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Anti-cancer effect of celecoxib and aerosolized docetaxel against human non-small cell lung cancer cell line, A549

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Abstract

Direct delivery of chemotherapeutic agents to the lung can increase both the drug concentration and exposure period to lung tumours. The objective of this study was to formulate docetaxel (DOC) into a metered dose inhaler (MDI), assess its aerodynamic characteristics and to evaluate the effect of celecoxib (CXB), a cyclooxygenase-2 (COX-2) inhibitor, on the in-vitro cytotoxicity and apoptotic response of aerosolized DOC against human lung adenocarcinoma cell line A549. A stable solution-type MDI formulation was developed with 0.25% DOC and 15% w/w ethyl alcohol using HFA 134a propellant. The formulation was evaluated for medication delivery, mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), percent throat deposition, respirable mass and respirable fraction. A six-stage viable impactor was used to assess the in-vitro cytotoxicity of DOC-MDI alone or in combination with CXB. Induction of apoptosis in A549 cells by DOC (non-aerosolized and aerosolized) in combination with CXB was evaluated by established techniques, such as caspase-3 estimation and terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) staining. The influence of different treatments on the expression of COX-2 and peroxisome proliferator-activated receptor-γ (PPAR-γ) in A549 cells was studied by RT-PCR. The DOC-MDI formulation had a MMAD of 1.58 μm, (GSD = 3.2) and a medication delivery of 80 μg/shot. DOC-MDI (one shot) in combination with CXB (10 μg mL^{-1}) had a cell kill of more than 80% as determined by in-vitro cytotoxicity assay. The specific caspase-3 activity in A549 cells treated with DOC (0.01 μg mL^{-1}) and CXB (10.0 μg mL^{-1}) combination was 4 times higher than CXB and untreated control group, respectively. Further, TUNEL staining showed significant apoptosis of A549 cells treated with aerosolized DOC alone or in combination with CXB when compared with CXB and untreated cells. The RT-PCR experiments showed similar expression of COX-2 in both control and treated groups. PPAR-γ expression was increased in the combination treatment (0.01 μg mL^{-1} DOC and 10 μg mL^{-1} CXB) as compared with control (untreated), DOC (0.01 μg mL^{-1}) and CXB (10 μg mL^{-1}) treatments. Our results indicate the potential of inhalation delivery of DOC in the treatment of lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related deaths and accounts for more deaths than colorectal, breast, prostate and pancreatic cancer combined (Tian et al 2004). The lungs are also the primary site of metastases from other types of cancer, including breast, colon, skin and prostate cancer. Conventional routes of drug delivery have shown limited results in the treatment of lung cancer (Zou et al 2004, Koshkina & Kleinerman 2005). This is partly due to the inability to provide effective concentrations at the tumour site without encountering dose-limiting toxicity.

Over the last few years, inhalation drug delivery for the treatment of lung cancer has received new attention from scientists. Nebulized liposome formulations of 9-nitrocamptothecin (9-NC) and paclitaxel have been studied in the treatment of lung cancer in animal models (Knight et al 1999). Aerosolized liposomes with an estimated pulmonary deposition of 76.7 μg kg^{-1} 9-NC were found to significantly ($P<0.001$) reduce the mean tumour growth when compared with oral treatment (100 μg kg^{-1} 9-NC per day) in human lung cancer xenograft growing over the thorax in nude mice. However, the studies showed that about 30% of aerosol inhaled by mice was deposited in the respiratory tract and the remaining 70% was exhaled. Hence, improvement in the respiratory deposition of anti-cancer drugs, camptothecin and paclitaxel, was achieved using 5% CO_2 enrichment of air. The increased pulmonary drug

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concentrations have been attributed to the changed respiratory patterns (Koshkina et al 2001a). However, this also followed increased drug accumulation in other organs, such as liver, spleen, kidney, blood and brain. Encapsulation of paclitaxel into lipid vehicles and administration by continuous aerosolization into mice bearing pulmonary renal carcinoma metastases (Koshkina et al 2001b) showed that the tumour growth was significantly reduced and survival time was increased when compared with control mice inhaling placebo liposome suspension. The total deposited dose of paclitaxel by liposome aerosol treatment was 5 mg kg^{-1}, which is substantially lower than doses used for intravenous administration (>20 mg kg^{-1}). Further, nebulized ciclosporin plus paclitaxel liposomes have been demonstrated to be suitable for aerosol treatment of lung cancer (Koshkina et al 2004). Tumour surface areas were significantly smaller in ciclosporin–paclitaxel treated animals when compared with untreated control and paclitaxel or ciclosporin treated animals ($P < 0.01$).

In another study, Wang et al (2003) demonstrated that nebulized aerosol formulation of the anti-cancer agent farnesol induced cell death of human lung cancer cells, H460 and A549, in-vitro. Further, gemcitabine has been shown to inhibit the growth of primary osteosarcoma and osteosarcoma lung metastases (Koshkina & Kleinerman 2005). The response of LM7 and LM8 lung metastases to aerosol gemcitabine was dose dependent. The efficacy of aerosol versus intraperitoneal gemcitabine was compared using a 0.5-mg kg^{-1} dose, with the treatment initiated when the primary tumour volume reached 130 mm^3. The number of micrometastases in the lung was significantly reduced only in mice receiving aerosol gemcitabine when compared with control and the intravenously treated group.

Difluromethylornithine and 5-flurouracil are reported to be effective chemopreventive compounds against carcinogenesis of the upper respiratory tract by using aerosol delivery (Wattenberg et al 2004). Both compounds increased the percentage of animals free of tumours and prevented infiltrating squamous cell carcinoma by >50%. Aerosolized delivery of chemopreventive agents, budesonide and isotretinoin, have been found to be effective in the chemoprevention of lung cancer in A/J mice (Wattenberg et al 1997).

One of the most widely used and convenient inhalation devices for the delivery of drugs to the lungs is the pressurized metered dose inhaler (MDI). Aerosolized delivery of all-*trans* retinoic acid using hydrofluroalkane (HFA)-134a based MDI has been shown to prolong the pulmonary half-life and lower the systemic levels of the drug (Brooks et al 2000). We have previously demonstrated the feasibility of using a methotrexate MDI formulation that could induce apoptosis in HL-60 cells and showed a cell kill greater than 50% with two actuations (Shaik et al 2002). Cyclooxygenase-2 (COX-2) inhibitors, nimesulide and celecoxib, when formulated into an MDI, are shown to significantly enhance the activity of anti-cancer drugs against human lung (A549 and H460) and colon (SW620) tumour cell lines (Haynes et al 2003, 2005).

In this study we aim to explore the efficacy of delivering docetaxel as an aerosol using a HFA propellant-based MDI. Docetaxel is an antineoplastic agent belonging to the taxoid family. It is the first chemotherapeutic agent shown to significantly improve survival for patients after failure of previous platinum-based chemotherapy and has also been recently approved by the FDA for the second-line treatment of advanced non-small cell lung cancer (NSCLC). Delivering docetaxel by inhalation may provide a novel delivery approach, possibly with improved therapeutic response due to its direct delivery to the target organ. Also the therapeutic effect can be obtained at a much lower dose by the inhalation delivery and it may be possible to reduce the adverse effects associated with intravenous docetaxel.

Further, various investigations have shown synergistic cytotoxic effects of COX-2 inhibitors with anti-cancer drugs (Maca 1991; Duffy et al 1998; Soriano et al 1999). 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. Previous investigations in our laboratory have shown that celecoxib potentiates the in-vitro and in-vivo anti-tumour activity of docetaxel, when administered by the conventional intravenous route (Shaik et al 2006). Hence, we also aim to examine the effect of celecoxib on the in-vitro cytotoxicity of aerosolized docetaxel against NSCLC, and determine the underlying mechanisms responsible for the anti-cancer activity.

Materials and Methods

Materials

HFA-134a and 227 were obtained from Du Pont (Ingleside, TX, USA) and Solvay Fluorides Inc. (Hanover, Germany), respectively. Docetaxel and celecoxib were provided as generous gifts from Sanofi-Aventis (Collegeville, PA, USA) and Pfizer (Skokie, IL, USA), respectively. All tissue culture chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA). DeadEnd Colorimetric Apoptosis Detection System and CaspACE Assay System colorimetric kits were obtained from Promega Corporation (Madison, WI, USA). DNA marker (100 bp ladder) was purchased from Fisher Scientific (Suwanee, GA, USA). All other chemicals were of reagent grade. The six-stage viable impactor and eight-stage Andersen cascade impactor, Mark II, were obtained from Graseby Andersen (Smyrna, GA, USA). Continuous and non-continuous valves were kindly provided by 3M Pharmaceuticals (St Paul, MN, USA). The human lung tumour 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 the tissue culture media contained penicillin (50 000 U mL^{-1}), streptomycin (0.1 mg mL^{-1}) and neomycin (0.2 mg mL^{-1}). The tumour cells were grown in standard tissue culture conditions, passaged at 80–90% confluence and cytotoxicity experiments were performed between 2 and 20 passages.

Solubility of docetaxel in HFA-134a and -227

The solubility of docetaxel, at various concentrations of ethanol, was determined in HFA propellants (134a and 227) after equilibration for 48 h. Excess docetaxel (at least 30 mg) 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 8.0 g. The vials were then placed on a platform shaker (Innova 2000; New Brunswick Scientific, Edison, NJ, USA) at 150 rev min^{-1} 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. 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 docetaxel in methanol was then determined by using a spectrophotometer (Beckman DU 640) at a wavelength of 228 nm, and the solubility of docetaxel in the propellant system was calculated as percent weight.

Formulation of docetaxel-MDI

Twenty milligrams of docetaxel was placed in a clean 15-mL glass vial containing 1.2 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 rev min^{-1} and allowed to shake overnight. The continuous valve was then replaced with a 50-μL non-continuous valve.

Medication delivery (ex-actuator dose) of docetaxel formulation

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^{-1}. The MDD chamber was then diluted with 25 mL of methanol and assayed with a spectrophotometer. The formulation was tested at least three times. Medication delivery (emitted dose) refers to the amount of drug delivered (μg/shot) from the actuator.

Aerodynamic particle size distribution

An eight-stage Andersen cascade impactor, Mark II, was used to assess the aerodynamic size distribution of the docetaxel formulation. The formulation was primed by firing five shots into waste. Then five shots (at 5-s intervals) were fired into the cascade impactor under a flow rate of 28.3 L min^{-1}. The deposited docetaxel 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 an appropriate volume of methanol. The samples were analysed on a spectrophotometer at a wavelength of 228 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 docetaxel alone and in combination with celecoxib against various cancer cell-lines

The tumour cells (A549 or H460) were seeded at a density of 10 000 per well in 96-well plates and incubated overnight. Subsequently, the cells were treated with docetaxel alone (0.0001–0.5 μg mL^{-1} or celecoxib alone (1–50 μg mL^{-1}). For the combined effect of docetaxel with celecoxib on cytotoxicity, celecoxib was employed at 6, 10, 12 and 15 μg mL^{-1} along with graded concentrations of docetaxel. Upon treatment, the cells were incubated for 72 h and the cytotoxicity was assayed by crystal violet dye uptake assay by measuring the absorbance at 540 nm. For 50% cytotoxicity, combination index (CI) values were calculated (Waskewich et al 2002):

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2 \quad (1)$$

where $(D_x)_1$ is the dose of drug 1 to produce 50% cell kill alone, $(D)_1$ is the dose of drug 1 to produce 50% cell kill in combination with $(D)_2$, $(D_x)_2$ is the dose of drug 2 to produce 50% cell kill alone and $(D)_2$ is the dose of drug 2 to produce 50% cell kill with $(D)_1$; $\alpha = 0$ for mutually exclusive or 1 for mutually non-exclusive modes of drug action.

In-vitro cytotoxicity of aerosolized docetaxel-MDI alone and its combination with celecoxib against A549 cells

A six-stage viable impactor was used to assess the in-vitro cytotoxicity of aerosolized docetaxel, alone or in combination with a known concentration of celecoxib (Haynes et al 2005). The viable impactor is similar to the Mark II impactor, but allows keeping sterile glass petri plates (outside diameter 9.5 cm) on its various stages. The impactor can be hooked up to the MDI through a USP throat and operated at a flow rate of 28.3 L min^{-1}. A549 cells (one 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 docetaxel formulation for 1 shot. After this exposure, the petri plates were taken from the impactor, covered with sterile aluminium 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 nimesulide, 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 since 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 phosphate-buffered saline (PBS), and detached by adding trypsin. The cells were spun down with a centrifuge and resuspended in an appropriate amount of medium. The viable cells were then counted with a haemocytometer 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 docetaxel-MDI in conjunction with celecoxib, except a fixed concentration of celecoxib ($10\,\mu g\,mL^{-1}$) was added to cells in the petri plate. To determine the aerosolized dose of the docetaxel-MDI in the six-stage viable impactor, 20 mL of 50% polyethylene glycol (PEG) 400 solution was used as the collection medium in petri plates in place of the tissue culture medium, and 1 shot was fired under a flow rate of $28.3\,L\,min^{-1}$. The concentration of docetaxel was determined by spectrophotometer, and the corresponding dose was calculated.

Detection of apoptosis by caspase-3 activity

A549 cells (10 000 cells/well) were plated in 96-well plates, each well containing 100 μL of media. Subsequently, they were treated with docetaxel ($0.01\,\mu g\,mL^{-1}$), celecoxib ($10\,\mu g\,mL^{-1}$) or a combination of docetaxel ($0.01\,\mu g\,mL^{-1}$) plus celecoxib ($10\,\mu g\,mL^{-1}$). Untreated cells were used as control. After 72 h, caspase 3 activity was determined by Caspase-Glo 3/7 kit (Promega Corp, Madison, WI) following the kit protocol.

Assessment of apoptosis in A549 cells induced by aerosolized docetaxel in combination with celecoxib by TUNEL staining

A549 cells (one million in 20 mL of medium with or without $10.0\,\mu g\,mL^{-1}$ celecoxib) were exposed to aerosolized docetaxel on the fifth stage of the viable impactor, in the same way as described above for the in-vitro cytotoxicity of aerosolized docetaxel. Upon exposure of the cells to the aerosolized docetaxel, 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 and washed twice with PBS. The slide was studied for TUNEL staining using a DeadEnd Colorimetric Apoptosis Detection System kit (Promega) as per the manufacturer's instructions.

Effect of the combination of celecoxib with docetaxel on mRNA levels of COX-2 and PPAR-γ

Total RNA was eluted from A549 cells treated with docetaxel ($0.01\,\mu g\,mL^{-1}$), celecoxib ($10\,\mu g\,mL^{-1}$) or a combination of docetaxel ($0.01\,\mu g\,mL^{-1}$) plus celecoxib ($10\,\mu g\,mL^{-1}$). Untreated cells were used as control. Reverse transcription was performed with Moloney-murine leukaemia virus reverse transcriptase (MuLV-RT) (Applied Biosystem, CA, USA) according to the manufacturer's protocol with some modifications. The polymerase chain reaction (PCR) was performed with COX-2 and PPARγ primer pairs and ATAQ DNA polymerase (Applied Biosystem). The PCR products were separated in a 1.5% agarose gel and the band intensities were quantified using ImageJ 1.33u software (Wayne Rasband, NIH, USA).

Statistical analysis

One-way analysis of variance followed by Tukey's Multiple Comparison Test was performed to determine the significance of difference between: in-vitro cytotoxicity of aerosolized docetaxel (one shot) alone and in combination with celecoxib ($10\,\mu g\,mL^{-1}$) against A549 cells on the third, fourth, fifth and sixth stages of viable impactor; in-vitro cytotoxicity of the combination of aerosolized docetaxel with celecoxib versus the combination of docetaxel (non-aerosolized) with celecoxib; and specific caspase-3 activity of different treatments. The statistical analysis was performed using GraphPad PRISM version 2.0 software (San Diego, CA, USA).

Results

Solubility of docetaxel in HFA-134a and -227

Figure 1A shows the solubility profiles of docetaxel in HFA propellants containing various concentrations (0–15% w/w) of ethyl alcohol. In all cases, the addition of ethanol increased the solubility of docetaxel in HFA-134a and –227. The highest solubility of docetaxel (0.30% w/w) was observed in HFA-134a with 15% ethyl alcohol. Therefore, a solution formulation of 0.25% w/w docetaxel, 15% w/w ethyl alcohol in HFA-134a was prepared and used as a model MDI formulation.

Characterization of docetaxel-MDI formulation

Using commercially available Proventil HFA actuators, the docetaxel-MDI had a medication delivery of $80.0 \pm 2.3\,\mu g$/shot with $42.2 \pm 3.2\%$ of the dose being respirable. The MMAD and GSD of the formulation were $1.58 \pm 0.4\,\mu m$ and 3.2 ± 0.5, respectively. The formulation showed a throat deposition of $27.2 \pm 5.6\%$ and a respirable mass of $44.4 \pm 5.6\,\mu g$/shot. The formulation was found to be chemically stable at both room temperature and at 40°C for one month (data not shown). The deposition pattern of the aerosolized docetaxel from the Andersen cascade impactor is illustrated in Figure 1B.

Analysis of combined drug effects

To verify the synergistic cell killing activity, the combined effect of docetaxel and celecoxib was assessed using the isobolographic analysis. Table 1 shows the Combination Index (CI) values of the interaction between docetaxel and celecoxib against human lung cancer cells. The CI values ranged from 0.4 to 1.2 for 50% cell kill level, suggesting a moderate synergism.

In-vitro cytotoxicity of aerosolized docetaxel formulation alone and in combination with celecoxib against A549 cells

Figure 2 shows the in-vitro cytotoxicity of the aerosolized docetaxel formulation alone and in combination with

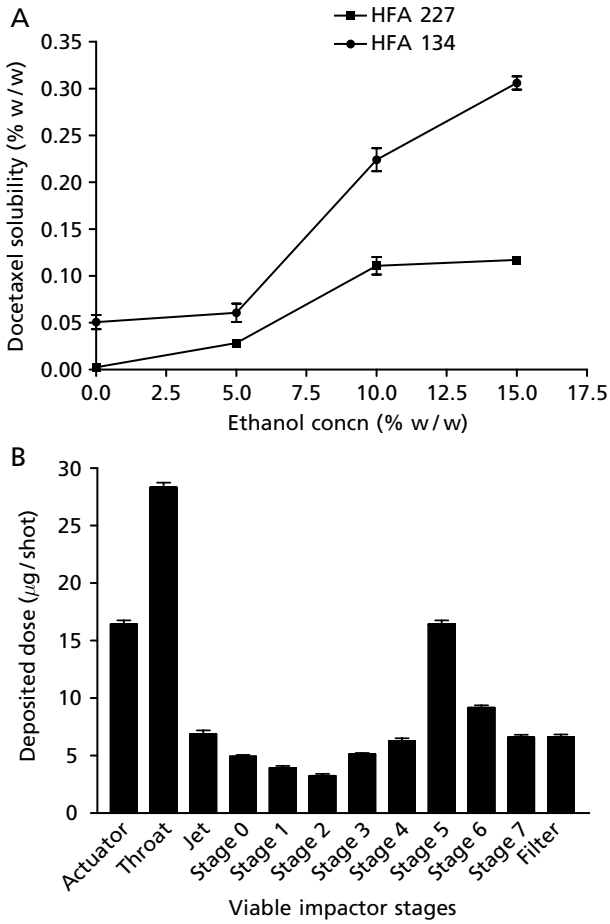

Figure 1 A. Solubility of docetaxel in HFA propellants as a function of ethanol concentration. Each point represents mean ± s.d., of three experiments. B. Anderson cascade impactor deposition profile of docetaxel-MDI. Data expressed as μg/shot of the total drug deposited on all stages of impactor, including actuator and throat. Values represent mean ± s.d. of three determinations.

celecoxib against A549 cells kept on stages 3–6 of the viable impactor. Stages 3–6 of the viable impactor were chosen because these stages correspond to a cut-off particle diameter of 4.7 μm employed in the calculation of respirable mass and respirable fraction values from Andersen impactor data. Aerosolized docetaxel (1 shot) had a cell kill of 71.5 ± 1.8% on stage 5. However, when A549 cells were treated with 10.0 μg mL^{-1} celecoxib and exposed to 1 shot of the aerosolized MDI, a cell kill as high as 92.5 ± 3.2% was observed on the 5th stage of the viable impactor. Subsequently, the cytotoxicity was determined using the non-aerosolized docetaxel (dose equal to the amount deposited on stages 5 and 6 of the viable impactor) in combination with celecoxib essentially in the same way as described above for the aerosolized docetaxel. Figure 3A shows the deposition profile of docetaxel on the various stages of the viable impactor after one shot of the MDI formulation. The experimentally determined dose of docetaxel deposited on stages 5 and 6 of the viable impactor following one actuation was 358 and 242 μg, respectively, which corresponds to 0.494 and 0.430 μg mL in 20 mL of collection medium employed in petri plates (Figure 3B). The comparison between in-vitro cytotoxicity of the aerosolized versus non-aerosolized docetaxel and its combination with celecoxib was performed for stages 5 and 6 of the viable impactor, because there was greater deposition of docetaxel on these stages as compared with the other stages (Figure 3A). Figure 3B shows the cytotoxicity profiles of non-aerosolized docetaxel (dose corresponding to that deposited on stages 5 and 6 of the viable impactor following one actuation of MDI formulation) either alone or in combination with celecoxib. It is evident that the enhanced cytotoxicity of aerosolized docetaxel with celecoxib is comparable to the combination of non-aerosolized docetaxel with celecoxib without any significant ($P < 0.05$) difference on stages 5 or 6 of the viable impactor. The results indicate that the process of aerosolization has not altered the activity of docetaxel and that the aerosolized docetaxel exhibits the same activity as that of the non-aerosolized form under equivalent dosing conditions.

Induction of apoptosis in A549 cells by the combination of celecoxib with docetaxel

It is evident from Figure 4 that the specific caspase-3 activity in A549 cells treated with docetaxel (0.01 μg mL^{-1}) + celecoxib (10 μg mL^{-1}) was four times higher in comparison with

Table 1 Combination Index (CI) values of the interaction between docetaxel and celecoxib against human lung cancer cells

Drug combination	A549		H460	
	CI at IC50	Interpretation	CI at IC50	Interpretation
Docetaxel + celecoxib 6 μg mL^{-1}	0.465	Synergism	0.723	Moderate synergism
Docetaxel + celecoxib 10 μg mL^{-1}	0.701	Moderate synergism	0.923	Additive effect
Docetaxel + celecoxib 12 μg mL^{-1}	0.654	Moderate synergism	1.026	Moderate antagonism
Docetaxel + celecoxib 15 μg mL^{-1}	1.041	Moderate antagonism	1.183	Antagonism

The human lung cancer cell lines A549 (adenocarcinoma) and large-cell carcinoma type H460 (large-cell carcinoma) were obtained from American Type Culture Collection (Rockville, MD, USA). Celecoxib was employed at 6, 10, 12 and 15 μg mL^{-1} to study the effect on IC50 of docetaxel. Variable ratios of drug concentrations were employed to determine the in-vitro cytotoxicity data and mutually non-exclusive equations were used to determine the CI. The CI values represent mean of four experiments. CI > 1.3, antagonism; CI 1.1–1.3, moderate antagonism; CI 0.9–1.1, additive effect; CI 0.8–0.9, slight synergism; CI 0.6–0.8, moderate synergism; CI 0.4–0.6, synergism; CI 0.2–0.4, strong synergism (Menendez et al 2001; Waskewich et al 2002).

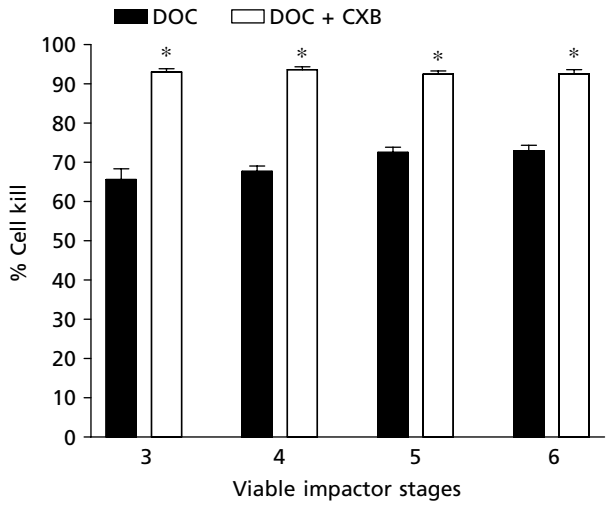

Figure 2 In-vitro cytotoxicity profiles of aerosolized docetaxel (DOC, one shot) alone and in combination with celecoxib (DOC + CXB, 10 μg mL^{-1}) against A549 cells on the 3rd, 4th, 5th and 6th stages of viable impactor. Data represent the mean ± s.d. of three experiments. *$P < 0.001$, cytotoxicity of aerosolized DOC vs aerosolized DOC with CXB.

celecoxib (10 μg mL^{-1}) and untreated control. Statistically, the caspase-3 activity in A549 cells treated with the combination of docetaxel plus celecoxib was significantly higher ($P < 0.001$) in comparison with either control or celecoxib-treated cells.

Apoptosis in A549 cells by the combination of aerosolized docetaxel with celecoxib

We evaluated the effect of the combined treatment of aerosolized docetaxel from MDI (1 shot) with celecoxib on the apoptosis in A549 cells by TUNEL assay (Figure 5). The results showed that there was negligible apoptosis in control and celecoxib-treated cells. However, treatment with docetaxel aerosol alone or in combination with celecoxib produced a significantly high apoptotic response. Our results show that apoptosis could be induced in A549 cells by employing sub-apoptotic concentrations of celecoxib with aerosolized docetaxel.

Effect of celecoxib with docetaxel on mRNA levels of COX-2 and PPAR-γ in A549 cells

The RT-PCR experiments showed that there was similar expression of COX-2 in both control and treated groups. The expression of PPAR-γ was elevated in the combination treatment of celecoxib with docetaxel as compared with the control, celecoxib or docetaxel treatments (Figure 6).

Discussion

New therapeutic agents, molecular targets and delivery approaches are needed to address the limited effectiveness of current treatment modalities for NSCLC (Zou et al 2004).

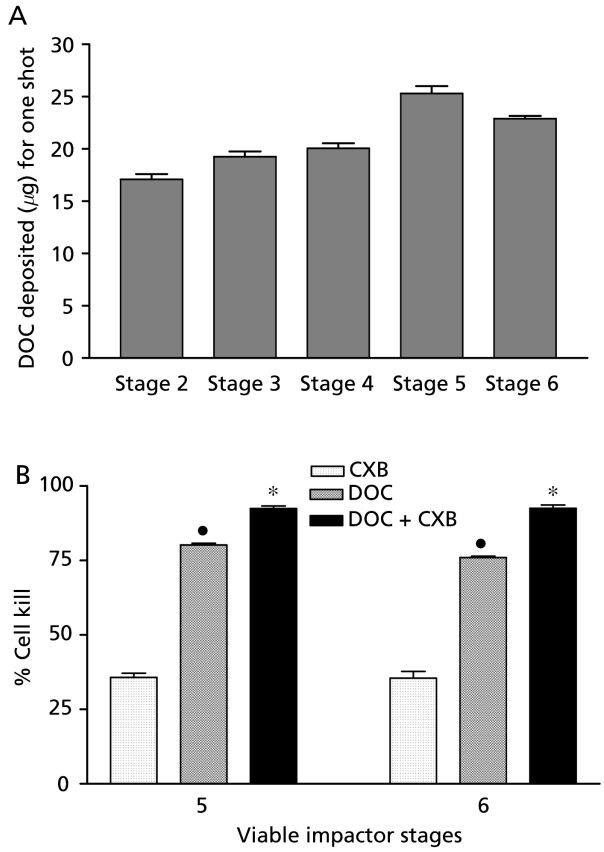

Figure 3 A. Docetaxel (DOC) deposition following single actuation of MDI on different stages of viable impactor. Data represent mean ± s.d. of three experiments. B. In-vitro cytotoxicity of DOC alone (nonaerosolized) and in combination with CXB (10 μg mL^{-1}) against A459 cells. The concentration of DOC used on stages 5 and 6 of the viable impactor corresponds to 0.494 μg mL^{-1} and 0.430 μg mL^{-1}, respectively. The concentration was based on the experimentally determined amount of DOC deposited for one shot of MDI formulation in 20 mL medium on stages 5 and 6 of viable impactor. Data represent the mean ± s.d. of three experiments. •$P < 0.001$, cytotoxicity of non-aerosolized DOC vs CXB; *$P < 0.001$, cytotoxicity of non-aerosolized DOC + CXB vs non-aerosolized DOC.

Among treatment avenues being explored as alternatives to systemic drug delivery, direct tumour-targeted aerosolized delivery of chemotherapeutic agent, alone or in combination with other drugs, seems a novel promising approach for the treatment of lung cancer (Kohlhaufl et al 2002; Gautam & Koshkina 2003; Estensen et al 2004). This method of delivery significantly alters the agent's biodistribution and pharmacokinetics in favour of pulmonary deposition. The drug is processed through the lungs before it gets into the systemic circulation when administered as aerosol. However, any drug administered systemically is first diluted in the blood before it reaches the lung.

To our knowledge, there is no cited report on the inhalation delivery of docetaxel, either as an MDI or any other aerosolized form. In this study, we evaluated the solubility of docetaxel in the HFA propellants 134a and 227. One of the most important factors dealing with the new HFA

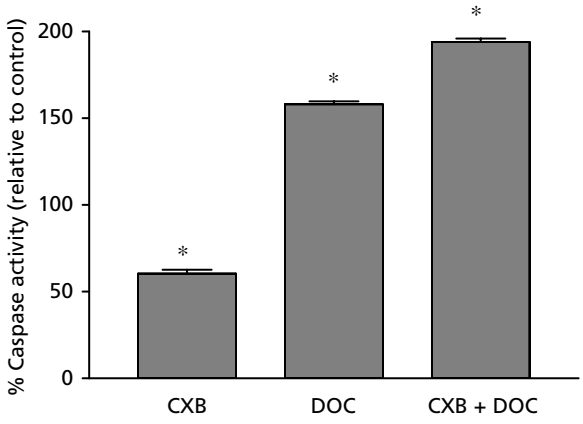

Figure 4 Caspase-3 activity in A549 cells treated with celecoxib (CXB, 10 μg mL^{-1}), docetaxel (DOC, 0.01 μg mL^{-1}) and CXB plus DOC. Data represent mean ± s.d. of three experiments. *$P < 0.001$, compared with control cells.

propellants is the need for ethanol in the system as a co-solvent to solubilize any surfactant or the drug itself. The ethanol concentration is also found to influence the aerodynamic characteristics of MDI formulations. Myrdal et al (2004) showed that as the ethanol concentration decreased from 10 to 3% by weight, the fine particle fraction of ciclosporin-MDI formulation increased from 34 to 68% for HFA-227 and from 33 to 52% for HFA-134a. Docetaxel is a highly lipophilic molecule, practically insoluble in water. Docetaxel injection concentrate is supplied in a single-dose vial as a sterile, pyrogen-free, non-aqueous, viscous solution with an accompanying sterile non-pyrogenic diluent (13% ethanol in Water for Injection) vial. Moreover, the drug solubility in HFA-134a does not follow correlations similar to aqueous systems (Dickinson et al 2000). Hence, initial pre-formulation work dealt with determining the solubility of docetaxel in the HFA propellants at various concentrations of ethanol.

We developed an MDI formulation of docetaxel based upon its maximum solubility (0.3% w/w, 15% ethanol) in HFA-134a in the presence of ethyl alcohol. The difference in the solubility of drugs in HFA propellants is attributed to the differences in their physiochemical properties and their interactions with the propellants (Haynes et al 2005). The high solubility of docetaxel in HFA-134a with ethanol enabled us to choose a model formulation (0.25% w/w docetaxel with 15% ethanol), which could deliver 80.0 ± 2.3 μg of drug per actuation. The physical stability of docetaxel-MDI was found to be satisfactory, without any precipitation, crystal growth and colour change, for one month at both room and elevated temperature (40°C). The docetaxel formulation was also found to be chemically stable at the above-mentioned conditions with no significant change in the aerodynamic characteristics evaluated in this study. However, HFA-based solution aerosols have been shown to have about 15% of their ex-actuator dose emitting via exhalation. This may pose a concern to

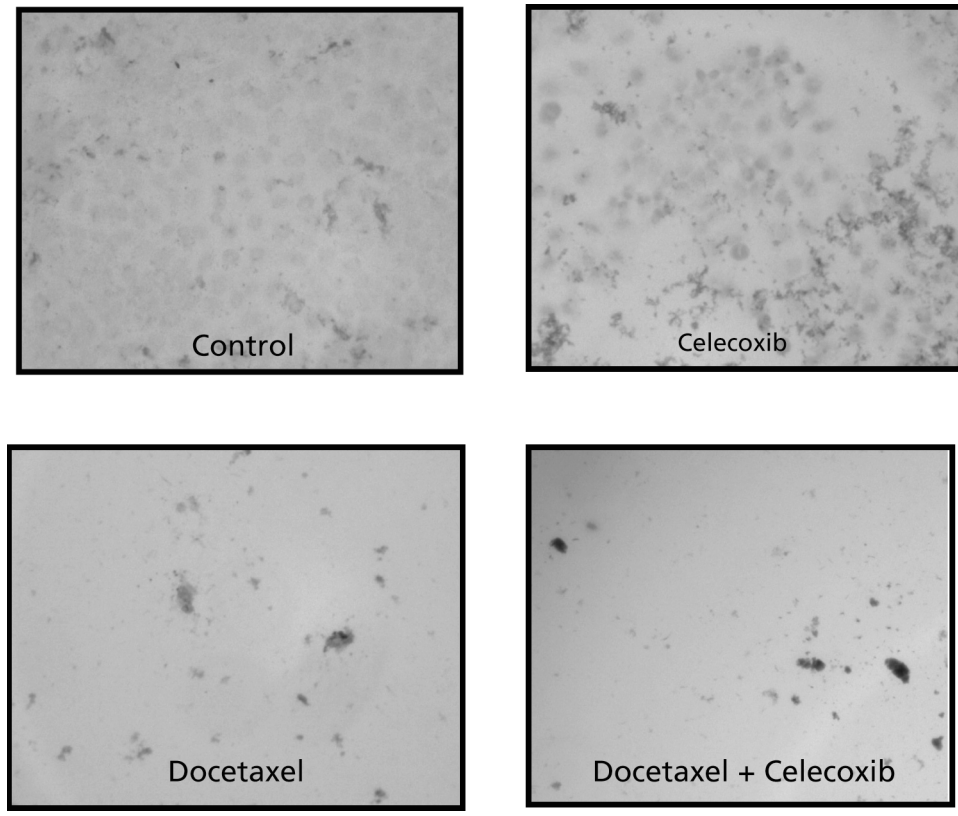

Figure 5 TUNEL staining of A549 cells. Cells were untreated (control) or were exposed to celecoxib (10 μg mL^{-1}), aerosolized docetaxel (one shot) or aerosolized docetaxel (1 shot) + celecoxib (10 μg mL^{-1}). Original magnification 40×.

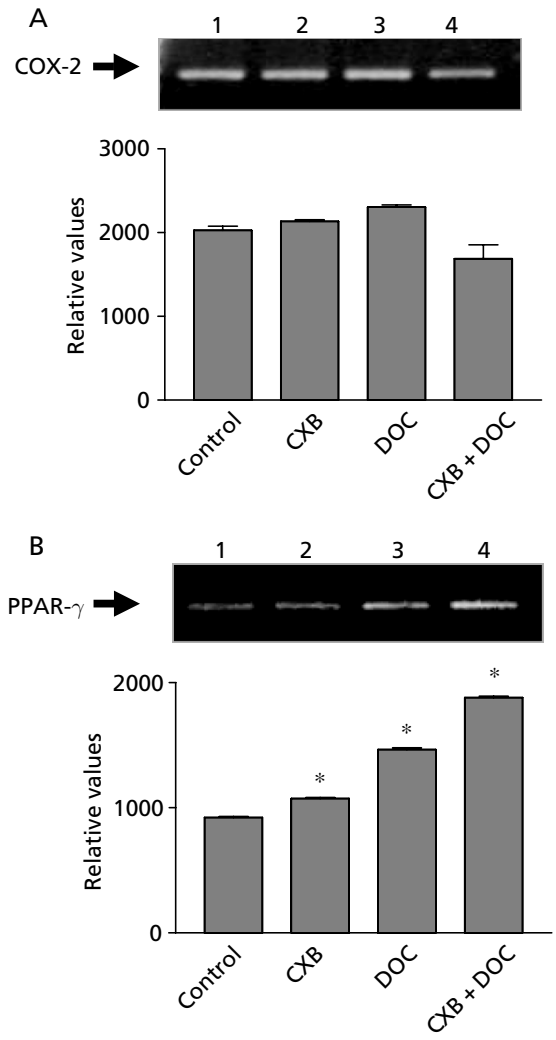

Figure 6 mRNA levels of COX-2 (A) and PPAR γ protein (B) expression in A549 cells by RT-PCR. Lane 1, untreated control cells; lane 2, celecoxib (CXB, 10 μg mL^{-1}); lane 3, docetaxel (DOC, 0.01 μg mL^{-1}); lane 4, CXB (10 μg mL^{-1}) + DOC (0.01 μg mL^{-1}). It is evident from figure 6A that the expression of COX-2 was not significantly altered by treatment with CXB, DOC or the combination of CXB with DOC. The human PPARγ1 and PPARγ2 proteins are 53 and 57 kDa proteins, respectively. In figure 6B, a band was seen in lane 4, located between 50 and 60 kDa for the protein markers, thus indicating the expression of PPARγ in A549 cells treated with the combination of CXB with DOC. *$P < 0.001$ compared with control cells.

health workers as inhaled anti-cancer drugs are delivered to the lung-cancer patients under medical supervision. Therefore, inhalation devices may need to be modified to collect the emitted drug either via a low-resistance filter/bacterial filter (Leach et al 2002) or in specialized hoods.

Investigation of the aerodynamic characteristics of the MDI formulation is imperative for its usefulness in man. The aerodynamic properties indicate a high respirable fraction (>40%), low MMAD (1.58 ± 0.4 μm) and GSD (3.2 ± 0.5), suggesting that nearly half of the actuated dose is deposited in the bronchioalveolar regions of the lungs. Aerosol particles with these MMAD characteristics are well-suited for pulmonary deposition, whether nebulized or administered by MDI (Phalen et al 1984). Formulation attempts are thus directed to delivering a therapeutically optimum dose to the lung. Shaik et al (2002) showed that it is possible to obtain a respirable mass of 29.0 μg/shot, respirable fraction of 35.5% and reduce the GSD and throat deposition with methotrexate-MDI when methotrexate is passed through a 5-μm sieve. Sommerville & Hickey (2003) demonstrated the utility of dimethyl ether/propane/lecithin microemulsion system to generate aerosols with particle size distributions suitable for pulmonary delivery by MDI (e.g., MMAD 3.1 micron, fine particle fraction (FPF) 59% for dimethyl ether with lecithin content 3%, water content 2.5% w/w). Increasing water concentration (up to 8% w/w) was correlated with a reduction in FPF. Freezing and rewarming had no adverse effect on MMAD, GSD or FPF. Storage of microemulsion samples for up to 3 weeks also did not adversely affect the MMAD, GSD or FPF.

Before evaluating the in-vitro cytotoxicity with aerosolized docetaxel, we studied the effect of celecoxib on docetaxel cytotoxicity against a panel of NSCLC cell lines. We analysed the combination effect by isobolographic analysis. It is evident from the data presented that celecoxib exhibits a dose-dependent potentiation of docetaxel activity as measured by CI values. The fact that celecoxib can interact synergistically with docetaxel with simultaneous exposure has important mechanistic implications (Waskewich et al 2002). The dose-dependent effect must be taken into consideration while extrapolating the in-vitro results into therapeutic practice. In a recent study, Nakata et al (2004) demonstrated that celecoxib enhanced the response of A431 human tumour xenograft in nude mice to the chemotherapeutic agent docetaxel by an enhancement factor (EF) of 2.07. Celecoxib also enhanced the tumour response when added to the combined docetaxel plus radiation treatment (EF = 2.13). Our data is in agreement with the work of Menendez et al (2001), who demonstrated by isobolographic analysis the ability of exogenous fatty acids to modulate the cytotoxic activity of anti-cancer drugs, like doxorubicin, in breast-cancer cell lines. The CI values ranged from 0.799 to 1.170 for 5–95% cell kill levels, suggesting slight to moderate synergism or nearly additive interactions.

Previously we have used the six-stage viable impactor for determining the cytotoxicity of aerosolized celecoxib and nimesulide against A549 cells (Haynes et al 2005). In this study, a similar method has been used for exposing A549 cells to aerosolized docetaxel and we could demonstrate that docetaxel-MDI (one shot) shows significant in-vitro cytotoxicity on stages 5 and 6 of the viable impactor. These results indicate that a sufficient in-vitro dose could be delivered with only one shot from a solution MDI. The in-vitro cytotoxicity of aerosolized docetaxel was corroborated by no significant difference in cytotoxicity between aerosolized docetaxel and non-aerosolized docetaxel (dose equal to the one shot of docetaxel-MDI) in combination with celecoxib. Based upon the data presented, it is evident that it requires one shot of aerosolized docetaxel (240 μg dose) to produce nearly 70% in-vitro cytotoxicity against A549 cells under the experimental conditions studied. As is known, with the MDI formulations,

this dose is based upon the solubility of drug in the formulation. We have previously demonstrated that the combination of celecoxib with docetaxel has a greater anti-tumour effect in-vitro (Haynes et al 2005) and in-vivo (Shaik et al 2006) than when used alone in NSCLC cells. In this study, we demonstrate that the formulation of docetaxel in MDI and aerosolization process has no effect on the in-vitro cytotoxicity of docetaxel by exposing A549 cells to both aerosolized and non-aerosolized docetaxel.

There have been studies on the in-vitro and in-vivo potentiation of cytotoxicity of anti-cancer drugs by COX-2 inhibitors. Previous results from our laboratory are in agreement with the current findings and suggest that celecoxib potentiates the anti-tumour activity of docetaxel via modulation of intratumour prostaglandin E2 (PGE_2) and 15d-PGJ_2 levels. The results showed increased expression of PPAR-γ, whereas there was no significant effect on the COX-2 expression. In this study, we attempted to investigate the mechanisms underlying the anti-tumour effect of aerosolized docetaxel with or without celecoxib.

Caspase-3 activation is an important event in the apoptosis cascade, where its activation triggers poly(ADP-ribose)polymerase (PARP) cleavage (Li et al 2001). In this study, we demonstrate that celecoxib at concentrations equal to, or below, its IC50 enhances the apoptotic response of docetaxel against A549 cells by increasing caspase 3 activity. Procaspase 8 leads to caspase-8 formation, which triggers caspase-3 release leading to apoptosis. Caspase 9, on the other hand, is activated from pro-caspase 9 by up regulation of APAF1, which forms a complex with cytochrome c. This active caspase 9 can also directly trigger caspase 3 release and apoptosis.

TUNEL staining confirmed that celecoxib enhances the apoptotic response of aerosolized docetaxel against A549 cells where both the compounds are employed at concentrations below their apoptotic concentrations (Figure 5). Apoptosis has been implicated for the in-vitro anti-proliferative effect of various non-steroidal anti-inflammatory drugs (NSAIDs) and cytotoxic compounds against lung-cancer cell lines (Sawaoka et al 1998; Leahy et al 2002). In this study, we demonstrate that celecoxib potentiates the cytotoxic effect of docetaxel at one-half of its IC50 value.

Further, we looked into the expression of PPAR-γ, as numerous studies have implicated its role in lung cancer. PPAR-γ is a nuclear receptor that plays a pivotal role in the regulation of gene transcription and cellular differentiation. The PPARs are activated by a large number of structurally diverse compounds, including prostanoids, long-chain fatty acids, hypolipidaemic drugs, leukotriene antagonists and antidiabetic thiazolidinediones. Although chemically diverse, these compounds share certain common structural features, including a lipophilic backbone and an acid moiety, usually a carboxylate. Several NSAIDs share some of these broad structural features and have been shown to activate PPARs (Lehmann et al 1997). Further, Nixon et al (2003) indicated that COX-2 inhibitors are potent activators of PPAR-γ. Our results are in agreement with the observation where PPAR-γ expression was significantly ($P<0.001$) enhanced by celecoxib, docetaxel and also a combination of docetaxel + celecoxib treatments, as compared with the control.

Conclusion

Results obtained from these studies indicate the potential of inhalation delivery of docetaxel in the treatment of lung cancer. However, further pre-clinical studies are needed to evaluate the advantages associated with inhalation delivery of docetaxel. Currently, studies are in progress in our laboratory to address this aspect. Further, a COX-2 inhibitor was found to potentiate the activity of aerosolized docetaxel against A549 cells, which may be useful as combination therapy for lung cancer. Based on this data, it can be concluded that an MDI formulation of docetaxel can deliver cytotoxic concentrations of the drug in the bronchoalveolar region of the lungs, which may be exploited for the treatment of lung cancer.

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