR-gamma and p53 protein, which may result in an increased apoptotic effect. Celecoxib and docetaxel combination also stimulated the caspase pathway. The caspase pathway involved stimulation of PARP cleavage, which in turn converted the pro-caspase to caspase-3, inducing apoptosis. The direct effect of the combination of celecoxib with docetaxel is indicated by bold arrow. The possible direct or indirect effects of reduced PGE_2 synthesis or decrease in the 5-LOX expression contributing to apoptosis is indicated by dotted arrows. The arachidonic acid pathway of prostaglandin biosynthesis is indicated by open arrow.

vidual treatments. $cPLA_2$ is the major intracellular form of PLA_2. PLA_2 is the major enzyme responsible for arachidonic acid release from membrane lipids and has shown to play a major role in mouse lung tumorigenesis (27). Further down the arachidonic acid pathway, we evaluated 5-LOX, another key enzyme (besides COX) which metabolizes arachidonic acid. 5-LOX converts arachidonic acid to 5-*S*-HETE, which in turn is converted to LTA_4 and then LTB_4 by LTA_4 hydrolase (28). Recent investigations have shown that 5-LOX and its metabolites are involved in various roles in tumorigenesis (29). In addition, inhibitors of 5-LOX have shown antitumor effects on murine bladder cancer *in vitro* (30). Our results support these finding, as there was a decrease in the expression of 5-LOX in combination treatments.

Further, we evaluated the roles of various other molecular targets outside the arachidonic acid pathway. Our studies showed an increase in PPAR-γ and p53 expression, as well as evidence of activation of the caspase pathway. Our previous studies have demonstrated increased expression of PPAR-γ in the enhanced cytotoxicty seen with combining nimesulide with doxorubicin in A549 cells (11). Similarly, PPAR-γ expression was significantly expressed in the combination of celecoxib with docetaxel, as compared to the control and treatment, further establishing the role of this protein in the inhibition lung cancer cell growth. In addition, we evaluated proteins in the caspase pathway, which may have also contributed to the observed enhanced apoptotic response seen in the combination therapy. In this combination treatment, we found an increased expression of PARP and a decreased expression of the pro-caspase-3. Decreased expression of the pro-active form of caspase-3 indicates a change leading to the active form. Caspase-3 activation is an early event in the apoptotic cascade and it triggers PARP cleavage, and induction of apoptosis. This pathway is confirmed by the parallel

increased expression of PARP, indicating increased PARP cleavage. Finally, we evaluated the role of the tumor suppressor protein, p53, and found that this protein was most significantly expressed in the combination treated cells. p53 regulates the response to various cellular associated stressors, including DNA damage and oncogenic stimulation. When p53 is activated it becomes localized in nucleus and induces cell cycle arrest or apoptosis via transcriptional activation of numerous effector genes. It has been reported that celecoxib facilitated the nuclear localization of p53 in colon cancer cells, which enhanced the responsiveness of p53 to DNA damage (31). This mechanism may help explain the enhanced cytotoxicity of the combination therapy, as celecoxib may sensitize the cells to the anticancer agent.

CONCLUSIONS

These results indicate the potential of inhalation delivery of COX-2 inhibitors for the enhancement of cytotoxic activity of anticancer agents used in the treatment of lung cancer. The current study showed that aerosolized celecoxib significantly enhanced the cytotoxic and apoptotic response of docetaxel against A549 and H460 cells. Furthermore, novel molecular targets involved in the enhanced cytotoxic and apoptotic response of the combination of celecoxib with docetaxel in lung cancer cell lines were elucidated.

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