JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 851–860
© 2005 The Authors
Received December 9, 2004
Accepted March 7, 2005
DOI 10.1211/0022357056334
ISSN 0022-3573

Intratracheal budesonide-poly(lactide-co-glycolide) microparticles reduce oxidative stress, VEGF expression, and vascular leakage in a benzo(a)pyrene-fed mouse model

Nagesh Bandi, Surya P. Ayalasomayajula, Devender S. Dhanda, Jun Iwakawa, Pi-Wan Cheng and Uday B. Kompella

Abstract

The purpose of this study was to determine whether intratracheally instilled polymeric budesonide microparticles could sustain lung budesonide levels for one week and inhibit early biochemical changes associated with benzo(a)pyrene (B[a]P) feeding in a mouse model for lung tumours. Polymeric microparticles of budesonide-poly (DL-lactide-co-glycolide) (PLGA 50:50) were prepared using a solvent evaporation technique and characterized for their size, morphology, encapsulation efficiency, and in-vitro release. The microparticles were administered intratracheally (i.t.) to B[a]P-fed A/J mice. At the end of one week drug levels in the lung tissue and bronchoalveolar lavage (BAL) were estimated using HPLC and compared with systemic (intramuscular) administration. In addition, in-vivo end points including malondialdehyde (MDA), glutathione (GSH), total protein levels and vascular endothelial growth factor (VEGF) in BAL, and VEGF and c-myc mRNA levels in the lung tissue were assessed at the end of one week following intratracheal administration of budesonide microparticles. Budesonide-PLGA microparticles (1–2 μm), with a budesonide loading efficiency of 69–94%, sustained in-vitro budesonide release for over 21 days. Compared with the intramuscular route, intratracheally administered budesonide-PLGA microparticles resulted in higher budesonide levels in the BAL and lung tissue. In-vivo, B[a]P-feeding increased BAL MDA, lung VEGF mRNA, lung c-myc mRNA, BAL total protein, and BAL VEGF levels by 60, 112, 71, 154, and 78%, respectively, and decreased BAL GSH by 62%. Interestingly, intratracheally administered budesonide-PLGA particles inhibited these biochemical changes. Thus, biodegradable budesonide microparticles sustained budesonide release and reduced MDA accumulation, GSH depletion, vascular leakage, and VEGF and c-myc expression in B[a]P-fed mice, indicating the potential of locally delivered sustained-release particles for inhibiting angiogenic factors in lung cancer.

Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE, USA

Nagesh Bandi, Surya P. Ayalasomayajula, Devender S. Dhanda, Uday B. Kompella

Department Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

Jun Iwakawa, Pi-Wan Cheng

Correspondence: U. B. Kompella, UNMC College of Pharmacy, Omaha, NE 68198-6025, USA. E-mail: ukompell@unmc.edu

Funding and acknowledgements: The authors acknowledge the University of Nebraska Medical Center for providing graduate research fellowships to Nagesh Bandi and Surya P. Ayalasomayajula. The authors thank Dr Carol Toris for critical review of the manuscript. This work was supported in part by NIH grants R01 DK064172 (U. B. Kompella), R03 EY013842 (U. B. Kompella), and R01 HL56190 (Pi-Wan Cheng) and state of Nebraska NRI-Gene Therapy Program (Pi-Wan Cheng).

Introduction

Lung cancer is the second most common cancer in the United States, accounting for approximately 28% of cancer deaths (Giovino 2002; Nelson & Kelsey 2002). Significant progress has been made in the last decade in understanding the pathogenesis of lung cancer. Abnormalities of proto-oncogenes, genetic and epigenetic changes of tumour suppressor genes, and tumour angiogenesis are some of the important factors that underlie the pathogenesis of lung cancer. The involvement of tumour angiogenesis in the initiation and/or progression of lung cancer is supported by a plethora of evidence (Giatromanolaki 2001).

Angiogenesis, defined as new blood vessel growth, is an important biological process that governs the nourishment and/or development of a variety of tumours including those of the lung (Kerbel et al 1998; Bunn et al 2000; Herbst & Fidler 2000). Although multiple factors are likely responsible for the tumour angiogenesis in lung cancer, substantial evidence suggests a key role for vascular endothelial growth factor (VEGF), a potent 42-kDa endothelial specific mitogen. Evidence indicates that VEGF expression is four to six times greater in lungs with tumours compared with normal

lungs (Koukourakis et al 2000). Also, a correlation exists between elevated lung VEGF expression and vascular hyper-permeability in lung tumours (Karmpaliotis et al 2002). VEGF, through its ability to bind to its receptor, VEGF-R2/fetal liver kinase (flk), induces capillary leakage, fluid accumulation, and hence oedema (Karmpaliotis et al 2002). In addition, VEGF induces endothelial cell proliferation and formation of vasculature, tumour invasion, and metastasis. Thus, inhibition of lung VEGF expression has been identified as an attractive therapeutic strategy to treat lung cancer (Bunn et al 2000; Cox et al 2000). To this end, new treatment approaches directed against VEGF and its receptors are under development for treating lung cancer as well as other cancers. It is noteworthy that recently an anti-VEGF antibody (Avastin, Genentech) has been approved for the treatment of colorectal cancer.

Budesonide is being investigated as a potential candidate for inhibiting VEGF expression in lung cancer. Budesonide (MW 430.5 Da; log P = 3.2), a potent non-halogenated corticosteroid, is currently in clinical use for treating asthma, allergic rhinitis, and inflammatory bowel disease. Budesonide reduces VEGF expression in respiratory (airway and alveolar) and ocular (retinal pigment) epithelial cells (Bandi & Kompella 2001; Kompella et al 2003). Budesonide is known to prevent lung tumours (adenomas) induced by vinyl carbamate (Pereira et al 2002; Tao et al 2002) and benzo[a]pyrene (Wattenberg & Estensen 1997; Wattenberg et al 1997, 2000) in A/J mice. Although these studies indicated that budesonide prevented lung tumour formation, its ability to inhibit VEGF expression was not addressed. If budesonide is capable of inhibiting VEGF expression in lung tumours, potentially it can be used as an adjuvant to potentiate the effects of cytotoxic drugs (Eskens 2004). Clinically, regional chemotherapy has been useful in selected situations for treating colon cancer, melanoma, ovarian cancer, carcinomatous meningitis, and superficial bladder cancer (Ghussen & Kruger 1989; Markman 1996, 1999; Kemeny et al 1999). Using a regional therapy approach, Wattenberg et al (1997, 2000) demonstrated that nasal administration of budesonide as an aerosol at a low dose ($72\,\mu g\,kg^{-1}$, six times a week for 16 weeks) was as potent as a high dose dietary budesonide ($300\,\mu g\,kg^{-1}$, administered in the feed for 16 weeks) in preventing pulmonary tumour formation in benzo(a)pyrene (B[a]P)-fed female A/J mice. Therefore, this study assessed inhibition of VEGF expression in response to regional budesonide delivery. Although budesonide has been delivered by the inhalation route to prevent lung tumour formation (Wattenberg et al 1997, 2000), no attempts have been made to sustain the respiratory delivery of budesonide in a lung tumour model. Colloidal drug delivery systems including liposomes and polymeric particles are useful in sustaining drug delivery to the lungs (Lai et al 1993; Suarez et al 2001; Konduri et al 2003). This study used a biodegradable polymeric particulate system to sustain lung delivery of budesonide, because such a system allows efficient encapsulation and sustained release ranging from weeks to months.

The B[a]P-fed mouse is a widely used model to examine the molecular and cellular mechanisms governing the pathogenesis of lung cancer. Upon inhalation and/or ingestion, B[a]P is metabolized to mutagenic derivatives, which form DNA adducts within the target cells. Considerable experimental evidence has suggested that B[a]P induces oxidative stress, as indicated by an increase of lipid peroxidation end products such as malondialdehyde (MDA) and a decrease of antioxidants such as glutathione (GSH) (Yuan et al 1994; Garcon et al 2001). The induced oxidative stress can act as a potent stimulus for the upregulation of the expression of a variety of proto-oncogenes such as c-myc (Li & Spector 1997) and angiogenic genes such as VEGF (Maulik & Das 2002; Ruddell et al 2003). In addition, B[a]P-fed A/J mice exhibit K-ras oncogene mutations (Gray et al 2001), which are associated with VEGF gene expression (Konishi et al 2000). Possibly due to these biochemical changes, B[a]P-fed mice develop lung tumours (Wattenberg & Estensen 1997; Wattenberg et al 2000). Thus, the purpose of this study was to prepare biodegradable polymeric microparticles of budesonide and to assess their ability to reduce early biochemical changes including MDA formation, GSH depletion, VEGF and c-myc expression, and vascular leakage in a B[a]P-fed mouse model of lung tumour.

Materials and Methods

Chemicals

RT-PCR kit was obtained from Promega Corporation (Madison, WI, USA) and the primers for VEGF, c-myc, and 18S rRNA were custom synthesized (DNA Core Facility, University of Nebraska Medical Center, Omaha, NE, USA). Budesonide and polyvinyl alcohol (PVA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Poly (lactide-co-glycolide) (50:50) (PLGA) of intrinsic viscosities 0.17 and $0.66\,dL\,g^{-1}$ were obtained from Birmingham Polymers, Inc. (Birmingham, AL, USA). The HPLC grade methylene chloride and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Budesonide-PLGA particle formulation

Polymeric-budesonide microparticles were formulated using a solvent evaporation method using PLGA (50:50) of intrinsic viscosity $0.17\,dL\,g^{-1}$ (formulation 1, F1) or $0.66\,dL\,g^{-1}$ (formulation 2, F2). Budesonide and the polymer were dissolved in 1 mL dichloromethane and this solution was added to 10 mL aqueous PVA (2% w/v) solution. The resulting mixture was sonicated for 1.5 min at 20 W using a probe sonicator (Misonix Inc., Farmingdale, NY) to obtain an oil/water emulsion. The oil/water emulsion was immediately added drop-wise to 100 mL aqueous PVA (2% w/v) solution. The contents were stirred overnight at room temperature to evaporate the methylene chloride, allowing the formation of a turbid particulate suspension. The microparticles were separated

using centrifugation (1000 *g* for 30 min). The pellets were washed twice, re-suspended in de-ionized water, and freeze-dried to obtain lyophilized particles.

Electron microscopy of budesonide-PLGA particles

The morphology of the budesonide-PLGA microparticles was analysed using scanning (SEM) and transmission (TEM) electron microscopes.

SEM

A small quantity of microparticles were layered on the SEM stubs and coated with gold-palladium under an argon atmosphere using a gold sputter module in a high vacuum evaporator. Samples were then observed for their surface morphology with a Phillips SEM 51S scanning electron microscope set at 10 KV.

TEM

Initially, carbon-coated grids were floated on a droplet of the microparticles suspension on Parafilm, to permit the adsorption of the particles onto the grid. After blotting the grid with a filter paper and air-drying for 5 min, the grid was transferred onto a drop of the negative stain. Following this, the grid was blotted with a filter paper and air-dried for 5 min. Ammonium molybdate was used as a negative stain in these experiments. Finally, the samples were examined with an EM410 Phillips electron microscope set at 60 kV.

Zeta potential measurements

The zeta potential of the microparticles was measured using a zeta plus instrument (Brookhaven Instruments Corporation, Huntsville, NY, USA) in the zeta potential analysis mode. A sample of microparticles weighing 1.5 mg was suspended in 5 mL distilled water. The suspended particles were then sonicated for 60 s and the zeta potential was measured using palladium electrodes.

Particle size and drug loading measurement of budesonide-PLGA particles

Size

The particle size of budesonide-PLGA microparticles was determined using a particle size analyser (Brookhaven Instruments Corporation, Huntsville, NY) set at an angle of detection of 90^0. A 0.5-mg sample of polymeric-budesonide particles was suspended in 5 mL distilled water and the diluted suspension was then subjected to particle size measurement.

Drug loading

The loading efficiency of budesonide in F1 and F2 was determined by extracting and quantifying the encapsulated budesonide. Budesonide-polymeric particles (2 mg) were dissolved in 2 mL methylene chloride and the resulting solution was evaporated to dryness under nitrogen. The dried residue was reconstituted with 1000 μL acetonitrile:water mixture (70:30). This reconstituted solution was vortexed for 1 min, centrifuged at 12 000 *g* for 5 min, and 100 μL supernatant was injected onto the HPLC column. Drug recovery in this method was > 93%.

In-vitro drug release

The in-vitro release of budesonide from F1 and F2 was carried out at 37°C using dialysis membrane bags (molecular weight cut-off: 10 000, Spectrum Laboratories, Rancho Dominiguez, CA, USA) (Kompella et al 2003). A 0.5-mL suspension of budesonide-PLGA particles containing 160 μg budesonide was taken into the dialysis bag and the unit was allowed to float in 40 mL release medium (phosphate-buffered saline (PBS; pH 7.4) containing 0.025% sodium azide as a preservative). At various time points up to 21 days, 1.5 mL of the release medium was removed and replaced with fresh release medium. A 100-μL sample was directly analysed using an HPLC assay.

Tissue levels of budesonide

Female A/J mice (Jackson Laboratories, Bar Harbor, ME, USA; approximately five-weeks old and 20–25 g) were used in all experiments. The A/J mice carry lung cancer susceptibility genes (Stoner & Shimkin 1991). Lung adenomas in this mouse progress to adenocarcinomas, with some metastasis. These adenocarcinomas are morphologically similar to alveolgenic carcinomas in man. Female mice were chosen for this study because they exhibit greater lung tumour incidence/multiplicity and develop tumours more rapidly compared with the male A/J mice following B[a]P administration. This is possibly due to lower levels of detoxifying enzymes such as glutathione S-transferase isoenzymes in the female mice (Singh et al 1998).

The animals were fed a normal diet (Purina, Inc., Richmond, IN, USA). The mice were housed in a constant temperature facility with controlled lighting: lights on at 0600 h and off at 1800 h. The mice were given the first of three administrations of 2 mg B[a]P in 0.2 mL cottonseed oil. The time interval between the first and second doses was four days and that between the second and third doses was three days. Budesonide administration was made one week following the last dose of B[a]P. The mice were randomized into three groups of five each. Group 1: B[a]P-fed mice administered with single intratracheal instillation of budesonide-PLGA particles (budesonide dose: 150 μg); group 2: B[a]P-fed mice administered with single intramuscular injection of budesonide suspension (budesonide dose: 500 μg); and group 3: B[a]P-fed mice administered with single intramuscular injection of budesonide-PLGA particles (budesonide dose: 500 μg). The animals were killed at the end of one week of budesonide treatment and the lungs were isolated. In all the in-vivo studies, particles from formulation F1 were used. The protocol for the various animal studies was approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center.

Intratracheal instillation
After anaesthetizing the animals using an intraperitoneal injection of avertin (5 mg/20 g), each mouse received an intratracheal instillation of budesonide-PLGA particles (containing 150 μg budesonide) suspended in sterile saline. The budesonide-PLGA particle suspension was delivered to the trachea by bolus injection using a 27-gauge, 0.75-in angiocath (Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT) (Yanagihara et al 2001).

Intramuscular administration
After anaesthetizing the animals using an intraperitoneal injection of avertin (5 mg/20 g), one group of mice received an intramuscular injection of budesonide suspension (500 μg budesonide) and the other group received an intramuscular injection of budesonide-PLGA particles (500 μg budesonide) suspended in sterile saline. Both forms of budesonide (100 μL) were administered intramuscularly into the tibial cranial muscle, longitudinally, using a 27-gauge needle.

Isolation of bronchoalveolar lavage (BAL) and lung tissues
After killing the animal, the trachea was cut at the oesophageal region and the lung lobes were isolated along with the trachea (Yanagihara et al 2001). Following isolation, a small incision was made on either of the lung lobes and 1 mL sterile PBS was perfused through the trachea using a 27-gauge needle and the lavage fluids were collected. Using the collected lavage, the procedure was repeated three times and the recovered fluids were pooled. Samples of the BAL were immediately processed for GSH, MDA, total protein, and VEGF protein estimations. Part of the lung tissue was immediately extracted for total RNA and the RNA was stored at -80°C for RT-PCR analysis within two days. Parts of BAL and tissue were stored at -80°C and used within a week for drug analysis.

HPLC assay of budesonide

The amount of budesonide in samples obtained from in-vitro release, drug loading measurement studies, and lung tissue and BAL obtained in disposition studies was determined using a previously reported HPLC assay (Chanoine et al 1991). Samples from in-vitro release and drug loading measurement studies were directly injected after processing as described above. For drug analysis in lung tissue, the isolated lung tissue was homogenized in 200 μL PBS buffer (Tissue Tearor, Fisher Scientific, Pittsburgh, PA). To the homogenate, 2.5 μL 40 μg mL^{-1} solution of celecoxib was added as an internal standard and mixed thoroughly. To this, methylene chloride (2 mL) was added and mixed thoroughly at room temperature for 5 min. The resulting extract was evaporated to dryness under nitrogen and the dried residue was reconstituted with 150 μL acetonitrile:water (70:30) mixture. This reconstituted solution was vortexed for 1 min, centrifuged at 12 000 g for 5 min, and 100 μL supernatant was injected onto a Waters HPLC equipped with a pump (Waters TM 616), a controller (Waters 600 S), an autoinjector (Waters 717 plus), and a PDA detector (Waters 996). For the BAL, an appropriate concentration of internal standard was added and a similar extraction procedure was followed. The drug was separated isocratically on a 25-cm long Discovery C-18 column (Supelco, Emeryville, CA) with a particle diameter of 5 μm and a pore size of 100 Å, using a mobile phase consisting of acetonitrile and aqueous buffer mixture (70:30 v/v). The buffer was 0.1% acetic acid in water at pH 3. The mobile phase flow rate was set at 1 mL min^{-1} and the drugs were detected using a photodiode array detector set at 250 nm. The peak areas were integrated using Millennium software (Version 2.15.01). The limit of detection of the HPLC assay was 1 ng and the budesonide recovery from the tissues with the above extraction procedure was $> 95\%$.

In-vivo effect study

For this study, the mice were randomized into three groups of four each. Group 1: untreated mice; group 2: B[a]P-fed mice; group 3: B[a]P-fed mice administered with single intratracheal instillation of budesonide-PLGA particles (budesonide dose: 150 μg). The animals were killed at the end of one week of budesonide treatment and the lungs were isolated. In all the effect studies, particles from formulation F1 were used. The B[a]P treatment for all the mice was similar to that described for the disposition study.

Measurement of biological end points after budesonide administration

BAL malondialdehyde (MDA) levels
BAL levels of MDA, the end product of lipid peroxidation, were determined using a TBARS (thiobarbituric acid reacting substances) assay (Janero 1990). MDA reacts with TBA to produce a fluorescence product, which can be measured using a spectrofluorometric assay. For this analysis, BAL was centrifuged at 1000 g for 10 min to remove cells, which were likely to interfere with the measurement. The supernatant was separated and treated with 10% trichloroacetic acid (TCA) to precipitate the proteins that were likely to interfere with the assay. MDA was measured in the supernatants (100 μL) of the TCA-treated samples. 1,1,3,3-Tetramethoxypropane was used as the standard and MDA was estimated at excitation and emission wavelengths of 525 and 547 nm, respectively.

BAL glutathione (GSH) levels
GSH level in BAL, a measure of antioxidant status, was determined using a spectrofluorometric method described by Ayalasomayajula & Kompella (2002). The assay is based on the reaction of GSH with O-phthalaldehyde to form a fluorescence product. The samples for the assay were prepared as described above for MDA analysis. GSH reaction product in the supernatants of the TCA-treated BAL was measured at excitation and emission wavelengths of 350 and 420 nm, respectively.

BAL protein
The isolated BAL was centrifuged at 1000 g for 10 min to separate any cells. The pellet was discarded and the supernatant was used for protein analysis. Determinations were performed using the bicinchoninic acid microassay method (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's instructions.

Semi-quantitative analysis of VEGF mRNA
Total RNA was extracted from the isolated lungs using RNA STAT-60 RNA isolation kit (TEL-TEST, Friendswood, TX, USA). The RNA pellet was dissolved in autoclaved water, quantified for RNA and a volume equivalent to 3 μg was taken to amplify VEGF mRNA using access RT-PCR kit as described by Ayalasomayajula & Kompella (2003). The PCR was done for 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s. Finally an extension step at 72°C was carried out for 5 min. The VEGF mRNA expressions were normalized to 18S rRNA, used as internal control. The products were separated by electrophoresis on a 2% agarose gel and quantified using densitometric analysis (Nucleovision Imaging System, Nucleotech, San Mateo, CA). A plot of band intensities of VEGF or 18S rRNA genes vs number of PCR cycles (10, 20, 30, 35, and 40 cycles) indicated that the products obtained at 30 cycles were in the linear range (r^2 values for VEGF and 18S rRNA were 0.98 and 0.92, respectively). The forward and reverse primer sequences for VEGF analysis were GACCCTGGTGGACATCTTCCAGGA and GGTGAGAGGTCTAGTTCCCGA, respectively. The forward and reverse primer sequences for 18S rRNA analysis were GGACCAGAGGCAAAGCATTTGCC and TCAATCTCGGGTGGCTGAACGC, respectively. The DNA sequencing of PCR products followed by a Blast sequence search (Genebank database Accession #: NM_03186.1) confirmed $VEGF_{188}$ (514 bp), $VEGF_{164}$ (462 bp), $VEGF_{120}$ (330 bp), and 18S rRNA (495 bp).

Semi-quantitative analysis of c-myc mRNA
RTPCR was performed using 3 μg RNA using a method described by Tao et al (2002). The amplification was done for 30 cycles at 95°C for 1 min, 57°C for 1 min, and 72°C for 6 min. Finally an extension step at 72°C was carried out for 5 min. The c-myc mRNA (393 bp) expression was normalized to 18S rRNA. A plot of band intensities of c-myc or 18S rRNA genes vs number of PCR cycles (10, 20, 30, 35, and 40 cycles) indicated that the products obtained at 30 cycles were in the linear range (r^2 values for c-myc and 18S rRNA were 0.98 and 0.92, respectively). The DNA sequencing of the PCR product of 393 bp followed by Blast sequence search (Genebank database Accession #: Z38066) identified the product as c-myc. The forward and reverse primer sequences for c-myc analysis were TGACGAGACCTTCGTGAAGA and ATTGATGTTATTTACACTTAAGGGT, respectively.

BAL VEGF levels
The VEGF protein levels in 50 μL BAL samples were estimated using an ELISA method according to manufacturer's recommendations (R & D systems, New Jersey). The kit measured $VEGF_{164}$ and $VEGF_{120}$ isoforms of VEGF.

Statistical analysis

Unless otherwise stated the results were expressed as mean ± s.d. Mean values between the different treatments were compared using analysis of variance followed by Tukey's post hoc analysis using SPSS (version 8.0) software. Differences were considered statistically significant at $P < 0.05$.

Results

Particle morphology, particle size, and encapsulation efficiencies of budesonide-PLGA particles

Particle morphology
The microscopy pictures indicated mostly spherical particles with smooth surfaces.

Particle size and zeta potential
The mean particle size of formulations F1 and F2 were 0.93 ± 0.03 and 2.3 ± 0.02 μm, respectively (Table 1). The zeta potentials of the particles were -32 ± 3.2 and -21 ± 1.6 mV, respectively. The significant variation in zeta potential could be explained by the differences in polymer molecular weight, which was inversely related to the number of carboxylate end groups in the polymer. Thus, the F1 employing a lower molecular weight (12 000) polymer gave a higher zeta potential compared with F2 with a higher molecular weight (66 000) polymer. The zeta potential was not significantly different between the formulations with and without the drug, indicating potential lack of interference of surface drug with zeta potential and encapsulation of the drug.

Encapsulation efficiency
Budesonide encapsulation studies indicated that the encapsulation efficiency of budesonide in F1 and F2 were 69 ± 7 and 94 ± 3%, respectively.

In-vitro drug release

The release of budesonide from F1 and F2 formulations into PBS was measured in-vitro at 37°C (Figure 1). For F1, following an initial burst release of 17% budesonide, the drug release was sustained over the three-week period. The cumulative budesonide release at the end of three

Table 1 The particle size and zeta potential of poly(lactide-co-glycolide) microparticles

	Formulation 1 (with drug)	Formulation 1 (without drug)	Formulation 2 (with drug)	Formulation 2 (without drug)
Particle size (μm)	0.93 ± 0.03	0.85 ± 0.04	2.3 ± 0.02	1.23 ± 0.05
Zeta potential (mV)	-32 ± 1.2	-34 ± 1.9	-21 ± 1.6	-24 ± 3.1

Mean ± s.d. is indicated for n = 3.

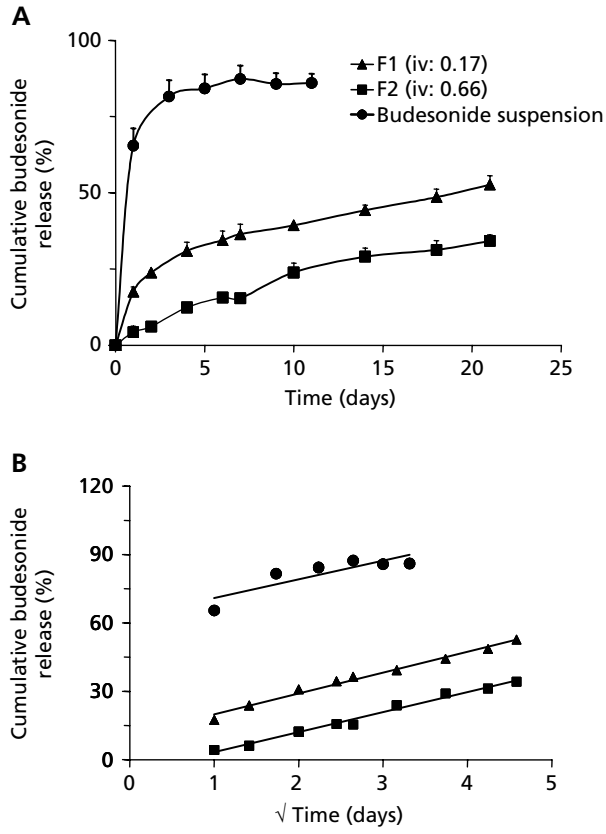

Figure 1 A. Percent budesonide released from microparticle formulations and budesonide suspension. Data are expressed as mean ± s.d. for n = 4. B. Percent budesonide released vs square root of time plot for various budesonide formulations. ▲, PLGA 50:50 (intrinsic viscosity: 0.17 dL g^{-1}) microparticles; ■, PLGA 50:50 (intrinsic viscosity: 0.66 dL g^{-1}) microparticles; ●, budesonide suspension.

weeks was ~53% of the initial drug loading. Budesonide release from F2 was devoid of any significant burst (~4% at the end of day 1). The budesonide release was also sustained over the three-week study period and the cumulative budesonide release at the end of three weeks was 35% of the initial drug loading. A Higuchi plot (cumulative amount released vs square root of time) was linear for formulations F1 ($r^2 = 0.99$) and F2 ($r^2 = 0.99$) unlike the plain budesonide suspension ($r^2 = 0.86$), indicating that budesonide was entrapped within the PLGA matrix. Budesonide-PLGA microparticles with 0.93 μm diameter (F1) were further assessed for budesonide delivery and effect in-vivo in B[a]P-fed A/J mice.

Budesonide levels in BAL and lung tissue

Budesonide levels in BAL and lung tissue were compared following a single intratracheal instillation of budesonide-PLGA particles (150 μg budesonide), or single intramuscular injection of budesonide suspension (500 μg budesonide), or single intramuscular injection of budesonide-PLGA particles (500 μg budesonide) at the end of one week (Table 2). In the lung, budesonide levels were

Table 2 Budesonide levels in the lung tissue and BAL following single dose administration of budesonide-PLGA 50:50 (intrinsic viscosity: 0.17 dL g^{-1}) microparticles or budesonide suspension to B[a]P-fed mice. Drug levels were quantified at the end of one week following drug administration

Drug levels	Intramuscular suspension	Intramuscular microparticles	Intratracheal microparticles
Lung tissue (ng mg^{-1} (mg dose)$^{-1}$)	6.14 ± 0.94	15.76 ± 4.30	224.93 ± 21.65*
Bronchoalveolar lavage (ng mL^{-1})	n.d.	22.91 ± 20.15	60.65 ± 20.85*

Budesonide was administered at a dose of 150, 500, and 500 μg for intratracheal microparticle, intramuscular suspension, and intramuscular microparticle groups, respectively. *$P < 0.05$, significance between intratracheal and intramuscular groups. Data are expressed as mean ± s.d. for n = 5 for lung tissue, n = 3 for BAL with intramuscular suspension, n = 4 for BAL with intratracheal and intramuscular microparticles. n.d. indicates drug levels were below detection limits in the intramuscular suspension group.

225 ± 22, 6 ± 1, and 16 ± 4 ng (mg tissue)$^{-1}$ (mg dose)$^{-1}$, for the intratracheal particle, intramuscular suspension, and intramuscular particle groups, respectively. In the BAL, budesonide levels were 61 ± 21 and 23 ± 10 ng mL^{-1} for the intratracheal particles and intramuscular particles groups, respectively. Budesonide was below the detection limit in the BAL for the budesonide suspension group.

Efficacy of intratracheal budesonide microparticles in B[a]P-fed mice

Budesonide-PLGA microparticles reduced MDA accumulation

The levels of MDA, an end product of lipid peroxidation, indicate oxidative stress in a biological system. B[a]P administration significantly increased MDA levels in BAL. Treatment with intratracheal budesonide microparticles significantly reduced this MDA accumulation (Figure 2A).

Budesonide-PLGA microparticles reduced GSH depletion

Since B[a]P-induced biological effects are associated with depletion of intracellular GSH, intracellular GSH levels in BAL were estimated with and without budesonide treatment. Compared with untreated controls in group 1, B[a]P-fed mice in group 2 exhibited significantly lower BAL GSH concentration. Treatment with intratracheal budesonide microparticles significantly inhibited this GSH depletion in B[a]P-fed mice (Figure 2B).

Budesonide-PLGA microparticles reduced capillary leakage

Protein content in BAL was used as a measure of capillary leakage. Compared with untreated controls in group 1, B[a]P-fed mice in group 2 indicated a significant increase in BAL protein content. Treatment of B[a]P-fed mice with

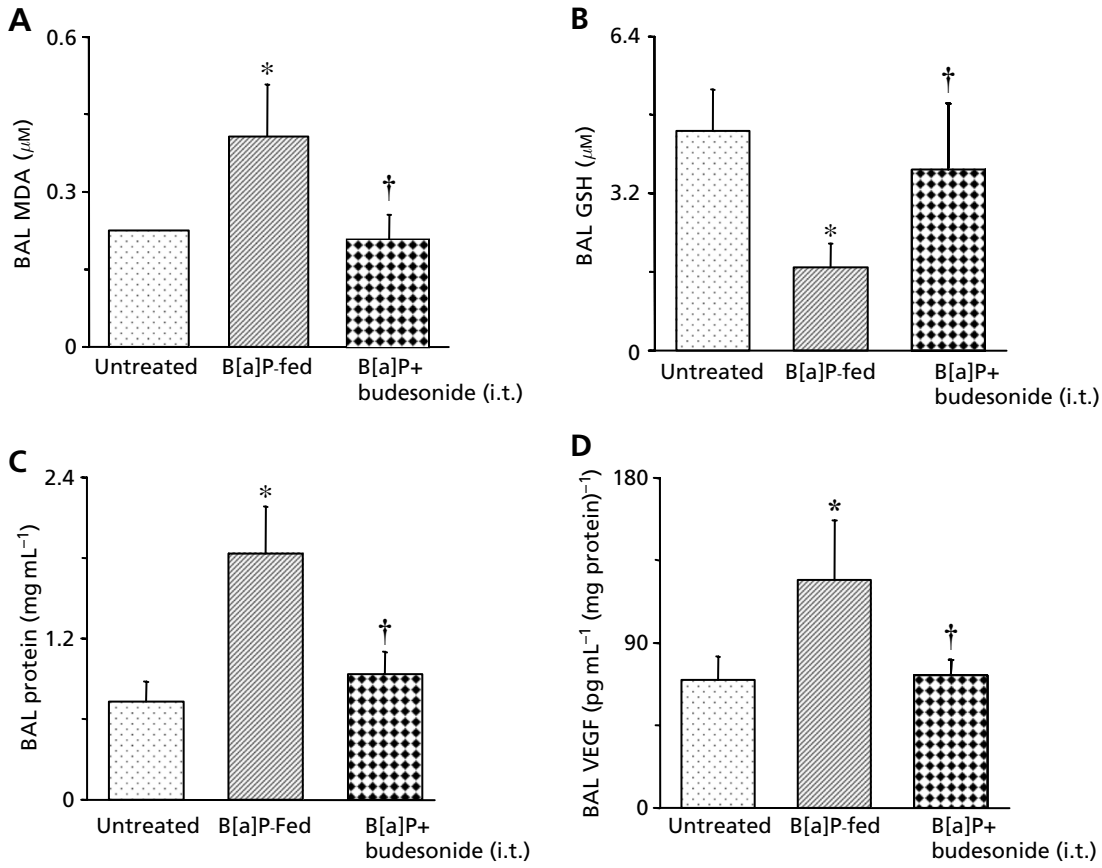

Figure 2 Malondialdehyde (MDA) (A), glutathione (GSH) (B), total protein (C), and VEGF protein (D) levels in BAL at the end of one week following drug administration. Equivalent of 150 μg budesonide in the form of budesonide-PLGA 50:50 (intrinsic viscosity: 0.17 dL g^{-1}) microparticles to B[a]P-fed mice. Data are expressed as mean ± s.d. for n = 4. *$P < 0.05$, between untreated and B[a]P-fed groups. †$P < 0.05$, compared with B[a]P-fed group.

intratracheal budesonide microparticles significantly reduced this elevated BAL protein content (Figure 2C).

Budesonide-PLGA microparticles reduced BAL VEGF levels

B[a]P treatment significantly increased BAL VEGF levels from 69.6 ± 13 to 124 ± 32 pg mL^{-1} (mg protein)$^{-1}$ (Figure 2D). Intratracheal administration of budesonide-PLGA microparticles significantly reduced the elevated BAL VEGF levels to 72.4 ± 8.2 pg mL^{-1} (mg protein)$^{-1}$. For BAL VEGF estimations, in addition to the above groups, we assessed the effect of intratracheal budesonide suspension in B[a]P-fed mice (n = 4). However, intratracheal administration of budesonide suspension did not reduce the BAL VEGF levels significantly (99.1 ± 45.8 pg mL^{-1} (mg protein)$^{-1}$).

Lung VEGF mRNA expression

VEGF mRNA expression in mouse lung tissues indicated bands at 514, 462, 330, and 495 bp corresponding to $VEGF_{188}$, $VEGF_{164}$, $VEGF_{120}$, and 18S rRNA, respectively (Figure 3). Compared with group 1 mice, B[a]P-fed mice in group 2 indicated 112% increase in VEGF mRNA expression and this effect was statistically significant ($P < 0.05$). Treatment of B[a]P-fed mice with intratracheal budesonide microparticles (group 3) significantly inhibited VEGF mRNA elevation in group 2, suggesting the ability of budesonide microparticles to inhibit elevated VEGF mRNA expression in-vivo ($P < 0.05$).

Lung c-myc mRNA expression

c-myc mRNA expression in mouse lung tissues was reflected as an RTPCR band at 393 bp (Figure 3). Compared with group 1 mice, B[a]P-fed mice in group 2 showed a 71% increase in c-myc mRNA expression and this effect was statistically significant ($P < 0.05$). Treatment of B[a]P-fed mice with intratracheal budesonide microparticles (group 3) significantly reduced the elevated c-myc mRNA in group 2, indicating the ability of budesonide to inhibit elevated c-myc mRNA expression in a B[a]P mouse model for lung tumours ($P < 0.05$).

Discussion

Local delivery of budesonide to the lungs is a desirable method to inhibit VEGF expression in lung tumours

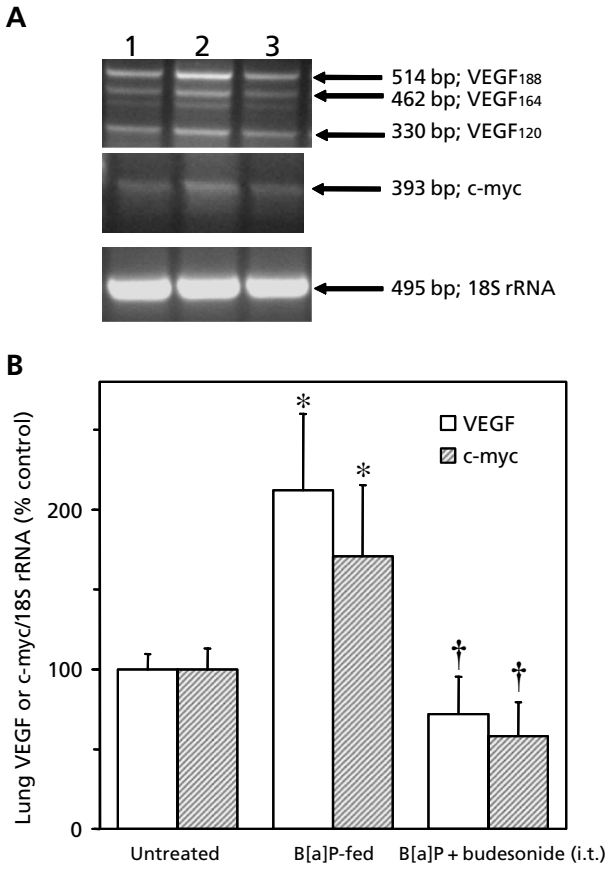

Figure 3 Effect of budesonide-PLGA microparticles on VEGF and c-myc mRNA expression in B[a]P-fed mice. A. VEGF, c-myc, and 18S rRNA expression at the end of one week following drug administration. B. Densitometric analysis of VEGF, c-myc, and 18S rRNA expression in lung tissues. Data are expressed as the mean ratio of VEGF or c-myc/18S rRNA band intensity. The lane assignments for VEGF, c-myc, and 18S expression in lung tissues are: lane 1: untreated mice; lane 2: B[a]P-fed mice; lane 3: single intratracheal administration of budesonide-PLGA 50:50 (intrinsic viscosity: 0.17 dL g^{-1}) microparticles (budesonide dose = 150 μg) to B[a]P-fed mice. Data are expressed as mean ± s.d. for n = 4 for VEGF and n = 3 for c-myc. *$P < 0.05$, between untreated and B[a]P-fed groups. †$P < 0.05$, compared with B[a]P-fed groups.

because oral administration of budesonide results in extensive hepatic first-pass metabolism (~85%) and because local delivery minimizes the systemic side effects of corticosteroids (Clissold & Heel 1984). Use of sustained release microparticle aerosols reduces the dosing frequency, which might be particularly useful in severely ill patients.

In this study, PLGA microparticles were used to sustain budesonide release. PLGA is used in the fabrication of several FDA-approved sustained-release injectable products including Trelstar Depot, Nutropin Depot, and Sandostatin LAR (Jain 2000), due to its biodegradability, biocompatibility, and formulation flexibility. When compared with small microparticles (F1), large microparticles (F2) resulted in lower burst release of the drug (Figure 1). This was consistent with the lower surface area and surface drug present in the larger particles. When encapsulated in polymers, budesonide loses its crystallinity (Kompella et al 2001). Despite any increase in drug solubility due to loss of crystallinity, budesonide release was sustained (Figure 1), indicating that the drug was entrapped in the polymeric matrix.

Following administration of budesonide-PLGA microparticles (F1) in A/J mice, the BAL and lung budesonide concentrations were higher with the intratracheal budesonide microparticles compared with the intramuscular microparticles, indicating the superiority of regional administration for budesonide delivery to the lungs (Table 2). At one week following intramuscular administration, microparticles resulted in higher drug levels compared with the drug suspension, consistent with the ability of microparticles to better sustain drug delivery. In other studies using CD1 mice, we observed that intratracheal administration of budesonide-poly(lactide) microparticle suspension resulted in 10-fold drug levels compared with budesonide suspension or budesonide-hydroxypropyl β-cyclodextrin solution administered by the same route (unpublished data). A human study indicated that the plasma half-life of plain budesonide micro- and nano-suspensions administered to lungs via nebulizers was approximately five to six hours, with a t_{max} value of 14 min for micro-suspension and 8 min for nano-suspension (Kraft et al 2004). These absorption profiles were likely reflective of lung tissue levels as well. Based on such lung disposition, budesonide particles are administered once or twice daily. The polymeric system proposed in our study would probably allow less frequent administrations. Therefore, intratracheally administered budesonide-PLGA microparticles were assessed for their ability to inhibit oxidative stress, VEGF and c-myc expression, and BAL protein levels in a B[a]P-fed A/J mouse model for lung tumours.

We observed a significant reduction of GSH and a concomitant increase in MDA in the BAL of B[a]P-fed mice (Figure 2A, B). These changes indicated an elevation of oxidative stress in the lung tissues. In-vitro studies have suggested that the GSH S-tranferase (GST)-catalysed GSH conjugation is an important mechanism for the detoxification of (+)-anti-7,8-dihydroxy-9,10-epoxy-7,8,8,10-tetrahydrobenzo(a)pyrene ((+)-anti-BPDE), which is the activated form of B[a]P (Srivastava et al 2000). Budesonide microparticles reduced GSH depletion and MDA concentration in BAL at one week post-dosing, suggesting their ability to reduce oxidative stress in the B[a]P-fed mouse model (Figure 2A, B). This protective effect of budesonide may be beneficial in detoxifying carcinogens such as B[a]P. In the B[a]P-fed mice, elevated oxidative stress was associated with an elevation in lavage fluid protein (Figure 2C), which was inhibited by budesonide-PLGA microparticle treatment. Elevated BAL protein can be explained by vascular leakage, which can be anticipated if VEGF expression in the lung tissue is upregulated in B[a]P-fed mice.

Inhibition of oxidative stress by budesonide-PLGA microparticles is particularly advantageous in treating

tumours. Oxidative stress, identified as an imbalance between oxidants and antioxidants, is a potent stimulus for the expression of several pro-inflammatory and tumour-inducing genes such as VEGF and c-myc. Although previous studies observed elevated lung expression of several pro-inflammatory genes including TNF-α, IL-1β, and iNOS mRNA in the B[a]P-fed mouse model (Garcon et al 2001), there were no reports of VEGF expression in this model. This is the first study to report the elevation of lung VEGF expression in a B[a]P-fed mouse model for lung tumours (Figure 3). Previous studies in a vinyl carbamate-fed mouse model (34 weeks) and the B[a]P-fed mouse model (24 weeks) indicated alteration in tumour-modifying genes (c-myc, p21, and p27) (Pereira et al 2002; Tao et al 2002). However, there were no reports of c-myc expression after short-term administration in the B[a]P-fed mouse model. Interestingly, budesonide-PLGA microparticle treatment significantly reduced the elevated lung expression of VEGF and c-myc (Figure 3). The possible mechanisms of budesonide action could be inhibition of transcriptional factors (Jonat et al 1990) or remethylation of DNA (Tao et al 2002).

To confirm the reduction of VEGF expression by budesonide-PLGA microparticles, we observed that these microparticles reduced VEGF levels in the B[a]P-fed mice (Figure 2D). Plain drug suspension of an equal dose did not result in a significant reduction of BAL VEGF levels, indicating the advantage of sustaining budesonide delivery for inhibiting lung VEGF expression. Evidence from previous studies and results from this study indicated that B[a]P treatment induced the cellular and/or tissue expression of pro-angiogenic cytokines (Garcon et al 2001) including VEGF. The elevated VEGF was likely responsible for vascular hyper-permeability and hence, elevated BAL protein levels observed in this study. Together, these observations confirmed that A/J mice developed inflammatory and/or pro-angiogenic responses to B[a]P, which could be inhibited by budesonide-PLGA microparticles.

Since VEGF contributes to the vascular complications of lung cancer including angiogenesis (Koukourakis et al 2000), budesonide-PLGA particles capable of inhibiting VEGF expression would serve as a useful chemotherapeutic adjuvant. Budesonide-PLGA microparticles potentially could be combined with sustained release systems for cytotoxic drugs such as paclitaxel to potentiate the cytotoxic effects and minimize the side effects of the paclitaxel by allowing dose reduction (Marchetti et al 2002). Thus, lung delivery of drug encapsulating polymeric microparticles could be an effective approach for inhibiting VEGF expression and possibly lung tumours. Such drug delivery approaches might provide an alternative to daily systemic therapy, with the potential to reduce toxicity and improve patient compliance in lung cancer therapy.

Conclusions

Budesonide-PLGA microparticles sustained drug release in-vitro and following intratracheal administration in-vivo. This is the first study to have demonstrated the delivery of budesonide to the lung tissue using polymeric microparticles. Furthermore, this is the first study to have demonstrated the ability of the budesonide-PLGA microparticles to normalize oxidative stress (accumulation of MDA and depletion of GSH), VEGF and c-myc expression, and vascular permeability in a B[a]P-fed mouse model for lung tumours. Since budesonide-PLGA microparticles inhibited the expression of VEGF as well as its potent stimulus, oxidative stress, they are of potential value in inhibiting angiogenesis and tumour growth in the lung.

References

Ayalasomayajula, S. P., Kompella, U. B. (2002) Induction of vascular endothelial growth factor by 4-hydroxynonenal and its prevention by glutathione precursors in retinal pigment epithelial cells. *Eur. J. Pharmacol.* **449**: 213–220

Ayalasomayajula, S. P., Kompella, U. B. (2003) Celecoxib, a selective cyclooxygenase-2 inhibitor, inhibits retinal vascular endothelial growth factor expression and vascular leakage in a streptozotocin-induced diabetic rat model. *Eur. J. Pharmacol.* **458**: 283–289

Bandi, N., Kompella, U. B. (2001) Budesonide reduces vascular endothelial growth factor secretion and expression in airway (Calu-1) and alveolar (A549) epithelial cells. *Eur. J. Pharmacol.* **425**: 109–116

Bunn, P. A., Soriano, A., Johnson, G., Heasley, L. (2000) New therapeutic strategies for lung cancer: biology and molecular biology come of age. *Chest* **117**: 163S–168S

Chanoine, F., Grenot, C., Heidmann, P., Junien, J. L. (1991) Pharmacokinetics of butixocort 21-propionate, budesonide, and beclomethasone dipropionate in the rat after intratracheal, intravenous, and oral treatments. *Drug Metab. Dispos.* **19**: 546–553

Clissold, S. P., Heel, R. C. (1984) Budesonide. A preliminary review of its pharmacodynamic properties and therapeutic efficacy in asthma and rhinitis. *Drugs* **28**: 485–518

Cox, G., Jones, J. L, Walker, R.A., Steward, W. P., O'Byrne, K. J. (2000) Angiogenesis and non-small cell lung cancer. *Lung Cancer* **27**: 81–100

Eskens, F. A. (2004) Angiogenesis inhibitors in clinical development; where are we now and where are we going? *Br. J. Cancer* **90**: 1–7

Garcon, G., Gosset, P., Garry, S., Marez, T., Hannothiaux, M. H., Shirali, P. (2001) Pulmonary induction of proinflammatory mediators following the rat exposure to benzo(a)pyrene-coated onto Fe_2O_3 particles. *Toxicol. Lett.* **121**: 107–117

Ghussen, F., Kruger, I. (1989) Technical aspects of isolation extremity perfusion: experimental studies and clinical experience. *J. Invest. Surg.* **2**: 487–496

Giatromanolaki, A. (2001) Prognostic role of angiogenesis in non-small cell lung cancer. *Anticancer Res.* **21**: 4373–4382

Giovino, G. A. (2002) Epidemiology of tobacco use in the United States. *Oncogene* **21**: 7326–7340

Gray, D. L., Warshawsky, D., Xue, W., Nines, R., Wang, Y., Yao, R., Stoner, G. D. (2001) The effects of a binary mixture of benzo(a)pyrene and 7H-dibenzo(c,g)carbazole on lung tumors and K-ras oncogene mutations in strain A/J mice. *Exp. Lung Res.* **27**: 245–253

Herbst, R. S., Fidler, I. J. (2000) Angiogenesis and lung cancer: potential for therapy. *Clin. Cancer Res.* **6**: 4604–4606

Jain, R. A. (2000) The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials* **21**: 2475–2490

Janero, D. R. (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **9**: 515–540

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H., Herrlich, P. (1990) Antitumor promotion and anti-inflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* **62**: 1189–1204

Karmpaliotis, D., Kosmidou, I., Ingenito, E. P., Hong, K., Malhotra, A., Sunday, M. E., Haley, K. J. (2002) Angiogenic growth factors in the pathophysiology of a murine model of acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**: L585–595

Kemeny, N., Huang, Y., Cohen, A. M., Shi, W., Conti, J. A., Brennan, M. F., Bertino, J. R., Turnbull, A. D., Sullivan, D., Stockman, J., Blumgart, L. H., Fong, Y. (1999) Hepatic arterial infusion of chemotherapy after resection of hepatic metastases from colorectal cancer. *N. Engl. J. Med.* **341**: 2039–2048

Kerbel, R. S., Viloria-Petit, A., Okada, F., Rak, J. (1998) Establishing a link between oncogenes and tumor angiogenesis. *Mol. Med.* **4**: 286–295

Kompella, U. B., Bandi, N., Ayalasomayajula, S. P. (2001) Poly(lactic acid) nanoparticles for sustained release of budesonide. *Drug Del. Technol.* **1**(1): 28–34

Kompella, U. B., Bandi, N., Ayalasomayajula, S. P. (2003) Subconjunctival nano- and microparticles sustain retinal delivery of budesonide, a corticosteroid capable of inhibiting VEGF expression. *Invest. Ophthalmol. Vis. Sci.* **44**: 1192–1201

Konduri, K. S., Nandedkar, S., Duzgunes, N., Suzara, V., Artwohl, J., Bunte, R., Gangadharam, P. R. (2003) Efficacy of liposomal budesonide in experimental asthma. *J. Allergy Clin. Immunol.* **111**: 321–327

Konishi, T., Huang, C. L., Adachi, M., Taki, T., Inufusa, H., Kodama, K., Kohno, N., Miyake, M. (2000) The K-ras gene regulates vascular endothelial growth factor gene expression in non-small cell lung cancers. *Int. J. Oncol.* **16**: 501–511

Koukourakis, M. I., Giatromanolaki, A., Thorpe, P. E., Brekken, R. A., Sivridis, E., Kakolyris, S., Georgoulias, V., Gatter, K. C., Harris, A. L. (2000) Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res.* **60**: 3088–3095

Kraft, W. K., Steiger, B., Beussink, D., Quiring, J. N., Fitzgerald, N., Greenberg, H. E., Waldman, S. A. (2004) The pharmacokinetics of nebulized nanocrystal budesonide suspension in healthy volunteers. *J. Clin. Pharmacol.* **44**: 67–72

Lai, Y. L., Mehta, R. C., Thacker, A. A., Yoo, S. D., McNamara, P. J., DeLuca, P. P. (1993) Sustained bronchodilation with isoproterenol poly(glycolide-co-lactide) microspheres. *Pharm. Res.* **10**: 119–125

Li, D. W., Spector, A. (1997) Hydrogen peroxide-induced expression of the proto-oncogenes, c-jun, c-fos and c-myc in rabbit lens epithelial cells. *Mol. Cell Biochem.* **173**: 59–69

Marchetti, P., Urien, S., Cappellini, G. A., Ronzino, G., Ficorella, C. (2002) Weekly administration of paclitaxel: theoretical and clinical basis. *Crit. Rev. Oncol. Hematol.* **44** (Suppl.): S3–13

Markman, M. (1996) Intraperitoneal therapy of ovarian cancer. *Oncologist* **1**: 18–21

Markman, M. (1999) Intraperitoneal chemotherapy in the management of colon cancer. *Semin. Oncol.* **26**: 536–539

Maulik, N., Das, D. K. (2002) Redox signaling in vascular angiogenesis. *Free Radic. Biol. Med.* **33**: 1047–1060

Nelson, H. H., Kelsey, K. T. (2002) The molecular epidemiology of asbestos and tobacco in lung cancer. *Oncogene* **21**: 7284–7288

Pereira, M. A., Li, Y., Gunning, W. T., Kramer, P. M., Al-Yaqoub, F., Lubet, R. A., Steele, V. E., Szabo, E., Tao, L. (2002) Prevention of mouse lung tumors by budesonide and its modulation of biomarkers. *Carcinogenesis* **23**: 1185–1192

Ruddell, A., Mezquita, P., Brandvold, K. A., Farr, A., Iritani, B. M. (2003) B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics during lymphomagenesis. *Am. J. Pathol.* **163**: 2233–2245

Singh, S. V., Benson, P. J., Hu, X., Pal, A., Xia, H., Srivastava, S. K., Awasthi, S., Zaren, H. A., Orchard, J. L., Awasthi, Y. C. (1998) Gender-related differences in susceptibility of A/J mouse to benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis. *Cancer Lett.* **128**: 197–204

Srivastava, S. K., Xia, H., Pal, A., Hu, X., Guo, J., Singh, S. V. (2000) Potentiation of benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis in mice by D, L-buthionine-S, R-sulfoximine-mediated tissue glutathione depletion. *Cancer Lett.* **153**: 35–39

Stoner, G. D., Shimkin, M. B. (1991) Lung tumors in strain A mice as a bioassay for carcinogenicity. In: Milman, H. A., Weisburger, E. K. (eds) *Handbook of carcinogen testing*. Noyes Publications, Park Ridge, NJ, pp 179–214

Suarez, S., O'Hara, P., Kazantseva, M., Newcomer, C. E., Hopfer, R., McMurray, D. N., Hickey, A. J. (2001) Airways delivery of rifampicin microparticles for the treatment of tuberculosis. *J. Antimicrob. Chemother.* **48**: 431–434

Tao, L., Li, Y., Wang, W., Kramer, P. M., Gunning, W. T., Lubet, R. A., Steele, V. E., Pereira, M. A. (2002) Effect of budesonide on the methylation and mRNA expression of the insulin-like growth factor 2 and c-myc genes in mouse lung tumors. *Mol. Carcinog.* **35**: 93–102

Wattenberg, L. W., Estensen, R. D. (1997) Studies of chemopreventive effects of budesonide on benzo[a]pyrene-induced neoplasia of the lung of female A/J mice. *Carcinogenesis* **18**: 2015–2017

Wattenberg, L. W., Wiedmann, T. S., Estensen, R. D., Zimmerman, C. L., Steele, V. E., Kelloff, G. J. (1997) Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. *Cancer Res.* **57**: 5489–5492

Wattenberg, L. W., Wiedmann, T. S., Estensen, R. D., Zimmerman, C. L., Galbraith, A. R., Steele, V. E., Kelloff, G. J. (2000) Chemoprevention of pulmonary carcinogenesis by brief exposures to aerosolized budesonide or beclomethasone dipropionate and by the combination of aerosolized budesonide and dietary myo-inositol. *Carcinogenesis* **21**: 179–182

Yanagihara, K., Seki, M., Cheng, P. W. (2001) Lipopolysaccharide induces mucus cell metaplasia in mouse lung. *Am. J. Respir. Cell Mol. Biol.* **24**: 66–73

Yuan, J. W., Krieger, J. A., Maples, K. R., Born, J. L., Burchiel, S. W. (1994) Polycyclic aromatic hydrocarbons decrease intracellular glutathione levels in the A20.1 murine B cell lymphoma. *Fundam. Appl. Toxicol.* **23**: 336–341