METABOLISM OF INHALED GLUCOCORTICOIDS AND CYP3A GENE REGULATION IN LUNG CELLS

by

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ABSTRACT

Asthma is a common disease that is most frequently treated with inhaled glucocorticoids which are used to decrease inflammation and mucus production in the airways. However, about 30% of asthma patients do not respond to treatment. A possible hypothesis for glucocorticoid insensitivity is increased metabolism of inhaled glucocorticoids by cytochrome P450 3A (CYP3A) enzymes, particularly in the lung. The objectives for this dissertation were to evaluate the metabolism of five inhaled glucocorticoids (budesonide, beclomethasone dipropionate, fluticasone propionate, triamcinolone acetonide, and flunisolide) by CYP3A enzymes, and to determine if treatment with glucocorticoids in lung cells induced CYP3A enzyme expression, further increasing the metabolism of glucocorticoids in the lung. All three CYP3A enzymes (CYP3A4, 3A5, and 3A7) metabolized the five glucocorticoids, but to varying degrees and with unique products. CYP3A4 and CYP3A5 were the most efficient at metabolizing the glucocorticoids; CYP3A7 had the lowest rates of metabolism. The most common metabolites produced by CYP3A enzymes with triamcinolone acetonide, budesonide, flunisolide, and beclomethasone dipropionate were 6β-hydroxylated and Δ^6-dehydrogenated product, all of which are believed to be clearance metabolites. Investigation into the metabolism of beclomethasone dipropionate by A549 lung cells showed that a dehydrogenated P450-mediated metabolite, [M5], was produced, decreasing bioavailability of the active drug. It was also demonstrated that CYP3A5 mRNA was

induced in A549 cells with glucocorticoid treatment. The induction of CYP3A mRNA was blocked when cells were co treated with esterase inhibitors and BDP, confirming the active metabolite, beclomethasone 17-monopropionate ([M1]), was mediating the induction of CYP3A5 mRNA, presumably through the glucocorticoid receptor (GR). CYP3A5 mRNA induction was also attenuated by inhibiting GR using the antifungal drug, ketoconazole, further supporting the hypothesis that glucocorticoids binding to GR was the mechanism of CYP3A5 induction in A549 cells. Additional experimentation with primary cells (NHBE, lobar, SAEC, BEAS-2B, and tracheal cells) demonstrated that only SAEC cells expressed CYP3A5. However, CYP3A5 mRNA was not induced in SAEC cells with glucocorticoid treatment despite extensive manipulation of cell culture conditions, such as removing hydrocortisone and utilizing charcoal-stripped FBS for treatment, which could have interfered with the mechanism observed in A549 cells. Overall, the collective results described in this dissertation support the hypothesis that increased metabolism of glucocorticoids in the lung could lead to decreased bioavailability of pharmacologically active drug, and that continued treatment with inhaled glucocorticoids could perpetuate the inefficacy by inducing CYP3A5 enzymes, potentially causing glucocorticoid insensitivity seen in patients.

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LIST OF ABBREVIATIONS

1-ABT	1-aminobenzotriazole
A549	human lung adenomacarcinoma
B2M	β2 microglobulin
BDP	beclomethasone dipropionate
BEAS-2B	immortalized bronchial epithelial cell line
BUD	budesonide
CAR	constitutive androstane receptor
COPD	chronic obstructive pulmonary disease
CYP	cytochrome P450
CYP3A	cytochrome P450 3A family
DMSO	dimethyl sulfoxide
DPX2	HepG2 background with PXR and CYP3A4 stably expressed
EI	esterase inhibitors
ESI	electrospray ionization
FEV_1	volume of air exhaled during the first second of exhalation
FLN	flunisolide
FLT	fluticasone propionate
FVC	volume of air forcibly exhaled from maximal inhalation
GR	glucocorticoid receptor

GSDML	gadsdermin like gene
IL1RL1	interleukin 1 receptor-like 1
IL18R1	interleukin 18 receptor 1
IL-33	interleukin 33
LC/MS/MS	liquid chromatography-tandem mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NHBE	normal human bronchial/tracheal epithelial cell line
ORMCL3	orosomucoid -like 3 gene
PXR	pregnane X receptor
qPCR	real time quantitative polymerase chain reaction
RXRα	retinoid X receptor alpha
SAEC	small airway epithelial cell line
SNP	single nucleotide polymorphism
TCL	triamcinolone acetonide
TSLP	thymic stromal lymphopoietin

CHAPTER 1

INTRODUCTION

Asthma

Asthma is a disease characterized by chronic inflammation of the airways that includes bronchoconstriction, increased mucus production and occasional airway obstruction (*1*). This disease accounts for an estimated 500,000 hospital visits per year (*2*) and in 2007 the National Heart, Lung, and Blood Institute estimated that asthma cost the United States of America $20 billion in lost productivity and healthcare cost (*3*). The most alarming statistic was in 2009 when the Center for Disease Control estimated there were 24.6 million people with asthma in this country, with 7 million under the age of 18 (*4, 5*). From these data, it is evident that asthma directly and indirectly affects the entire U.S. population through increasing healthcare cost, loss of productivity, as well as having an enormous impact on the health of our pediatric and neonatal populations, which will continue to impact their health and livelihood throughout their lives.

Asthma Is a Complex Disease

Asthma is a complex disease because it has diverse genetic and environmental components. Twin studies have shown a strong genetic component to asthma (*6, 7*), with heritability estimated to be from 35% - 95% (*8-14*). Although inheritance does not follow

Mendelian genetics, it is commonly believed that multiple genes are responsible for asthma (*15, 16*). Genetic studies have found over 120 genes associated with asthma, all of which can be grouped into mutations affecting the structural components of the airways, increased levels and/or aberrant regulation of inflammatory proteins, or a decrease in antiinflammatory proteins (*17*).

Recent genome-wide association studies have implicated *four* loci that are consistently associated with asthma across different ethnicities; 17q21, 2q12, 5q22.1 and interleukin 33 (IL33) (*15, 18*). These loci are all associated with an increase in the endogenous inflammatory response. The first locus implicated in asthma susceptibility is the orosomucoid-like 3 gene/gadsdermin like gene (ORMCL3/GSDML) located on chromosome 17q21 (*19*). This locus has been associated with early childhood onset of asthma, particularly in children with frequent exacerbations, respiratory viral infections, and exposure to tobacco smoke (*20-22*). Interestingly, children that have one of the many single nucleotide polymorphisms (SNP) in this region do not have any asthma attacks associated with direct contact with known common allergens or atopy, but experience symptoms sporadically (*23*). The functions of ORMCL3 and GSDML are still poorly understood, but SNPs are believed to decrease endoplasmic reticulum Ca^{2+}, which results in an induced endogenous inflammatory response (*24*). The second locus implicated in asthma susceptibility is interleukin 1 receptor-like 1/interleukin 18 receptor 1 gene (IL1RL1/IL18R1) located on chromosome 2q12 (*25*). This locus has been implicated in atopic asthma patients comorbid and codes for the IL1RL1 receptor, which IL-33 binds to on mast cells, T helper 2 cells, T-regulatory cells, and macrophages (*18*). This locus is most commonly seen in patients that have asthma attacks from multiple environmental

factors and requires vigilant control of their environments (no pets, no carpet, etc). The third locus is TSLP (thymic stromal lymphopoietin) located on chromosome 5q22.1 (*25*). TSLP is an epithelial cell derived cytokine involved in the inflammatory process associated with asthma. SNPs in this region have also been highly associated with food allergies (*26*). The fourth locus is IL33, which has been implicated in allergy induced asthma. IL33 activates mast cells, T helper 2 cells, T-regulatory cells, and macrophages (*18*). This locus has also been implicated in children requiring vigilant control of their environments. Though these are the four most common loci associated with asthma, it has also been documented that children can be afflicted with asthma without having any family history of the disease (*15*), suggesting a strong environmental component.

The most common environmental instigators of asthma are a severe allergic reaction to an airborne allergen, such as cigarette smoke or a pesticide, and exposure to a viral infection in infancy or early childhood, such as the paramyxovirus or the respiratory syncytial virus (*27*). There is also been evidence to suggest that children born in winter months in colder regions of the world also have a higher incidence of asthma, possibly due to decreases in Vitamin D exposure (*28*).

Asthma patients suffer most commonly from acute attacks brought on by an environmental trigger. Some environmental triggers include dust mite exposure, exercise, pet dander, cigarette smoke inhalation, and air pollution (*29*). Studies have also shown that there are genetic factors that predispose patients to having asthma symptoms when exposed to certain environmental triggers (*30*), meaning not every patient has an asthma attack when exposed to the same triggers as another patient. This inherent variability in

patient susceptibility to triggers complicates studying the genetic basis for asthma, as well as diagnosis.

Diagnosis of Asthma

Symptoms of asthma include chest tightness, wheezing, and breathlessness that often occur several times in a day or week (*31*). Asthma diagnosis begins with an extensive family history questionnaire and a physical examination in which physicians evaluate hyperexpansion of the thorax, wheezing occurring during normal breathing and forced exhalation, increased nasal swelling, and a manifestation of any allergic skin condition, such as eczema (*32*). Spirometry tests are then used to evaluate the maximal volume of air forcibly exhaled from maximal inhalation (FVC) and the volume of air exhaled during the first second of exhalation (FEV_1) (*32*). Ratios of FEV_1/FVC before and after a short acting bronchodilator are used to determine how much obstruction is seen in patients and to determine if there is improvement or significant reversibility after a short acting bronchodilator (*29, 32*). Significant reversibility is defined as > 200mL increase in FEV_1 and a > 10% increase in FEV_1/FVC ratio as compared to baseline (*32*). Severity of asthma is also divided into four categories upon diagnosis: intermittent, mild persistent, moderate persistent, and severe persistent (*29*). Intermittent is defined as symptoms less than once per week with brief exacerbations. Mild persistent is defined as symptoms more than twice a week, but less than once a day, and exacerbations affecting activity or sleep. Moderate persistent is defined as daily symptoms with exacerbations affecting activity and sleep. Severe persistent is defined as daily symptoms, with frequent exacerbations and frequent sleep exacerbations. Defining severity of asthma aids the

physician in determining the most appropriate therapeutic paradigm based on recommendations from the National Heart, Lung, and Blood Institute (*32*).

Diagnosis of asthma is difficult because many of the characteristic symptoms of asthma can also occur in other conditions and disease states, such as chronic obstructive pulmonary disease (COPD), tuberculosis, allergic rhinitis, and pneumothorax (*18*). Chest x-rays may also be performed to rule out these disorders (*32*), but even after physical examination and performing the diagnostic procedures outlined above, patients are not considered to have asthma unless improvement is seen after a 2-3 week glucocorticoid treatment regiment (*32*). However, this theory is flawed based on reports of patient insensitivity to glucocorticoid treatment (*33*), and persistent symptoms of asthma, lending to potential misdiagnosis of asthma.

Inhaled Glucocorticoid Therapy

The first-line treatment for asthma is glucocorticoid therapy (*29, 32, 34*). These drugs work by activating the glucocorticoid receptor (GR) in the cytosol of bronchiolar lung cells, forming a steroid-receptor complex, which translocates to the nucleus, and decreases the expression of proinflammatory genes and mucus production pathways in the lung (*35-38*). The five most common inhaled glucocorticoids used in the clinic are flunisolide, triamcinolone acetonide, beclomethasone dipropionate, budesonide, and fluticasone propionate (*32*). Clinical studies over the last two decades have compared the potency of these five drugs, and laboratories have ranked them as follows (least potent to most potent): flunisolide = triamcinolone acetonide < beclomethasone dipropionate (BDP) < budesonide < fluticasone propionate (*38*). This ranking has been determined by

quantifying glucocorticoid receptor complex half-lives, glucocorticoid receptor binding affinities, and the topical potency skin blanching test (*38*), which measures the vasoconstriction of skin where steroids have been applied topically (*39*).

Glucocorticoid Resistance

Although glucocorticoids are the first-line treatment for chronic asthma, about 30% of the asthmatic population does not respond to treatment due to glucocorticoid resistance or insensitivity (*36*). Patients are defined as being resistant to steroids if they exhibit less than a 15% improvement in baseline FEV_1 after a ten to fourteen day course of high dose steroids (*40, 41*). Some factors contributing to steroid resistance are believed to be genetic abnormalities in the glucocorticoid receptor, abnormalities in histone acetylation, the ability of patients to control oxidative stress, and latent viral infections (*41, 42*). However, the exact mechanisms of glucocorticoid resistance is still unknown (*43*). A possible mechanism for resistance may be due to the differential metabolism and accelerated clearance of glucocorticoids in target cells and tissues by tissue-specific expression of catabolic enzymes, including cytochrome P450s.

Cytochrome P450 Enzymes

Cytochrome P450s (P450s) are heme-containing monooxygenases found in all organisms; *Cyto* stands for microsomal vesicles, chrome for colored, *P* for pigmented and *450* for their characteristic absorbance at 450 nm when treated with a reductant and carbon monoxide (*44-46*). They are biological catalysts that perform many different chemical reactions, such as oxidation of xenobiotics, and synthesis of steroids and

cholesterol (*47-50*). Over 58 human P450 enzymes have been identified since their initial discovery in 1962 (*45*). Table 1.1 summarizes all human cytochrome P450 enzymes identified to date, their tissue distribution, and their ability to metabolize glucocorticoids (*51-61*). Cytochrome P450 enzymes are expressed predominately in the liver, but are also expressed in other such as the kidney, lung, brain, intestine, pancreas, bone marrow, mast cells, skin, ovary, testis, nose, and blood cells (*62, 63*). Endogenous cytochrome P450 reactions include vitamin metabolism, fatty acid metabolism, sterol metabolism and synthesis, and eicosanoid metabolism (*62*). About 26% of cytochrome P450s are involved in the metabolism of xenobiotics. These specific P450s have been studied as factors regulating drug bioavailability and pharmacokinetics, determinants of deleterious drug/drug interactions, and causes of toxicity associated with different xenobiotics in humans (*62*).

Cytochrome P450 Catalytic Cycle

The P450 catalytic cycle has been well studied and is reviewed in detail (Figure 1.1) (*64*). In brief, the P450 catalytic cycle begins in the "resting state" in which a water molecule is bound to the ferric iron in the P450 heme cofactor. A substrate displaces the water from the heme, leaving a pentacoordinated-ferric heme. This induces the iron to adopt a high-spin state with an increased reductive potential which allows the ferric complex to become a better electron acceptor from a nicotinamide adenine dinucleotide phosphate (NADPH) cofactor via cytochrome P450 reductase. Oxygen then binds to the reduced ferrous iron, producing a ferrous dioxygen complex. A second reduction of the system takes place to form the ferric peroxo anion species, which is the rate limiting step

Table 1.1. Human cytochrome P450 enzymes separated by families and its members.

Family	Members	Glucocorticoid Metabolism	Tissue Predominately Expressed
CYP1A	1A1	Possible, Testosterone	Adult Liver > Parenchyma
	1A2	Unknown	Adult Liver
CYP1B	1B1	Possible, Testosterone	Adult Parenchyma, Bronchi
CYP2A	2A6	Unknown	Adult Liver > Parenchyma, Bronchi
	2A7	Unknown	Adult Liver > Parenchyma, Bronchi
	2A13	Possible, Testosterone	Adult Bronchi
CYP2B	2B6	Possible, Testosterone	Adult Liver > Parenchyma, Bronchi
CYP2C	2C8	Possible, Testosterone	Adult Liver > Parenchyma, Bronchi
	2C9	Possible, Testosterone	Adult Liver > Bronchi
	2C18	Unknown	Adult Liver > Bronchi
	2C19	Possible, Testosterone	Adult Liver > Parenchyma, Bronchi
CYP2D	2D6	Unknown	Adult Liver > Bronchi
CYP2E	2E1	Unknown	Adult Liver > Parenchyma, Bronchi
CYP2F	2F1	Unknown	Adult Bronchi
CYP2J	2J2	Unknown	Adult Liver > Bronchi
CYP2R	2R1	Unknown	Adult Liver
CYP2S	2S1	Unknown	Adult Bronchi, Parenchyma
CYP2U	2U1	Unknown	Brain
CYP2W	2W1	Unknown	Colon and Adrenal Tumors
CYP3A	3A4	Yes, Flunisolide	Adult Liver and Gut
	3A5	Yes, Budesonide	Adult Parenchyma
	3A7	Yes, Budesonide	Fetal Lung and Liver, Adult Parenchyma
	3A43	Possible, Testosterone	Adult Liver
CYP4A	4A11	Unknown	Adult Liver
	4A20	Unknown	Unknown
	4A22	Unknown	Kidney
CYP4B	4B1	Possible, Testosterone	Adult Parenchyma and Bronchi
CYP4F	4F2	Unknown	Adult Liver
	4F3	Unknown	Adult Liver > Parenchyma
	4F8	Unknown	Prostate
	4F11	Unknown	Adult Liver > Bronchi
	4F12	Unknown	Adult Liver > Parenchyma and Bronchi
	4F22	Unknown	Skin

Glucocorticoid metabolism and the tissue they are predominately expressed in is listed for each enzyme.

Table 1.1. (continued)

Family	Members	Glucocorticoid Metabolism	Tissue Predominately Expressed
CYP4V	4V2	Unknown	Eye
CYP4X	4X1	Unknown	Trachea, Bronchi, and Aorta
CYP4Z	4Z1	Unknown	Mammary Tissue
CYP5A	5A1	Unknown	Small Intestine > Bronchi
CYP7A	7A1	Unknown	Adult Liver
CYP7B	7B1	Unknown	Adult Liver > Parenchyma and Bronchi
CYP8A	8A1	Unknown	Adult Parenchyma and Bronchi
CYP8B	8B1	Unknown	Adult Liver > Parenchyma and Bronchi
CYP11A	11A	Unknown	Adrenal Gland
CYP11B	11B1	Probable, Hydrocortisone	Adrenal Gland
	11B2	Probable, Hydrocortisone	Adrenal Gland
CYP17A	17A	Unknown	Adrenal Gland
CYP19A	19A	Possible, Testosterone	Placenta
CYP20A	20A	Unknown	Adult Liver
CYP21A	21A1	Unknown	Adrenal Gland
	21A2	Unknown	Adrenal Gland
CYP24A	24A	Unknown	Kidney
CYP26A	26A	Unknown	Adult Liver > Bronchi
CYP26B	26B	Unknown	Adult Liver
CYP26C	26C	Unknown	Unknown
CYP27A	27A	Unknown	Adult Liver and Parenchyma
CYP27B	27B	Unknown	Kidney
CYP27C	27C	Unknown	Unknown
CYP39A	39A	Unknown	Adult Liver > Parenchyma and Bronchi
CYP46A	46A	Possible, Testosterone	Brain
CYP51A	51A	Unknown	Testis

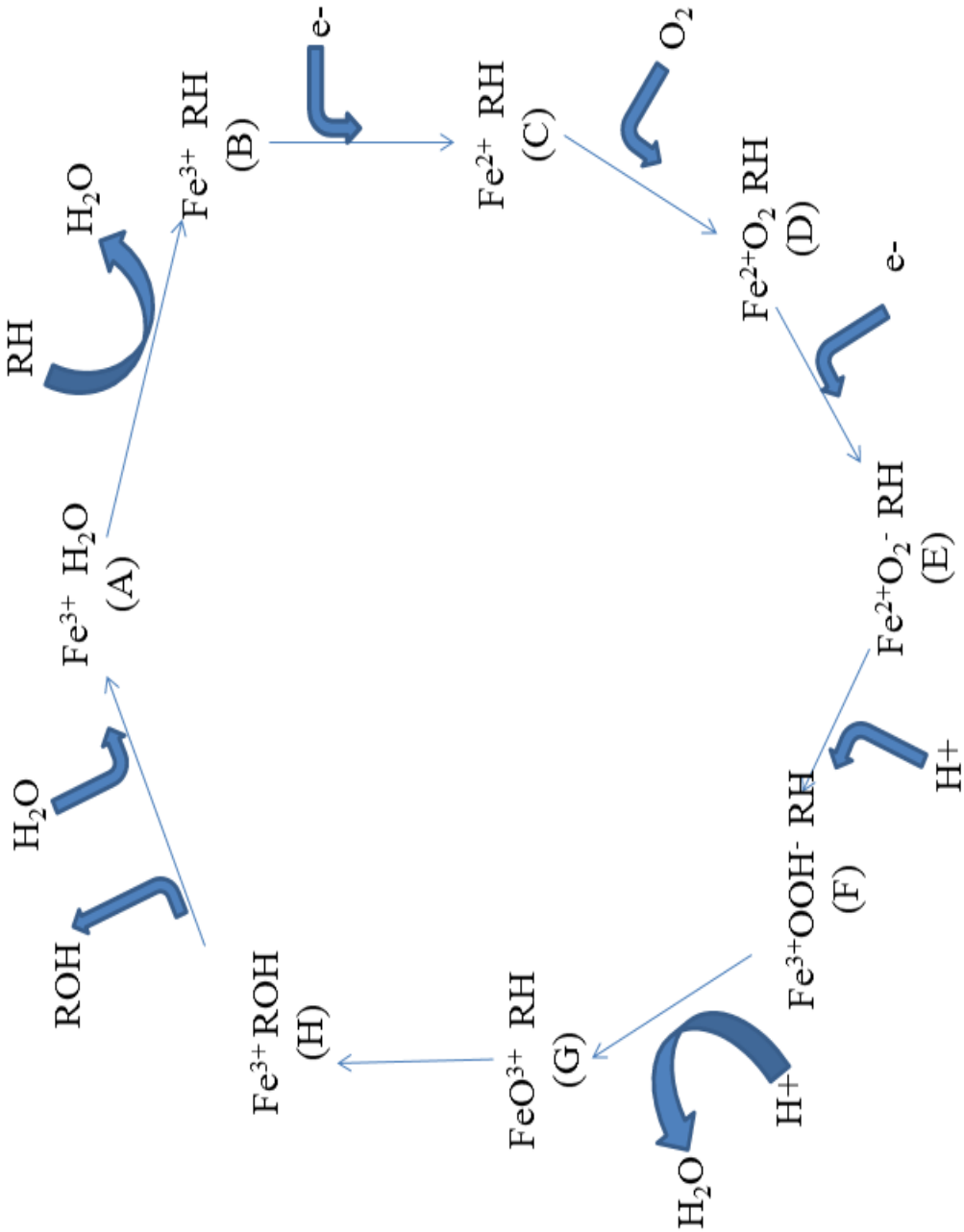

Figure 1.1. Cytochrome P450 catalytic cycle.

in the catalytic cycle of P450 enzymes. The ferric peroxo complex is a good Lewis base and is rapidly protonated to form a ferric-hydroperoxide species, also called compound 0. Compound 0 extracts an additional proton to form compound I and water. Compound I then oxidizes the substrate via the activated oxygen atom (*65*). This catalytic step is followed by product release, in which oxygen is incorporated, and the heme returns to the resting state.

Cytochrome P450-Mediated Reactions

Numerous endogenous and exogenous compounds that can produce physiological effects are hydrophobic enough to facilitate their diffusion through biological barriers and to reach their molecular targets. In order to avoid accumulation of such substances and to avoid toxicities associated with the accumulation of such chemicals, organisms have evolved the P450 enzymes, which metabolize a myriad of chemicals to more hydrophilic products that can be more easily excreted or further metabolized to conjugates that are actively excreted. Phase I enzymes, such as esterases, P450 enzymes, and monoamine oxidases introduce (e.g., hydroxylation) or expose (e.g., heteroatom dealkylation) more polar functional groups to increase water solubility and to facilitate phase 2 conjugation reactions and ultimately clearance. P450 enzymes are able to catalyze numerous different chemical reaction mechanisms to achieve this goal, including hydroxylation and dehydrogenation, which are central to the work presented in this dissertation.

Hydroxylation is the best studied and well understood P450 catalyzed reaction mechanism that is commonly accepted to occur via hydrogen radical abstraction followed by a hydroxyl radical rebound mechanism to produce an alcohol (*48*); this hydroxylation

reaction has been confirmed using other glucocorticoid substrates, but not with beclomethasone dipropionate (*66-68*).

Dehydrogenation reactions are less studied P450-catalyzed chemical reactions that produce a desaturated product (*69*); this reaction mechanism has been shown to occur during the metabolism of the glucocorticoids triamcinolone, flunisolide, and budesonide (*70-72*), but not beclomethasone dipropionate.

Cytochrome P450 3A (CYP3A) Family Enzymes

The 58 human P450s are named and arranged into families based on their sequence homology (*73-75*). One of the most important classes of P450 enzymes involved in drug metabolism, particularly glucocorticoid metabolism, is the CYP3A family of enzymes (*76*). The CYP3A family is located on chromosome 7 (*77*) and is the most abundant of the P450s in many tissues, primarily the liver, intestine, and lung and are differentially expressed based on which transcription factors are present (*78-80*). The CYP3A enzyme isoforms most pertinent to this dissertation include CYP3A4, CYP3A5, and CYP3A7. CYP3A4 is the predominant CYP3A isoform found in the liver and intestine (*61, 66, 81*), whereas CYP3A5 predominates in the lung (*61, 82, 83*). CYP3A4 and CYP3A5 together account for over 50% of the metabolism of therapeutic molecules on the market today (*84*). CYP3A7 is the hepatic fetal isoform (*85, 86*) and Lacroix *et al.* discovered that fetal CYP3A7 predominates at birth, but is replaced by the adult CYP3A4 or 3A5 isoforms shortly after birth (*85*), This phenomenon may be important to consider when treating neonatal versus pediatric and adult patients with glucocorticoids.

CYP3A Expression in the Lung

Analysis of CYP3A mRNA expression in adult lung tissue found that there are anatomical and age related differences in the level of expression of CYP3A enzymes (*61*). Leclerc *et al.* found that CYP3A5 and CYP3A7 message were detected in the pulmonary parenchyma, but CYP3A4 message was not detected. It was also noted that only a very low amount of CYP3A5 message could be detected in the bronchial mucosa (bronchial epithelial cells), while no CYP3A7 or CYP3A4 message was found. Therefore, in the context of asthma, CYP3A5 may be the most important CYP3A enzyme because it is expressed in the target tissue and it is capable of altering therapeutic concentrations of glucocorticoids through metabolic clearance, pharmacophore modification, and inactivation. There also is a difference in how CYP3A enzymes are expressed in whole lung tissue during development. Preliminary work by our laboratory has shown that CYP3A enzyme expression profiles are different between neonates, pediatric and adult patients. Neonatal tracheal washes revealed that CYP3A7 was expressed at very high levels, with CYP3A5 being expressed at moderate levels. Other research has shown that these higher CYP3A7 levels decline in the first year of life (*86*) with CYP3A5 remaining at moderate levels and even increasing to higher levels in some patients. Adult lung continues to show high levels of CYP3A5, but very low to nondetectable levels of CYP3A7 (unpublished data). Although these preliminary data show differences in the relative expression of CYP3A enzymes, little is still known about changes in the developmental expression and transcription of pulmonary CYP3A enzymes. It is possible that variations of CYP3A enzymes might influence the behavior of therapeutic agents.

Cytochrome P450 3A Enzyme Regulation

Transcriptional regulation of hepatic CYP3A genes has been extensively studied in liver cell and tissues, and it is commonly accepted that many xenobiotics, including glucocorticoids, induce changes in expression of drug-clearing enzymes via activation of the nuclear pregnane X receptor (PXR) (*87-91*). However, PXR-mediated changes of gene expression have not been detected in human lung (*92*). Although there is increasing evidence that drug treatment will alter pulmonary CYP3A expression, including a study by Hukkanen *et al.* that showed a 4-fold increase in CYP3A5 in response to BDP treatment in A549 lung carcinoma cells (*83*), the mechanism responsible for this physiological response has not been well studied and remains poorly understood. Likewise, the significance of this induction phenomenon with respect to glucocorticoid pharmacotherapy is also unknown.

Recent reviews have shown that the glucocorticoid receptor (GR) and the constitutive androstane receptor (CAR) also play important roles in regulation of gene expression in the liver (*93, 94*). Hepatocytes treated with submicromolar concentrations of dexamethasone have been shown to increase the expression of CAR via GR (*95, 96*). Furthermore, the induction and activation of CAR has also been shown to increase the expression of P450 enzymes, including CYP3A enzymes (*94*). Therefore, it is possible that the regulation of the CYP3A enzymes in the lung is mediated through GR and CAR upon treatment. Studies presented in this dissertation investigate the possibility that glucocorticoid insensitivity observed in 30% of asthmatics may be due to glucocorticoid induced CYP3A expression, causing increased rates of glucocorticoid clearance, and thus

reduced drug concentrations in the target tissue. Specifically, the roles of GR and CAR in pulmonary CYP3A expression are evaluated.

CYP3A4 and CYP3A5 Polymorphisms

Because almost 50%-90% of inhaled glucocorticoids are swallowed when inhaled (*97*), CYP3A4 will play a role in the metabolism of glucocorticoids in the liver. To date, there have been 22 polymorphisms of CYP3A4 described (*98*). Though most of the polymorphisms have no effect on steroid metabolism and are not robustly expressed in populations (*98*), one that could have an impact on steroid metabolism is *CYP3A4*22*, which codes for a null enzyme (*99-101*). Patients who do express this polymorphism could demonstrate decreased metabolism of glucocorticoids.

Many glucocorticoids are inhaled and exhibit their efficacy in the lung. Therefore, CYP3A5 polymorphisms that change glucocorticoid metabolism in lung cells would alter glucocorticoid efficacy. Many polymorphisms of CYP3A5 have been reported and two variants, *CYP3A5*3* and *CYP3A5*1*, are of key interest to the work presented in this dissertation. The most common polymorphism is *CYP3A5*3*, which codes for an inactive form of CYP3A5 in the liver due to a defective splice site (*81, 102*), and presumably in the lung. The majority of Caucasians are homozygotes for the *CYP3A5*3* allele and therefore do not express active CYP3A5 in the lung or elsewhere in the body. *CYP3A5*1* codes for an active enzyme (*102*). Patients expressing the *CYP3A5*1* polymorphism would presumably have increased metabolism of glucocorticoids in the lung and therefore would be predicted to exhibit decreased glucocorticoid efficacy compared to individuals

with the *CYP3A5*3* genotype. Both genotypes were assessed throughout the studies reported herein.

Metabolism of Beclomethasone Dipropionate

BDP is administered as an inhaled prodrug and becomes pharmacologically active in the lung by hydrolysis of an ester at the C-21 position, forming beclomethasone 17-monopropionate [M1] (Figure 1.2) (*103*). Two other metabolites previously documented in the literature are beclomethasone 21-monopropionate [M2], which requires the hydrolysis of an ester at the C-17 position, and beclomethasone [M3], which requires the hydrolysis of both esters (*104-108*). Cell culture experiments in A549 cells attributed product formation to esterases (*109*). However, these experiments did not contain P450 inhibitors, and thus, the possibility that P450 enzymes also contributed to the bioactivation of beclomethasone dipropionate was not determined. Preliminary research by our laboratory, as well as by others, has shown that P450 enzymes may be able to catalyze the de-esterification of BDP and thus potentially could contribute to the biological efficacy of this drug. At the time this research project began, it was not known whether CYP3A enzymes were capable of producing the pharmacologically active BDP metabolite [M1] or any other metabolites (e.g., [M4] and [M5]), that were predicted from the studies of the metabolism of other structurally similar glucocorticoids (*66-68, 70, 71*). Therefore, the relative rates of metabolism by CYP3A isoforms and the metabolites they produced were investigated and quantified in this dissertation.

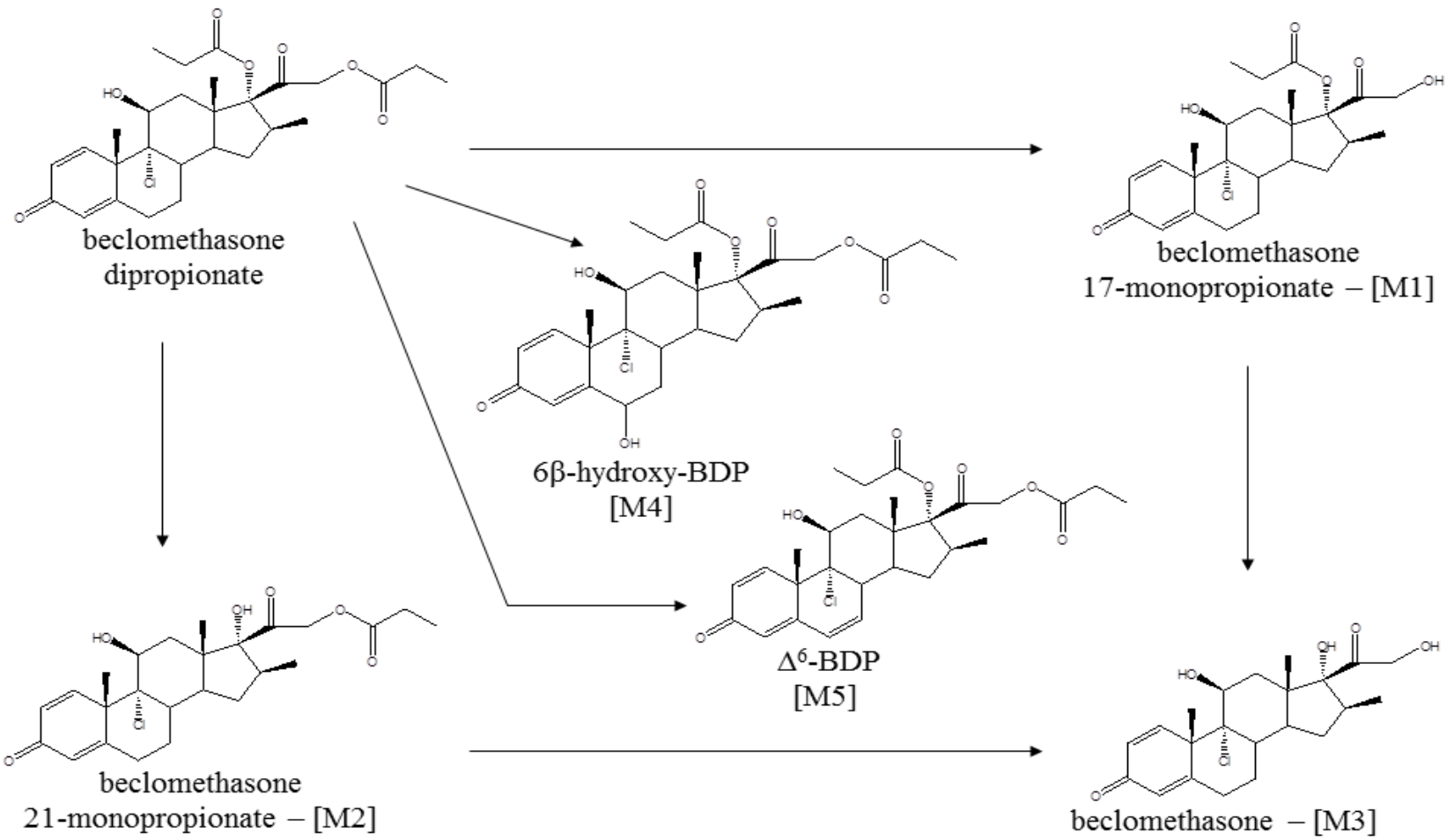

Figure 1.2. Proposed metabolic pathway of beclomethasone dipropionate. Esterase enzymes have been shown to contribute to the activation of beclomethasone dipropionate to [M1] as well as clearance metabolites [M2] and [M3]. P450 enzymes, specifically CYP3A enzymes, are the main mediators of the other clearance metabolites [M4] and [M5].

Research Objectives

The hypothesis for this work is that CYP3A enzymes produce the active metabolite as well as major clearance metabolites leading to glucocorticoid insensitivity, and beclomethasone dipropionate binds to GR, induces CAR, which induces the expression of CYP3A enzymes. The metabolism of glucocorticoids by the CYP3A isoforms and the regulation of CYP3A enzymes in the lung in response to glucocorticoid treatment were the main objectives for this dissertation.

Major Findings by Chapter

Chapter 2

The ability of CYP3A isoforms to metabolize four major glucocorticoids, triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate, had not been previously documented and was evaluated. CYP3A5 was able to metabolize all four glucocorticoids. CYP3A4 metabolized triamcinolone acetonide, budesonide, and fluticasone. CYP3A7 metabolized budesonide, fluticasone propionate, and triamcinolone acetonide, but at lower rates than CYP3A4 and CYP3A5. The major metabolites produced for triamcinolone acetonide, flunisolide, and budesonide were 6β-hydroxylation and Δ^6-dehydrogenation. Hydrolysis of an ester on the D-ring of the glucocorticoid by CYP3A enzymes occurred for fluticasone propionate and triamcinolone acetonide. Novel metabolites, 21-nortriamcinolone acetonide and Δ^6-flunisolide, were isolated and identified using NMR. This work had been published in the journal *Drug Metabolism and Disposition* (*72*).

Chapter 3

The ability of CYP3A isoforms to metabolize BDP was first evaluated. The results indicated that only CYP3A4 and CYP3A5 are able to metabolize BDP, exhibiting similar rates, while CYP3A7 does not metabolize BDP. Studies with *in vitro* incubations, including recombinant P450 enzyme, demonstrated that CYP3A4 and CYP3A5 produce clearance compounds, [M4] and [M5], and that the combined action of esterases and CYP3A4 produced [M6], a de-esterified, hydroxylated product. Further investigation using cell culture experiments, showed only the formation of [M5] by A549 cells (lung adenocarcinoma cells). However, both [M4] and [M5] were detected in liver cell culture (DPX2 cells). [M6] was not detected in cell culture experiments. This work has been published in the *Journal of Pharmacology and Experimental Therapeutics (110)*.

Chapter 4

Studies focused on the regulation of CYP3A enzymes in response to glucocorticoid treatment in various lung cell culture models. In A549 cells there was a 2-fold induction of CYP3A5 mRNA upon BDP treatment, but neither CYP3A4 nor CYP3A7 mRNA were detected, even with BDP (and other glucocorticoid treatment). The induction of CYP3A5 was blocked by inhibiting the formation of the active metabolite [M1] through inhibition of esterase activity. Furthermore, inhibition of all P450 and esterase activity (through 1-ABT and esterase inhibitors) and treatment with [M1] was sufficient to induce CYP3A5 mRNA. Blocking the glucocorticoid receptor using a competitive antagonist, ketoconazole, or knocking down GR expression using siRNA, blocked the induction of CYP3A5, suggesting this induction occurred through the

glucocorticoid receptor. The constitutive androstane receptor did not participate in the induction of CYP3A5. Additional investigations of this regulatory pathway in primary cell cultures, such as NHBE, SAEC, and lobar cells, showed that it could not be replicated with these model systems. This suggests that there is a deficit in current lung models used to study not only the regulation of CYP3A5 in the lung in response to glucocorticoid treatment, but also the metabolism of glucocorticoids by CYP3A enzymes in the lung. This work was not published prior to submission of the final version of this dissertation.

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CHAPTER 2

METABOLIC PATHWAYS OF INHALED GLUCOCORTICOIDS BY THE CYP3A ENZYMES

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Metabolic Pathways of Inhaled Glucocorticoids by the CYP3A Enzymes

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ABSTRACT

Asthma is one of the most prevalent diseases in the world, for which the mainstay treatment has been inhaled glucocorticoids (GCs). Despite the widespread use of these drugs, approximately 30% of asthma sufferers exhibit some degree of steroid insensitivity or are refractory to inhaled GCs. One hypothesis to explain this phenomenon is interpatient variability in the clearance of these compounds. The objective of this research is to determine how metabolism of GCs by the CYP3A family of enzymes could affect their effectiveness in asthmatic patients. In this work, the metabolism of four frequently prescribed inhaled GCs, triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate, by the CYP3A family of enzymes was studied to identify differences in their rates of clearance and to identify their metabolites. Both interenzyme and interdrug variability in rates of metabolism and metabolic fate were observed. CYP3A4 was the most efficient metabolic catalyst for all the compounds, and CYP3A7 had the slowest rates. CYP3A5, which is particularly relevant to GC metabolism in the lungs, was also shown to efficiently metabolize triamcinolone acetonide, budesonide, and fluticasone propionate. In contrast, flunisolide was only metabolized via CYP3A4, with no significant turnover by CYP3A5 or CYP3A7. Common metabolites included 6β-hydroxylation and Δ^6-dehydrogenation for triamcinolone acetonide, budesonide, and flunisolide. The structure of Δ^6-flunisolide was unambiguously established by NMR analysis. Metabolism also occurred on the D-ring substituents, including the 21-carboxy metabolites for triamcinolone acetonide and flunisolide. The novel metabolite 21-nortriamcinolone acetonide was also identified by liquid chromatography–mass spectrometry and NMR analysis.

Introduction

Asthma is a chronic lung disease characterized by recurring episodes of wheezing, shortness of breath, chest tightness, and coughing. These symptoms are caused by bronchial constriction due to hyperreactivity, inflammation, eosinophilic infiltration, and increased mucus production, causing intermittent airway obstruction. Asthma is a multifactorial disease that can be exacerbated by genetics, respiratory infections, allergens, air pollutants, temperature changes, exercise, and stress. Asthma is one of the most prevalent diseases in the world, with an estimated 300 million sufferers (World Health Organization, 2007). Many studies have demonstrated a genetic contribution to asthma, with a 36%–79% heritability risk (Los et al., 1999). The incidence of asthma in the US population alone continues to increase, with 1 in 12 people reported to have asthma in 2009, an increase from 1 in 14 in 2001. This increase comes with an increase in associated annual medical costs from $48.6 billion in 2002 to $50.1 billion in 2007 (http://www.cdc.gov/VitalSigns/Asthma/).

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Inhaled glucocorticoids (GCs) are potent anti-inflammatory agents that are the mainstay of treatment of patients with persistent asthma. Therapeutic agents include triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate. These compounds bind to the GC nuclear receptors in the relevant airway epithelial cells, which decreases migration and survival of inflammatory cells in the lung, decreases mucus production, and reduces action of proinflammatory cytokines (Barnes, 2011). Although GCs are currently the most effective therapy for controlling asthma, approximately 30% of asthmatics have some degree of steroid resistance or insensitivity (Chan et al., 1998; Szefler et al., 2002). Current proposed mechanisms explaining GC insensitivity/resistance include defective immune responses that correlate with clinical resistance to GC therapy, genetic abnormalities that result in the inactivation of the GCs, mutations of the GR gene, and molecular mechanisms involving inflammatory cytokines, the exact mechanism of which is unknown (Leung and Bloom, 2003).

Only 2%–10% of the inhaled dose of GCs is deposited in the lungs; the majority of a dose is swallowed and absorbed into systemic circulation (Taburet and Schmit, 1994). GCs are metabolized in the lung and liver by members of the cytochrome P450 (P450) CYP3A family of enzymes. The CYP3A family consists of CYP3A4, -5, -7, and -43, whose expression is differentiated by tissue and age (Koch et al., 2002; Leclerc et al., 2010). CYP3A4 is the predominate isoform found in adult intestine and liver, whereas CYP3A5 is found primarily

ABBREVIATIONS: amu, atomic mass unit; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; GC, glucocorticoid; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; LC/MS, liquid chromatography–mass spectrometry; MS/MS, tandem mass spectrometry; P450, cytochrome P450; RT, retention time.

 Approximately 15 % of total work was completed by the second author.

TABLE 1

^{1}H NMR of flunisolide and Δ^6-flunisolide in chloroform-d_3

Compound	H_1	H_2	H_4	H_6	H_{7a}	H_{7b}	H_{11}	H_{16}	H_{21a}	H_{21b}
					ppm					
Flunisolide	7.16, d	6.29, d	6.31, s	5.25, dd, 5.35, dd	1.31, ddd	2.47, ddd	4.49, ddd	5.04, dd	4.14, d	4.64, d
Δ^6-Flunisolide	7.23, d	6.31, d	6.32, s	—	5.61, d	—	4.54. ddd	5.07, dd	4.17, d	4.65, d

in pulmonary tissue. CYP3A7 is the primary P450 expressed in fetal liver tissue; however, it is silenced within 6–12 months of birth, when CYP3A4 is transcriptionally activated (Wrighton et al., 1988; Schuetz et al., 1994; Lacroix et al., 1997; Leeder et al., 2005). Recent studies suggest that transcripts of CYP3A5 and CYP3A7 are found in adult liver (Hustert et al., 2001; Koch et al., 2002) and CYP3A7 in adult lung (Leclerc et al., 2010), but the lack of specific antibodies has precluded definitive confirmation of expression. Furthermore, GCs induce CYP3A expression, and mRNAs of all three isoforms are induced by dexamethasone in HepG2 liver cells (Krusekopf et al., 2003), and CYP3A5 mRNA is induced up to 4- to 6-fold by dexamethasone, budesonide, and beclomethasone in A549 lung cells (Hukkanen et al., 2003). CYP3A43 is the most recently discovered CYP3A gene and has demonstrated low testosterone hydroxylase activity (Domanski et al., 2001). However, it is only expressed at levels of about 0.1% of CYP3A4 in the liver and is not found in the lung ((Domanski et al., 2001; Westlind et al., 2001). Therefore, CYP3A43 was not investigated in this study.

Tissue- and age-dependent expression of the specific members of the CYP3A family of enzymes could lead to differences in the pharmacokinetics of GCs and could affect the therapeutic outcome and toxicities of these compounds. Because the majority of inhaled GCs are swallowed and absorbed systemically, CYP3A4 in the liver is primarily responsible for the efficient systemic clearance. However, in neonatal patients, CYP3A7 is highly expressed in the liver and would play a vital role in systemic clearance. Although only 2%–10% of inhaled GCs remain in the lungs, it is this small portion of the drug at the target site that is most likely responsible for the therapeutic effect of inhaled GCs. Therefore, small changes in the concentration at this site via CYP3A5 or CYP3A7 metabolism could substantially alter the ability of specific GCs to control asthma symptoms. The purpose of this study was to evaluate the relative ability of the CYP3A family of enzymes to metabolize four commonly used inhaled GCs with similar structures, via known P450 pathways, and to identify new metabolites. This information could substantially improve therapeutic outcomes with GCs.

Materials and Methods

Chemicals, Reagents, and Treatments. All GCs (fluticasone propionate, flunisolide, budesonide, and triamcinolone acetonide), internal standard (prednisolone), deuterated chloroform, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), and reagents were purchased from Sigma-Aldrich (St. Louis, MO). GC stocks were prepared in dimethylsulfoxide, internal standard was prepared in 50:50 chloroform/methanol. All other chemicals for synthesis or analysis were of analytical grade or equivalent and obtained at the highest grade commercially available.

Instrumentation. Liquid chromatography–tandem mass spectrometry (LC/MS) was conducted on a Thermo LCQ Advantage Max ion trap instrument equipped with a Finnigan Surveyor LC pump, Surveyor Autosampler, and universal Ion Max source operated with Thermo Xcalibur software version 2.0 (Thermo Fisher Scientific, Waltham, MA). Triamcinolone acetonide, flunisolide, budesonide, fluticasone propionate, and their metabolites were resolved on a 150 × 2 mm Gemini 5-μm C6-Phenyl high-performance liquid chromatography (HPLC) column (Phenomenex, Torrance, CA). The mobile phase consisted of solvent A: acetonitrile; and solvent B: 0.1% formic acid (v/v). The mobile phase was increased from 5% to 27% solvent A for 5 minutes, increasing to 70% over 20 minutes, and finally held at 100% for 5 minutes, with a flow rate of 0.2 ml/min. Selected ion monitoring and tandem mass spectrometry (MS/MS) fragmentation were used to identify each GC compound and their respective CYP3A-dependent metabolites, using previously published material and established P450-dependent mechanisms to predict potential metabolites. Each method also scanned for the internal standard prednisolone (*m/z* 361). Novel GC metabolites were identified and verified by the predicted mass shifts relative to parent compounds, and MS/MS spectra were compared with previously published material and verified using predictive software tools in ChemBioDraw version 11.0.1 (CambridgeSoft, Cambridge, MA) and Mass Frontier 4.0 (HighChem, Bratislava, Slovakia).

Determination of Rates of Elimination. Recombinant P450s, containing P450 reductase and cytochrome b_5, were purchased from BD Biosciences (San Jose, CA). In vitro incubations contained 10 pmol P450, 1 μM substrate, 30 mM potassium phosphate buffer (pH 7.4), and 1.3 mM NADPH in a final reaction volume of 0.5 ml. Negative control incubations omitted NADPH. Incubations were initiated by the addition of NADPH and allowed to proceed at 37°C. Aliquots (50 μl) were removed at 0, 2, 4, 6, 10, 15, and 20 minutes. Aliquots from the incubations were added to 2× volume ice-cold acetonitrile containing internal standard (1 nmol prednisolone). Protein was removed by centrifugation at 21,000*g* for 15 minutes. Supernatant was removed and dried to completion under nitrogen gas and reconstituted in 60 μl of initial mobile phase for analysis via LC/MS. Kinetic curves were generated by plotting the parent/internal standard ratios versus time, and kinetic parameters (half-life [$t_{1/2}$]) were calculated by fitting the data to one-phase exponential decay [$Y = (Y_0 - \text{Plateau})(e^{-k_{obs}X}) + \text{Plateau}$; $t_{1/2} = 0.69/k_{obs}$)] with GraphPad Prism 4.02 (GraphPad Software, La Jolla, CA).

Identification of GC Metabolites. Recombinant P450s, containing P450 reductase and cytochrome b_5, were purchased from BD Biosciences. In vitro incubations contained 50 pmol P450, 100 μM substrate, 30 mM potassium phosphate buffer (pH 7.4), and 1.3 mM NADPH in a final reaction volume of 0.5 ml. Negative control incubations omitted NADPH. Incubations were initiated by the addition of NADPH and allowed to proceed at 37°C. Reactions were terminated by the addition of 2× volume of ice-cold acetonitrile. Protein was removed by centrifugation at 21,000*g* for 15 minutes. GC metabolites were extracted using C-18 Sep-Pak cartridges (Waters, Taunton, MA). The resulting eluate was dried to completion under nitrogen gas and reconstituted in 60 μl of initial mobile phase for analysis via LC/MS.

TABLE 2

^{1}H NMR of triamcinolone acetonide and 21-nortriamcinolone acetonide in chloroform-d_3

Compound	H_1	H_2	H_4	H_{11}	H_{16}	H_{21a}	H_{21b}
				ppm			
Triamcinolone acetonide	7.16, d, 1H	6.32, d, 1H	6.11, s, 1H	5.03, d, 1H	4.40, d, 1H	4.65, d, 1H	4.15, d, 1H
21-Nortriamcinolone acetonide	7.15, d, 1H	6.33, d, 1H	6.12, s, 1H	5.07, d, 1H	4.42, d, 1H	—	—

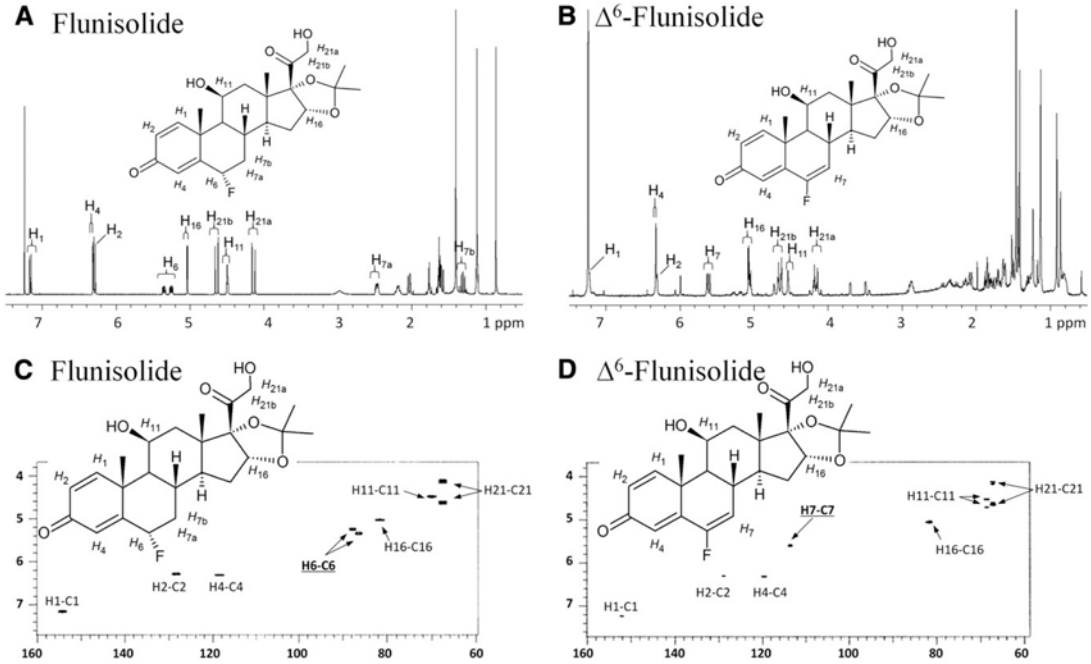


Fig. 1. ^{1}H NMR spectra of (A) flunisolide and (B) Δ^6-flunisolide in chloroform-d_3. HSQC spectra of (C) flunisolide and (D) Δ^6-flunisolide in chloroform-d_3, showing key differences between the two compounds. H_6-C_6 signals of flunisolide are split due to the coupling with the neighboring fluoride atom.

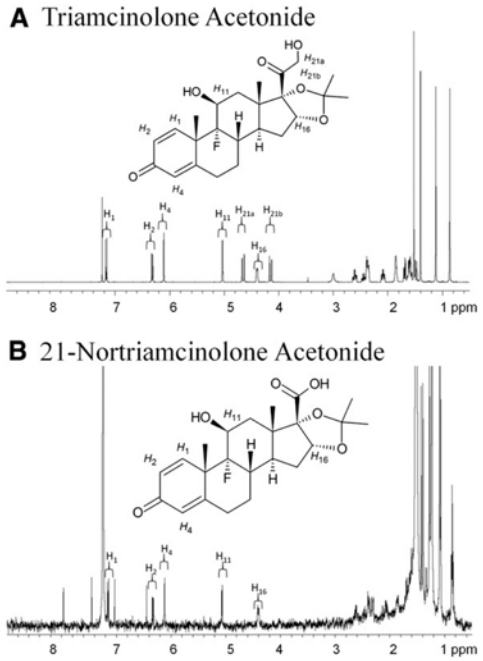


Fig. 2. ^{1}H NMR of (A) triamcinolone acetonide and (B) 21-nortriamcinolone acetonide in chloroform-d_3. The loss of H_{21a} and H_{21B} indicates the loss of C_{21} to form 21-nortriamcinolone acetonide.

Δ^6-Flunisolide and 21-Nortriamcinolone Acetonide Synthesis. Triamcinolone acetonide or flunisolide (18 mg) was dissolved in 15 ml of dry acetonitrile. DDQ (15 mg) was added, and the reaction mixture was refluxed for 4 hours at 90°C with consistent stirring. The mixture was allowed to cool to 25°C, after which glutathione (30.7 mg in 0.48 ml of phosphate-buffered saline, pH 7.4) was added and the mixture stirred for 0.5 hours. The synthesized products were extracted using C-18 Sep-Pak cartridges. The products were eluted off the cartridges with 100% methanol, and the eluate was evaporated to dryness under nitrogen and reconstituted in 1:1 acetonitrile/H_2O (v/v). The product of interest was isolated via HPLC, conducted on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) including an autosampler and a diode-array UV/VIS detector. Chromatography was performed on a Phenomenex Luna 5-μm C18 (250 $\times$ 4.60 mm) reverse-phase column, with the mobile phase consisting of linear gradient from 20% to 80% acetonitrile over 20 minutes with water as the countersolvent and a flow rate of 1 ml/min. Collected fractions were pooled and lyophilized for 48 hours. Products were solubilized in deuterated chloroform for analysis via NMR on an Inova500 NMR (Agilent Technologies). The chemical shifts of the parent compounds and the synthesized standards are found in Tables 1 and 2.

Results

Synthesis of Δ^6-Flunisolide and 21-Nortriamcinolone Acetonide. Dehydrogenation of flunisolide could occur at several places, so to establish the site of desaturation and provide a synthetic standard of this metabolite, flunisolide was chemically oxidized with DDQ to form a dehydrogenated product. Following isolation of the product with *m/z* 433 (−2 atomic mass units [amu] of parent compound) via HPLC, the ^{1}H NMR and the heteronuclear single quantum coherence (HSQC) spectra of both the parent and the dehydrogenated compound were determined (Fig. 1; Table 1). From the flunisolide ^{1}H and HSQC spectra, the H_6 proton was identified by its downfield shift and

TABLE 3

Observed half-lives for clinically relevant inhaled glucocorticoids by CYP3A isoforms in vitro

Drug	Half-Life of Drug (Mean ± S.E.) in Isoform		
	3A4	3A5	3A7
	min		
Fluticasone propionate	0.86 ± 0.08	16 ± 2	58 ± 9
Flunisolide	16 ± 1	N.A.	N.A.
Triamcinolone acetonide	12 ± 1	130 ± 43	213 ± 100
Budesonide	11 ± 1	40 ± 5	71 ± 16

N.A., not applicable.

splitting due to coupling to the fluorine (Fig. 1, A and C). The protons H_{7a} and H_{7b} were located at 1.31 and 2.47 ppm. However, the spectra of the dehydrogenated product showed that the H_6 signal was no longer present. Furthermore, although the protons at 1.31 and 2.47 ppm (H_{7a} and H_{7b}, respectively) were no longer detected, there was now a doublet at 5.61 ppm (Fig. 1, B and D). This doublet signal is consistent with a single proton on C_7, which has been shifted downfield because it is on an sp_2 carbon of an alkene. The loss of protons on C_6 and C_7, and the addition of an alkene at C_6-C_7, unambiguously established the structure as Δ^6-flunisolide.

Although the original intent of the chemical oxidation of triamcinolone acetonide was to obtain a dehydrogenated product, fortuitously a product with *m/z* 421 was discovered. Because a novel metabolite of *m/z* 421 was observed from the CYP3A-mediated metabolism of triamcinolone acetonide (see *Identification of Metabolites*), this product was collected for analysis by proton NMR. Pure triamcinolone acetonide was used as a comparison with obtain proton NMR data (Table 2). From these data, the doublet signals at 4.15 and 4.65 ppm were assigned to the two protons (H_{21b} and H_{21a}, respectively) on C_{21} (Fig. 2A). The NMR spectrum of the *m/z* 421 product was similar to that of the parent; however, the doublet signals for H_{21b} and H_{21a} were lost (Fig. 2B). Due to the loss of these protons and the mass decrease of 14 amu from the parent, we identified this chemical oxidation product as 21-nortriamcinolone acetonide.

Rates of Elimination of GC. Beginning with an initial concentration of 1 μM, the initial clearance rate of each GC was evaluated for each CYP3A isoforms. Despite the structural similarities among these GCs, significant variability between the rates of elimination was observed for each enzyme (Table 3). Furthermore, there was notable variability in the rate of disappearance catalyzed by different members of the CYP3A family (Fig. 3). CYP3A4 was the most efficient at metabolism of all four GCs, and fluticasone propionate had the shortest half-life. CYP3A5 was the second-most-efficient metabolizer of fluticasone propionate and budesonide, and CYP3A7 was the least

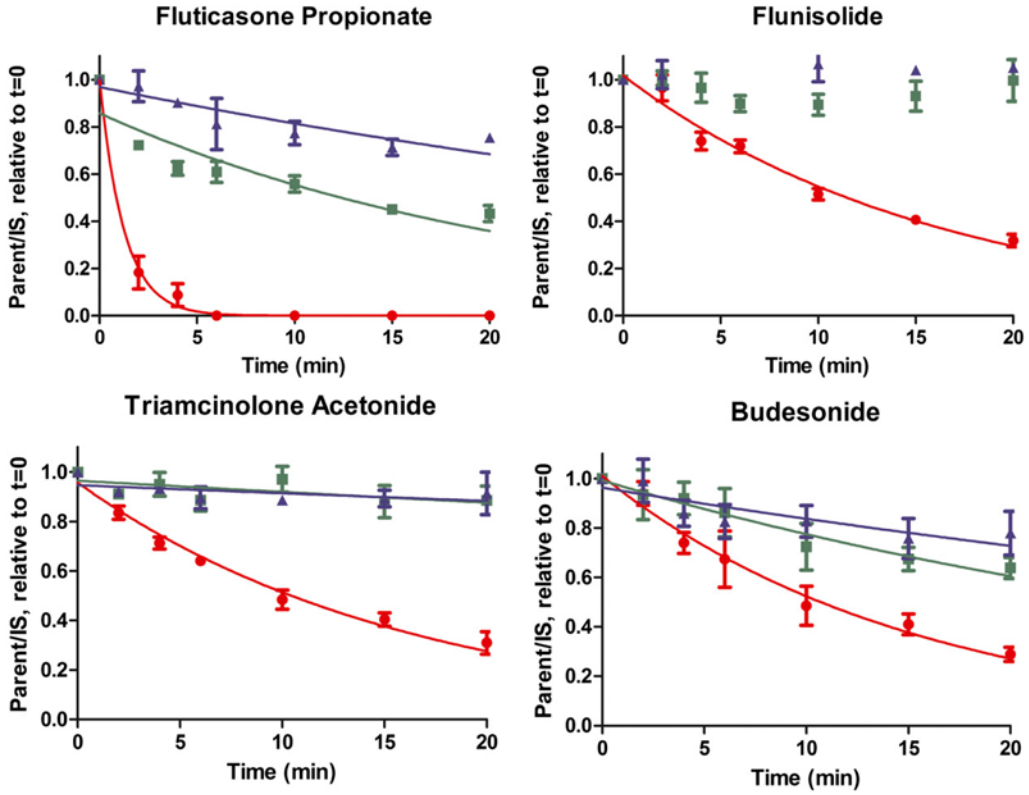
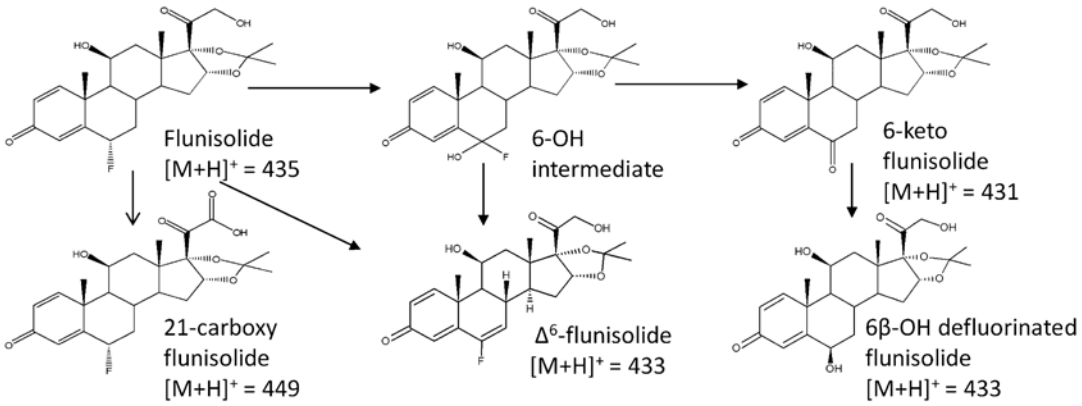


Fig. 3. Plots representing the initial rate of elimination for fluticasone propionate, flunisolide, triamcinolone acetonide, and budesonide by CYP3A4 (red circles), CYP3A5 (green squares), and CYP3A7 (blue triangles). All time points were run in triplicate. IS, internal standard.



Scheme 1. Scheme of CYP3A-mediated metabolism of flunisolide.

efficient enzyme. CYP3A5 and CYP3A7 were poor metabolizers of triamcinolone acetonide. Although flunisolide metabolites were detected for CYP3A5 and CYP3A7 at time points greater than 15 minutes, the initial rates of elimination were negligible.

Identification of Metabolites. Incubations with recombinant CYP3A enzymes were used to identify new and previously identified GC metabolites. Teitelbaum et al. (1981) previously demonstrated that flunisolide is metabolized to a 6-keto metabolite (*m/z* 431), a 6β-hydroxy-defluorinated metabolite (*m/z* 433), and Δ^6-flunisolide (*m/z* 433) using human liver microsomes (Scheme 1). From our incubations with recombinant CYP3As, four major flunisolide metabolites were identified with *m/z* 433, *m/z* 449, and two at *m/z* 431 (Fig. 4). The 433 *m/z* metabolite was identified as Δ^6-flunisolide, because it had the identical retention time (RT = 20.6 minutes) and MS/MS fragmentation as chemically synthesized Δ^6-flunisolide (Figs. 4 and 5). The novel *m/z* 449 metabolite (RT = 23.1 minutes) was determined to be 21-carboxy flunisolide based on the comparison of the MS/MS fragmentation of the *m/z* 449 metabolite to that of the parent (Fig. 5). Unfortunately, the core steroid structures of all the GCs tested are very stable and do not fragment to any predictable ions. Therefore, it is difficult to determine the site of oxygenation on these compounds by MS/MS. However, for all of the GCs tested, and others not in this study, the major sites of metabolism occur at the C_6 position or on the D-ring substituents. The *m/z* 449 metabolite is 14 amu greater than that of the parent, suggesting the formation of a ketone without the loss of the fluorine. The fluorine is at the C_6 position, which was the only major site of GC metabolism on the core steroid structure. Therefore, oxygenation at this site would most likely result in the loss of the fluorine and the formation of the 6-keto metabolilte. Because the fluorine was retained, the ketone must be on one of the D-ring substituents of flunisolide. The fragmentation of the m/z 449 metabolite produced daughter ions at *m/z* 335 and 353, corresponding to the loss of the acetonide group, while retaining the ketone on the molecule. Together, these data strongly suggest that the ketone is found on the hydroxyacetone group of flunsiolide. Furthermore, P450s are known to catalyze the formation of acetic acid from alcohol (Bell-Parikh and Guengerich, 1999), and triamcinolone acetonide has previously been shown to form the same P450-mediated 21-carboxy metabolite (Argenti et al., 2000). Two metabolites with *m/z* 431 (RT = 15.8 and 17.6 minutes) were also observed, consistent with the formation of a ketone, and the additional loss of HF. From the work of Teitelbaum et al., one of the *m/z* 431 metabolites is assumed to be 6-keto flunisolide. Again, the lack of fragmentation of the core steroid structure prevents precise identification of the site of oxygenation. However, the daughter ions at *m/z* 355 and 377 correspond to the loss of acetonide, while retaining the ketone on the molecule (Fig. 5). In addition, the loss of HF on the molecule suggests that oxygenation is occurring on the B-ring of flunisolide. Unfortunately, the MS/MS fragmentations of both *m/z* 431 metabolites were identical, and so 6-keto flunisolide could not be assigned to either peak. Teitelbaum et al. identified 6β-hydroxy-defluorinated flunisolide in their studies with human liver microsomes. This metabolite was not detected using

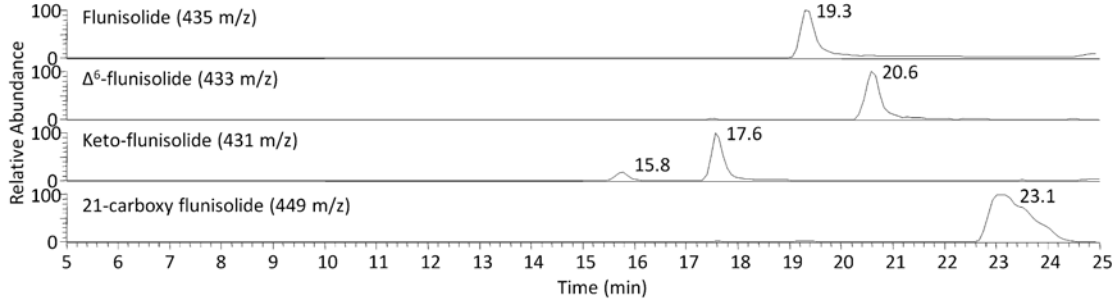


Fig. 4. LC/MS chromatograms of flunisoide and its CYP3A-mediated metabolites.

recombinant CYP3A enzymes, and therefore it was concluded that the oxidation of 6-keto flunisolide to 6β-hydroxy-defluorinated flunisolide is mediated via enzymes other than the CYP3As. The Δ^6-flunisolide and 6-keto flunisolide metabolites were probably both formed through a 6-OH, 6-F intermediate, which could lose HF to form the 6-keto metabolite or lose H_2O to form Δ^6-flunisolide (Scheme 1). It is theoretically possible that Δ^6-flunisolide was formed directly via P450-mediated dehydrogenation, but our results could not differentiate this pathway from the dehydration of the 6-OH, 6-F intermediate.

Interestingly, fluticasone propionate, which is structurally similar to flunisolide, was reported in a previous study to only be oxidized to 17β-carboxy fluticasone propionate (Scheme 2) (Pearce et al., 2006). However, more recent work using fluticasone furoate has detected defluorinated and several hydroxylated metabolites in plasma and fecal samples (Hughes et al., 2008). Therefore, it was concluded that fluticasone propionate was metabolized by oxidative defluorination and hydroxylation by CYP3A enzymes. Analysis of fluticasone propionate incubations detected the previously reported 17β-carboxy fluticasone metabolite (RT = 19.7 minutes), but no additional metabolites were detected. Interestingly, in the absence of NADPH, incubating fluticasone propionate with either CYP3A supersomes or human liver microsomes did not yield any 17β-carboxy fluticasone propionate. This suggests that esterases do not cleave the thioester of

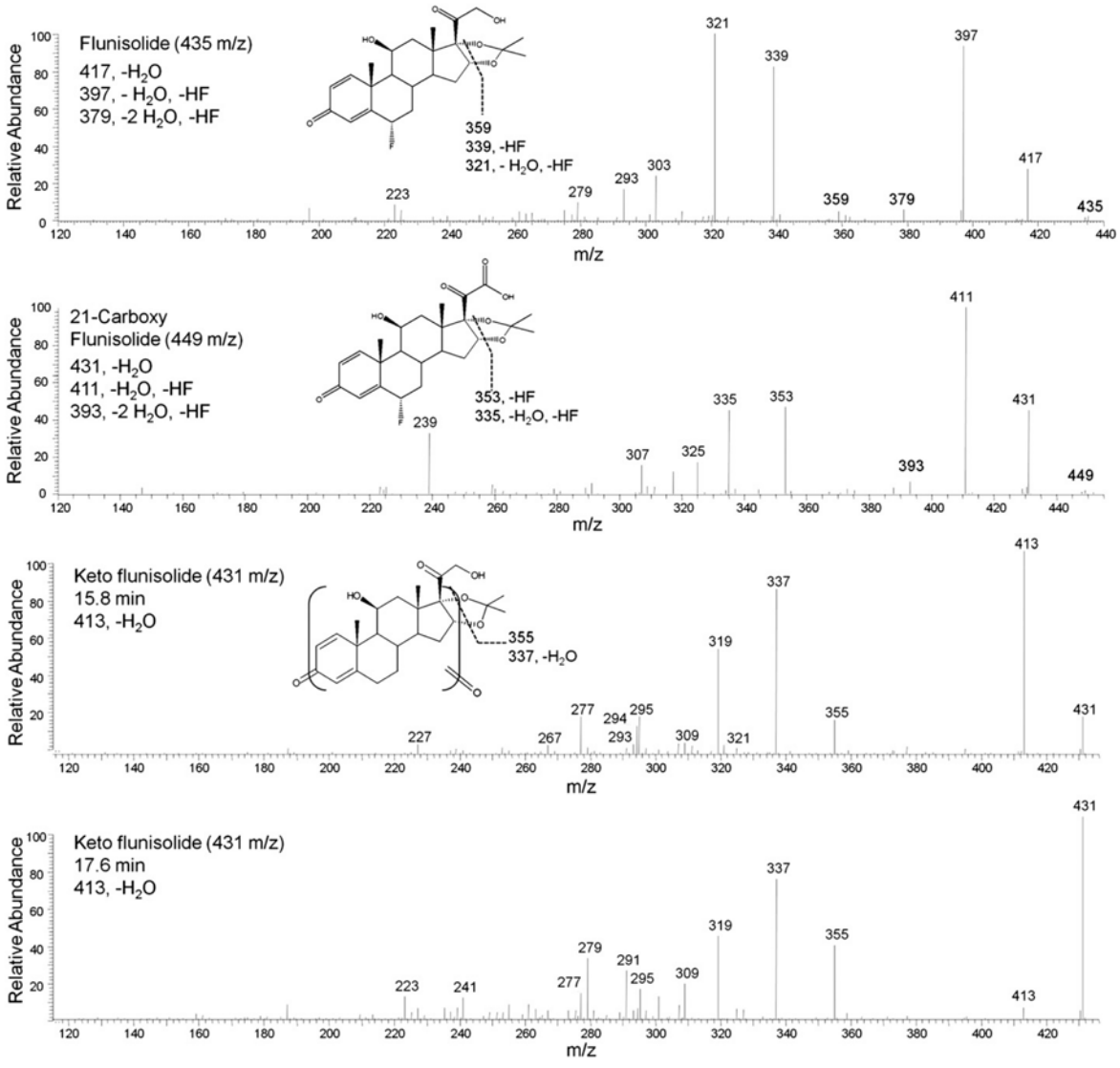
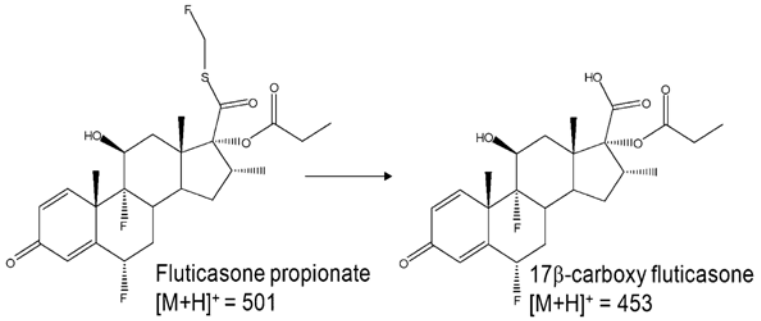


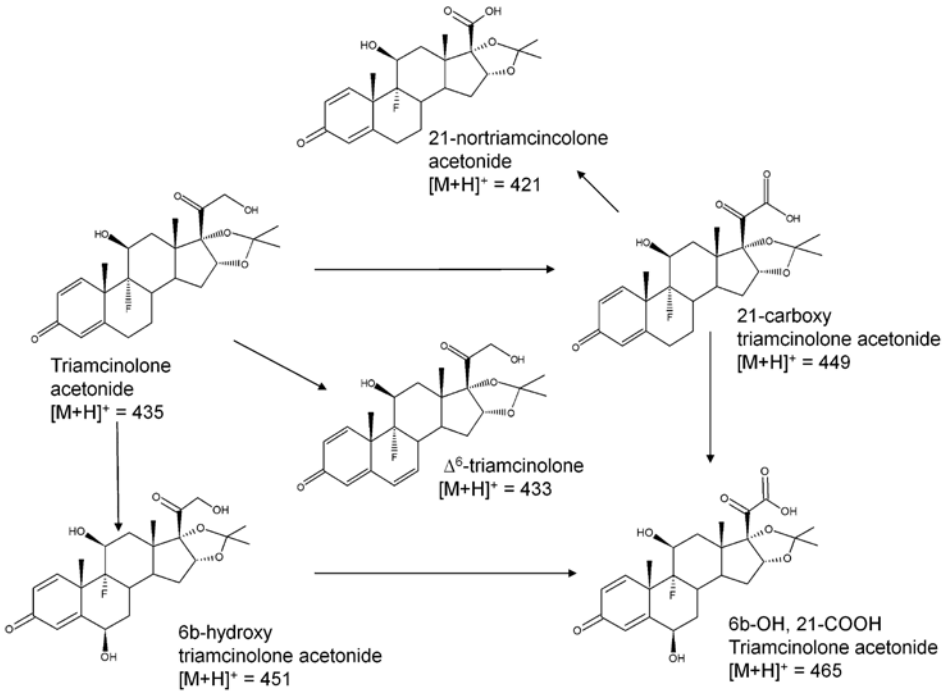
Fig. 5. MS/MS fragmentation spectra of flunisolide and its CYP3A-mediated metabolites.



Scheme 2. Scheme of CYP3A-mediated metabolism of fluticasone propionate.

fluticasone propionate, but that this hydrolytic process is catalyzed selectively by P450 enzymes.

Previous work by Argenti et al. (2000) demonstrated that after oral administration triamcinolone acetonide (435 *m/z*) was metabolized to the 6β-OH (*m/z* 451) and 21-carboxy metabolites (*m/z* 449) and sequentially oxidized to 6β-OH, 21-carboxy triamcinolone acetonide (*m/z* 465) in human subjects (Scheme 3). From the analysis of triamcinolone acetonide incubated with the CYP3A enzymes, we were able to confirm the presence of all three metabolites (RTs: 6β-hydroxy triamcinolone acetonide = 14.9 minutes; 21-carboxy triamcinolone actinide = 23.7 minutes; and 6β-OH, 21-carboxy triamcinolone acetonide = 16.4 minutes) (Fig. 6). In addition, two new metabolite peaks with *m/z* 421 and 433 were observed. By means of MS/MS fragmentation, the *m/z* 421 peak (RT = 18.9 minutes) was identified as 21-nortriamcincolone acetonide (Fig. 7). The major daughter ions at *m/z* 401 and 383 correspond to the loss of HF and the additional loss of water, respectively. The ion at *m/z* 355 corresponded to the loss of the carboxyl group, resulting in a similar fragment from the parent. The ions at *m/z* 343 and 325 were attributed to fragmentation of the acetonide group similar to the parent, with the resulting ions differing by the loss of C_{21} (i.e., 14 amu). Furthermore, comparison with the authentic 21-nortriamcinolone acetonide synthetic standard showed identical retention time and MS/MS fragmentation pattern (Figs. 6 and 7). 21-Nortriamcinolone is likely produced by the CYP3A-mediated decarboxylation of 21-carboxy triamcinolone acetonide. Although not a common mechanism, P450s have been shown to catalyze this type of decarboxylation (Fukuda et al., 1994; Komuro et al., 1995). By MS/MS fragmentation, the *m/z* 433 analyte (RT = 19.1 minutes) was determined to be Δ^6-triamcinolone acetonide (Fig. 7). The *m/z* 433 peak had a similar fragmentation pattern to triamcinolone acetonide,



Scheme 3. Scheme of CYP3A-mediated metabolism of triamcinolone acetonide.

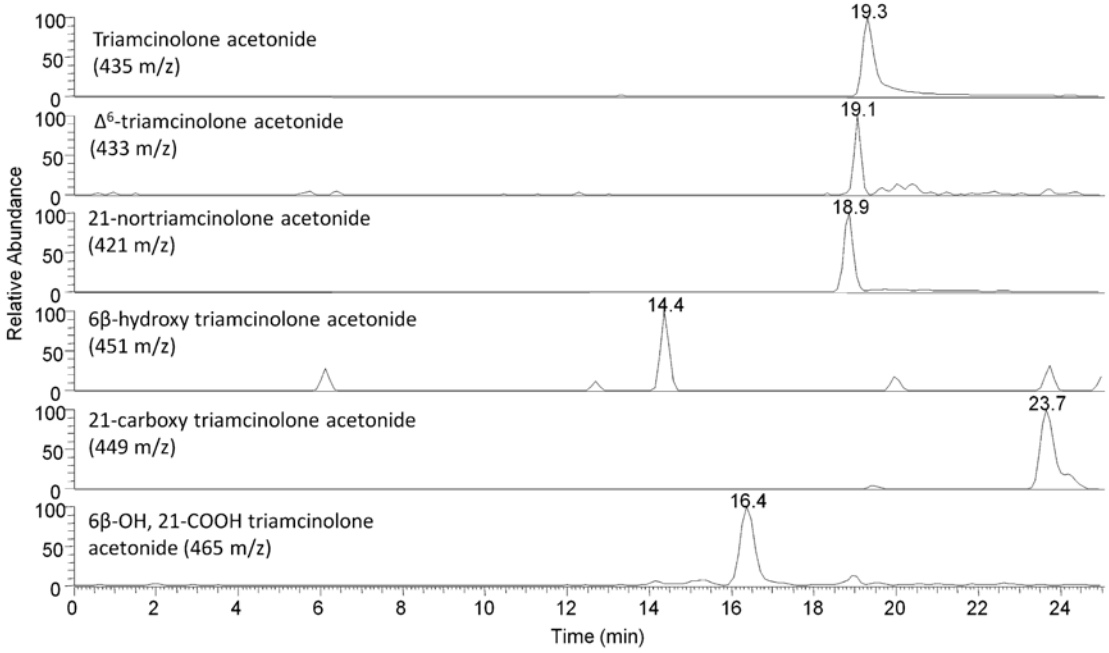


Fig. 6. LC/MS chromatograms of triamcinolone acetonide and its CYP3A-mediated metabolites.

but the major daughter ions were decreased by 2 amu, characteristic of dehydrogenated products.

Budesonide has been extensively studied and showed multiple hydroxylation products by human liver microsomes, including a 6β-hydroxy metabolite, a 23-hydroxy metabolite, and hydroxylation at C_{22}, which is probably rearranged to an ester, followed by cleavage to 16α-hydroxy prednisolone (Scheme 4) (Edsbacker et al., 1987a,b; Jonsson et al., 1995). Budesonide was also efficiently dehydrogenated to Δ^6-budesonide. We were able to detect these metabolites from all three CYP3A enzymes. Interestingly, preincubating the recombinant CYP3A supersomes with esterase inhibitors prevented the formation of 16α-hydroxy prednisolone, leading us to conclude that esterases, and not P450s, are responsible for cleavage of the ester. No novel metabolites were identified from the CYP3As' metabolism of budesonide.

Discussion

Inhaled GCs are potent anti-inflammatory drugs that have become the mainstay for the treatment of persistent asthma. Despite this fact, approximately 30% of asthmatics have some degree of steroid resistance or insensitivity (Chan et al., 1998; Szefler et al., 2002). The CYP3A enzymes are the major P450 enzymes known to metabolize these compounds. Therefore, interpatient variability in the metabolism of inhaled GCs could play a role in steroid resistance and insensitivity. Furthermore, GCs have demonstrated the ability to induce CYP3A expression in liver and lung cells (Hukkanen et al., 2003; Krusekopf et al., 2003), which could increase the rate of clearance at the site of action and/or systemically. For these reasons, it is vital to understand the role of CYP3As in the metabolism of inhaled GCs.

Our goal in this study was to investigate initial rates of clearance of the GCs with the CYP3As. The rates of metabolism of the inhaled GCs demonstrated both interenzyme and interdrug variability (Fig. 3; Table 3). CYP3A4 is the most efficient enzyme at eliminating all of the GCs tested. Although CYP3A5 is usually less efficient than CYP3A4, results here demonstrate that CYP3A5 is still an effective metabolizer, while CYP3A7 was the least efficient of the CYP3As. Fluticasone propionate is the most rapidly metabolized of all the GCs with all three enzymes. This is of particular importance because it has recently been demonstrated that fluticasone propionate was an efficient mechanism-based inactivator of CYP3A5, but CYP3A4 and CYP3A7 were inactivated to lesser extents (Murai et al., 2010). Interestingly, both CYP3A5 and CYP3A7 did not significantly metabolize flunisolide. Although CYP3A5 did appear to produce some hydroxylated flunisolide metabolites when incubated for long periods of time (30 minutes or more), the initial rate of metabolism was too slow to measure. Being the predominate lung CYP3A isoform, CYP3A5 activity will have the most profound effect on the GC concentration at the site of action. Furthermore, chronic GC use could result in increased levels of CYP3A5, resulting in more rapid GC elimination and a potential corresponding decrease in drug efficacy, with the exception of fluticasone propionate, which inactivates CYP3A5 and inhibits its own metabolism. This inactivation process could be a potential mechanism through which patients might develop steroid resistance and insensitivity.

In addition to induction, CYP3A5 has been shown to be polymorphically expressed, with significant interracial differences in prevalence (Hustert et al., 2001; Kuehl et al., 2001). It has been demonstrated that people with at least one *CYP3A5*1* allele express large amounts of enzyme, while the single-nucleotide polymorphisms in *CYP3A5*3* and **6* exhibit alternative splicing and protein truncation. Consequently, those with *CYP3A5*3* genotype have

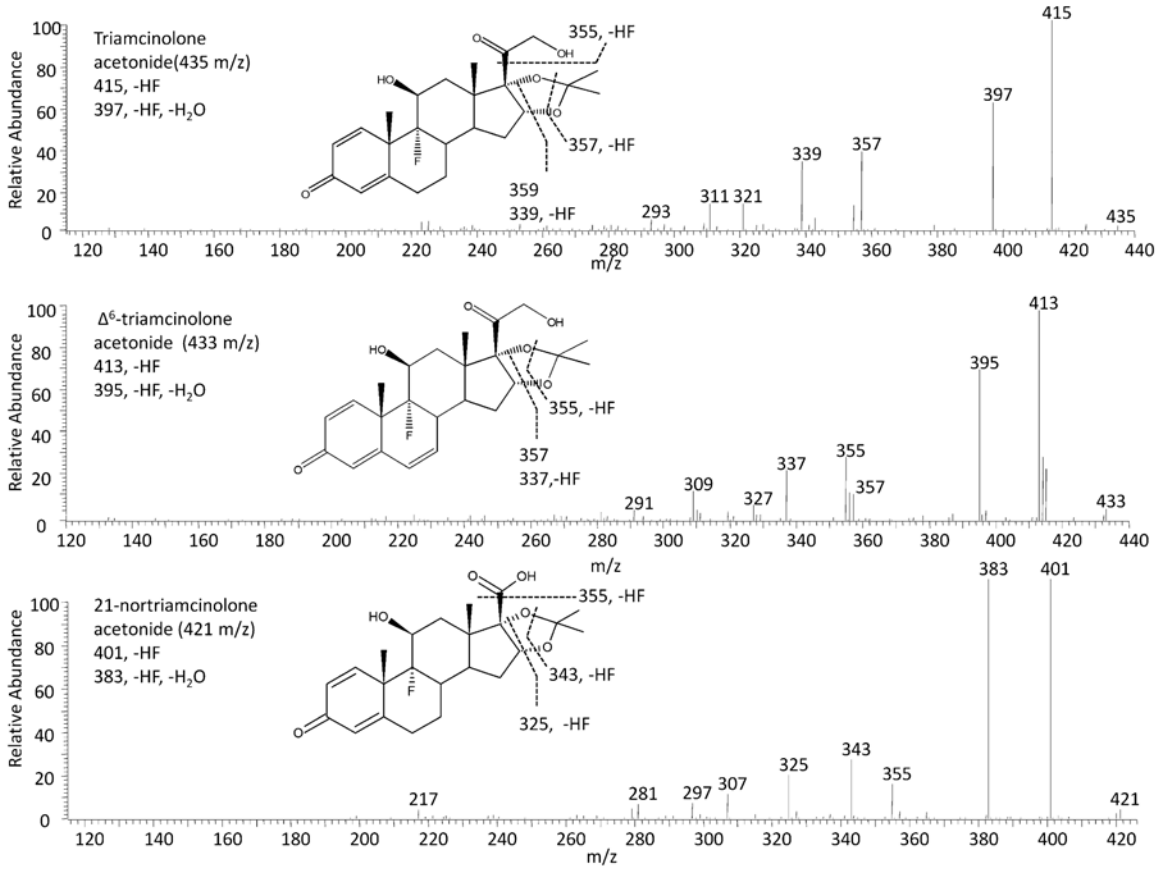
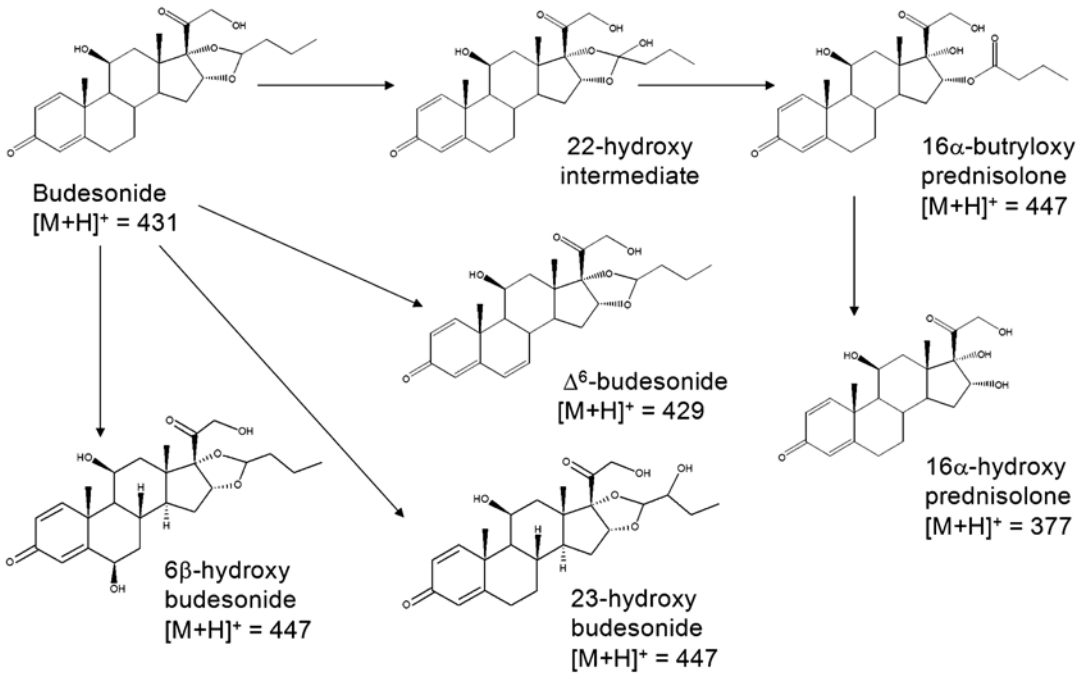


Fig. 7. MS/MS fragmentation spectra of triamcinolone acetonide and its CYP3A-mediated metabolites.

variable drug metabolism compared with the *CYP3A5*1* populace (Kim et al., 2009). It is likely that those with *CYP3A5*3* or **6* would have decreased metabolism of GCs, and therefore higher levels of GCs at the therapeutic site of action. Conversely, those who are *CYP3A5*1/*1* carriers would have increased levels of GC metabolism, which could result in insensitivity to GCs as a result of more efficient clearance in lung cells. Alternatively, flunisolide is not metabolized by CYP3A5 and would not be affected by polymorphic differences in CYP3A5 expression.

The four inhaled GCs tested in this study share the same basic steroid structure, except for the site of fluorination on the B-ring. CYP3A-mediated oxygenation of steroids often occurs at the C_6 on the B-ring. This regiospecific reaction is stereospecific as well, because it produces a hydroxyl group in the β-orientation. Among the GCs tested, CYP3A4 was able to oxygenate the C_6 position of the all the compounds except fluticasone propionate. Absence of fluorines (budesonide), fluorination at C_6 (flunisolide), or fluorination at C_9 (triamcinolone acetonide) did not affect hydroxylation of these compounds. Fluticasone propionate is unique as it is fluorinated at both the C_6 and C_9 positions, which may impede C_6 hydroxylation by causing steric impediments in the enzyme active site, which may block binding and catalysis of the C_6 above the heme. Alternatively, the two strong electron-withdrawing fluorines on C_6 and C_9 may impede the initial abstraction of hydrogen from the C_6 position, preventing subsequent hydroxyl rebound, or subsequent electron abstraction to produce the dehydrogenated product. Furthermore, whereas CYP3A5 and CYP3A7 catalyzed hydroxylation at the C_6 position of triamcinolone acetonide and budesonide, fluticasone propionate was not metabolized at this position. Further study is needed to determine the reason(s) why only the double-fluorinated GC was not hydroxylated at the C_6 position.

In addition to hydroxylation at the C_6 position, P450s were previously found to dehydrogenate GC at the C_6-C_7 position (Teitelbaum et al., 1981; Edsbacker et al., 1987a). Although Δ^6-flunisolide could be formed via a dehydrogenation reaction or loss of water from the 6-OH intermediate (Scheme 1), Δ^6-budesonide can only arise from a dehydrogenation reaction. Therefore, we sought to determine if the CYP3As could form dehydrogenated products. Our studies detected CYP3A4-mediated dehydrogenated metabolites of triamcinolone acetonide, flunisolide, and budesonide. CYP3A5- and CYP3A7-mediated dehydrogenated metabolites of triamcinolone acetonide and budesonide were also detected. It was not possible to unequivocally differentiate Δ^6-flunisolide formation by a dehydrogenation reaction or from the loss of water through the 6-OH intermediate. The dehydrogenation of flunisolide at the C_6-C_7 position was confirmed by comparison with an NMR-authenticated synthetic



Scheme 4. Scheme of CYP3A-mediated metabolism of budesonide.

standard. Unlike flunisolide, the Δ^6-budesonide and novel Δ^6-triamcinolone acetonide metabolites could only be formed by a dehydrogenation reaction. Although MS/MS fragmentation could not identify the site of dehydrogenation, it likely occurs at the C_6-C_7 position because this is the site of hydroxylation, and budesonide and flunisolide are dehydrogenated at this position. Interestingly, only fluticasone propionate did not form a detectable CYP3A-mediated dehydrogenated metabolite. However, if the double fluorination prevents hydroxylation at this site, it would also prevent dehydrogenation by the same process. Conversely, we have shown that fluticasone propionate is an efficient mechanism-based inactivator of CYP3A5, but the inactivating reactive intermediate was not identified (Murai et al., 2010). With the CYP3A-mediated metabolism producing triamcinolone acetonide and budesonide dehydrogenated products at the C_6–C_7 position, it is reasonable to speculate that CYP3A5 dehydrogenates fluticasone to a reactive intermediate at this vulnerable position. Dehydrogenated products are often unstable and can only be detected by trapping with nucleophiles. The dehydrogenated products that were identified in this study were stabile enough to be detected without trapping agents, suggesting that they are not highly electrophilic. Unfortunately, these dehydrogenated metabolites were labile in aqueous solutions, which precluded isolation and identification, except for the relatively stabile Δ^6-flunisolide product. However, our preliminary studies have discovered NADPH-dependent glutathione adducts with masses corresponding to the parent + glutathione for all the GCs tested (data not shown). These results strongly suggest that the GCs are susceptible to CYP3A-mediated dehydrogenation. Future studies with trapping agents will help to identify dehydrogenated products and elucidate the reactivity of these intermediates.

Other than the fluoridation on the B-ring, the most significant structural differences between the inhaled GCs were the modifications found on the D-ring. In addition to the C_6 position, these D-ring modifications were the sites of the majority of the metabolism. The only CYP3A-mediated metabolite of fluticasone propionate was formed by the cleavage of the thioester attached to the D-ring. Interestingly, the adjacent ester moiety was not cleaved by any of the CYP3As. Incubating fluticasone propionate with human liver microsomes, without NADPH, did not produce any fluticasone propionate metabolites. This suggests that neither the thioester nor the carboxylester were cleaved by esterases. Flunisolide, budesonide, and triamcinolone acetonide share a hydroxyacetone moiety on C_{17}. However, metabolism on this moiety varied greatly. Both flunisolide and triamcinolone acetonide were metabolized by CYP3A oxygenation at the C_{21} position to form similar 21-carboxy metabolites (Figs. 4 and 7). Triamicinolone acetonide was also metabolized to a 21-nortriamcinolone acetonide metabolite, which was confirmed by comparison with an NMR-authenticated standard (Fig. 2). In contrast, the hydroxyacetone moiety of budesonide was not oxidized by any of the CYP3As. We speculated that the carbon chain on the 16,17-butylidenebis(oxy) moiety of budesonide prevents positioning of hydroxyacetone for oxygenation at the C_{21} position. The CYP3As are able to oxygenate both C_{22} and C_{23} of budesonide (Scheme 4), suggesting that the butylidenebis(oxy) moiety prevents the hydroxyacetone moiety from occupying the correct position above the catalytic center.

This study demonstrates both the interenzyme and interdrug variability of the CYP3A metabolism of four commonly prescribed inhaled GCs. Although CYP3A4 was the most efficient metabolizer of all the compounds tested, CYP3A5 was also shown to be an efficient

metabolizer of all the compounds except flunisolide. All the CYP3A isoforms produced previously identified metabolites, and the novel metabolites Δ^6-triamcinolone acetonide, 21-carboxy triamcinolone acetonide, 21-nortriamcinolone acetonide, and 21-carboxy flunisolide were identified. These studies will aid in elucidating the mechanisms of steroid insensitivity and resistance via differential metabolism, which is critical to improving the clinical therapeutic use of inhaled GCs.

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Authorship Contributions

Participated in research design: Moore, Orton, Reilly, Ward, Yost.

Conducted experiments: Moore, Roberts, Orton, Murai, Fidler.

Contributed new reagents or analytic tools: Moore, Murai, Fidler.

Performed data analysis: Moore.

Wrote or contributed to the writing of the manuscript: Moore, Orton, Reilly, Ward, Yost.

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CHAPTER 3

METABOLISM OF BECLOMETHASONE DIPROPIONATE BY CYTOCHROME P450 3A ENZYMES

Metabolism of Beclomethasone Dipropionate by Cytochrome P450 3A Enzymes

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ABSTRACT

Inhaled glucocorticoids, such as beclomethasone dipropionate (BDP), are the mainstay treatment of asthma. However, ~30% of patients exhibit little to no benefit from treatment. It has been postulated that glucocorticoid resistance, or insensitivity, is attributable to individual differences in glucocorticoid receptor-mediated processes. It is possible that variations in cytochrome P450 3A enzyme-mediated metabolism of BDP may contribute to this phenomenon. This hypothesis was explored by evaluating the contributions of CYP3A4, 3A5, 3A7, and esterase enzymes in the metabolism of BDP in vitro and relating metabolism to changes in CYP3A enzyme mRNA expression via the glucocorticoid receptor in lung and liver cells. CYP3A4 and CYP3A5 metabolized BDP via hydroxylation ([M4] and [M6]) and dehydrogenation ([M5]) at similar rates; CYP3A7 did not metabolize BDP. A new metabolite [M6], formed by the combined action of esterases and CYP3A4 hydroxylation, was also characterized. To validate the results observed using microsomes and recombinant enzymes, studies were also conducted using A549 lung and DPX2 liver cells. Both liver and lung cells produced esterase-dependent metabolites [M1–M3], with [M1] correlating with CYP3A5 mRNA induction in A549 cells. Liver cells produced both hydroxylated and dehydrogenated metabolites [M4, M5, and M6], but lung cells produced only the dehydrogenated metabolite [M5]. These studies show that CYP3A4 and CYP3A5 metabolize BDP to inactive metabolites and suggest that differences in the expression or function of these enzymes in the lung and/or liver could influence BDP disposition in humans.

Introduction

Beclomethasone dipropionate (BDP) (Fig. 1) is a glucocorticoid administered by inhalation to treat asthma. BDP is administered as a prodrug that requires cleavage of the C-21 ester by esterase enzymes to be pharmacologically active (Wilcox and Avery, 1973; Brogden et al., 1984). Prior studies have described the production and pharmacokinetic properties of beclomethasone-17-monopropionate ([M1] in Fig. 2) and the pharmacologically less active metabolites beclomethasone 21-monopropionate [M2] and beclomethasone [M3] in human plasma and human lung homogenates (Foe et al., 1998a,b, 2000a,b; Daley-Yates et al., 2001). All three BDP metabolites are glucocorticoid receptor (GR) agonists, but beclomethasone 17-monopropionate [M1] exhibits 30-fold greater affinity for GR than BDP, whereas beclomethasone 21-monopropionate [M2] has ~50-fold lower affinity than BDP (Wurthwein and Rohdewald, 1990; Chanoine et al., 1991).

Prior studies demonstrated that de-esterification and activation of BDP was principally mediated by esterases (Mutch et al., 2007). Additionally, three other metabolites, designated as D-1, D-2, and D-3, have been documented (Foe et al., 1998a, b). D-2 and D-3 contained an epoxide on the C-ring at positions 9 and 11 (Fig. 1), presumably arising from cytochrome P450-mediated oxygenation and hydrolysis of both the 17- and 21-esters (D-2) or the 21-ester only (D-3); these metabolites are presumably inactive/clearance metabolites. Metabolism arising from the A- and B-rings of BDP (Fig. 1) has not been reported in the literature, nor has the production of specific metabolites by individual cytochrome P450 enzymes.

Xenobiotic metabolism in humans and animals frequently involves the action of cytochrome P450 enzymes. The most abundant subfamily of cytochrome P450 enzymes contributing to the clearance of the largest number of different xenobiotics is the CYP3A enzymes (Thummel and Wilkinson, 1998). CYP3A4, 3A5, and 3A7 are the most pertinent cytochrome P450 enzymes for this study because they are the most prominent cytochrome P450 enzymes involved in glucocorticoid

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ABBREVIATIONS: A549, human lung adenocarcinoma; B2M, β2 macroglobulin; BDP, beclomethasone dipropionate; DPX2, HepG2 background with PXR and a CYP3A4 reporter construct stably expressed; GR, glucocorticoid receptor; LC/MS/MS, liquid chromatography-tandem mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; PXR, pregnane X receptor; qPCR, real-time quantitative polymerase chain reaction.

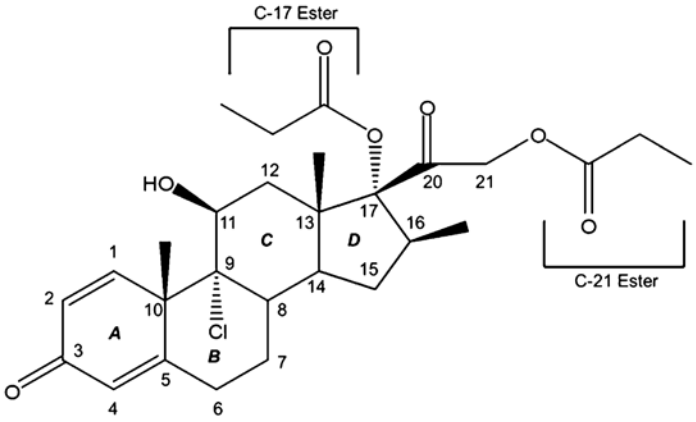

Fig. 1. Structure of and atom numbering for BDP.

metabolism (Jonsson et al., 1995; Pearce et al., 2006). CYP3A4 is the most abundant CYP3A enzyme in the liver and intestines (Jonsson et al., 1995; Westlind-Johnsson et al., 2003; Leclerc et al., 2010), and CYP3A5 is preferentially expressed in the lung (Hukkanen et al., 1997, 2003; Leclerc et al., 2010). CYP3A7 is expressed in fetal liver, but diminishes after birth as CYP3A4 becomes the dominant CYP3A enzyme (Schuetz et al., 1994; Lacroix et al., 1997). To date, the metabolism of BDP by these three human CYP3A enzymes and the contribution of cytochrome P450-dependent metabolism to the overall metabolism of BDP in lung and liver cells have not been reported.

The purpose of this study was to evaluate CYP3A-mediated metabolism of BDP to provide insights into how cytochrome P450 enzymes might affect the disposition of BDP in lung cells, which are the target for inhaled glucocorticoids. These studies are part of a larger study having an overarching hypothesis that variations in pulmonary metabolic clearance of glucocorticoids by CYP3A enzymes may impact the therapeutic efficacy of inhaled glucocorticoids in humans.

Materials and Methods

Chemicals. Beclomethasone dipropionate (BDP), prednisolone, NADPH, ammonium acetate, eserine, benzoic acid, ketoconazole, and methanol were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Paraoxon was purchased from Chem Service (West Chester, PA). Recombinant cytochrome P450 3A4, 3A5, and 3A7 enzymes were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Human liver microsomes were purchased from Celsis In Vitro Technologies (Baltimore, MD). Beclomethasone 17-monopropionate, beclomethasone 21-monopropionate, and beclomethasone were purchased from Steraloids, Inc. (Newport, RI).

Liquid Chromatography-Tandem Mass Spectrometry. Liquid chromatography-tandem mass spectrometry (LC/MS/MS) was conducted using a Thermo LCQ Advantage Max ion trap instrument equipped with a Finnigan Surveyor LC pump, Surveyor Autosampler, and universal Ion Max source operated with Thermo Xcalibur software version 2.0 (Thermo Fisher Scientific, Waltham, MA). Positive electrospray ionization (ESI) was used. The mass spectrometer was optimized for the detection of BDP. The source temperature was 290°C, ionization voltage was 4.5 kV, capillary voltage was 6 V, and the sheath gas (N_2) was 60 units. Parameters for MS/MS analysis were as follows: a collision energy of 27.5% for BDP, [M1], [M2], [M3],

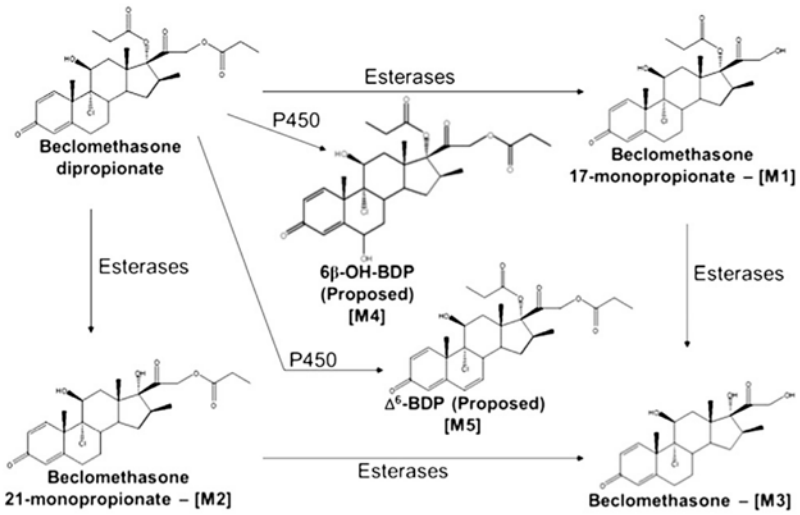

Fig. 2. Proposed metabolic scheme for beclomethasone dipropionate. [M1] is the active form of the drug. [M1], [M2], and [M3] metabolites are produced by esterases, and [M4] and [M5] are produced by cytochrome P450 enzymes.

and prednisolone (internal standard), and 30% for [M4], [M5], and [M6]; activation Q = 0.25; activation time = 30 ms; and an isolation width of 2 amu. BDP, BDP metabolites, and prednisolone (internal standard) were resolved on a 150 × 2-mm Gemini 5-μm C18 reverse-phase HPLC column (Phenomenex Inc., Torrance, CA) and eluted with a linear gradient of 45–90% methanol over 17 minutes and holding at 90% methanol for 8 minutes. The aqueous solvent was 2 mM ammonium acetate (pH 6.4), the flow rate was 0.2 ml/min, and the column temperature was 30°C. BDP, BDP metabolites, and prednisolone (internal standard) were identified by the $[M+H]^+$ ions m/z 521 (BDP), m/z 465 ([M1] and [M2]), m/z 409 ([M3]), m/z 537 ([M4]), m/z 519 ([M5]), m/z 481 ([M6]), and m/z 361 (prednisolone), in addition to the presence or absence of diagnostic product ions in the MS/MS spectra, LC retention time, and comparison with authentic standards ([M1] and [M2] only). A representative chromatogram obtained from the analysis of BDP, BDP metabolites, and prednisolone recovered from an in vitro incubation of BDP with CYP3A4 is shown for reference as Fig. 3. Comparison of the rates and extent of BDP metabolism and metabolite formation by CYP3A enzymes was based on the analyte-to-internal standard (prednisolone) peak area ratios obtained from data analysis using Thermo Xcalibur 2.0 software.

Characterization of BDP Metabolites. In vitro incubations using human liver microsomes contained 100 pmol of cytochrome P450, 20 μM BDP, and 2 mM NADPH in 50 mM potassium phosphate buffer, pH 7.4 (0.5 ml total volume). Esterase inhibitors (eserine, paraoxon, and benzoic acid in a 1:1:1 mixture, each at 35 μM) were added to inhibit esterase-mediated metabolism and to ascertain the relative contributions of cytochrome P450 enzymes and esterases in BDP metabolism. Reactions were initiated by addition of NADPH and continued at 37°C for 20 minutes. Reactions were terminated by adding 0.5 ml methanol containing prednisolone (5 nM). The samples were cooled on ice for 5 minutes, and the insoluble material was pelleted by centrifugation for 10 minutes at 21,000*g*. The supernatant was collected, loaded onto C18 Sep-Pak cartridges (Waters, Taunton, MA), washed with water, and eluted with 100% methanol. The eluates containing BDP and metabolites were dried under forced air, reconstituted in 60 μl H_2O:methanol (1:1 v/v), centrifuged at 21,000*g* for 5 minutes, and transferred to auto sampler vials for analysis.

Metabolism of BDP by Recombinant CYP3A Enzymes. Incubations contained 2.5 pmol recombinant CYP3A enzyme, 1 μM BDP, and 1.3 mM NADPH in 30 mM potassium phosphate buffer, pH 7.4 (0.5 ml total volume). Control incubations did not contain NADPH. The esterase inhibitors described above were also included in selected incubations, except at concentrations of 28 μM to account for the lower esterase content associated with the recombinant CYP3A microsomes relative to human lung and liver microsomes. Incubations were performed at 37°C, with aliquots of 50 μl removed at 0, 5, 10, 15, 20, 30, 40, and 60 minutes. Aliquots were mixed with an equal volume of methanol containing prednisolone (1 nM) and prepared for LC/MS/MS analysis as described above. The ratio of the peak area for BDP relative to the internal standard at each time point was plotted versus time. Data were fit using a one-phase exponential decay model [$Y = \text{Span}(e^{-k_{obs}X}) + \text{Plateau}$; plateau = 0, $t_{1/2} = 0.69/k_{obs}$] using GraphPad Prism 4.02 software for Windows (San Diego, CA) and approximate $t_{1/2}$ values are reported.

Characterization of BDP Metabolites Produced by Individual CYP3A Enzymes. Incubations were performed as described above, except 25 pmol of recombinant CYP3A4, 3A5, or 3A7 and 50 μM BDP (0.5 ml total volume) were used.

Analysis of BDP Metabolites in Cell Culture. A549 cells (human lung adenocarcinoma) (ATCC, Manassas, VA) were cultured

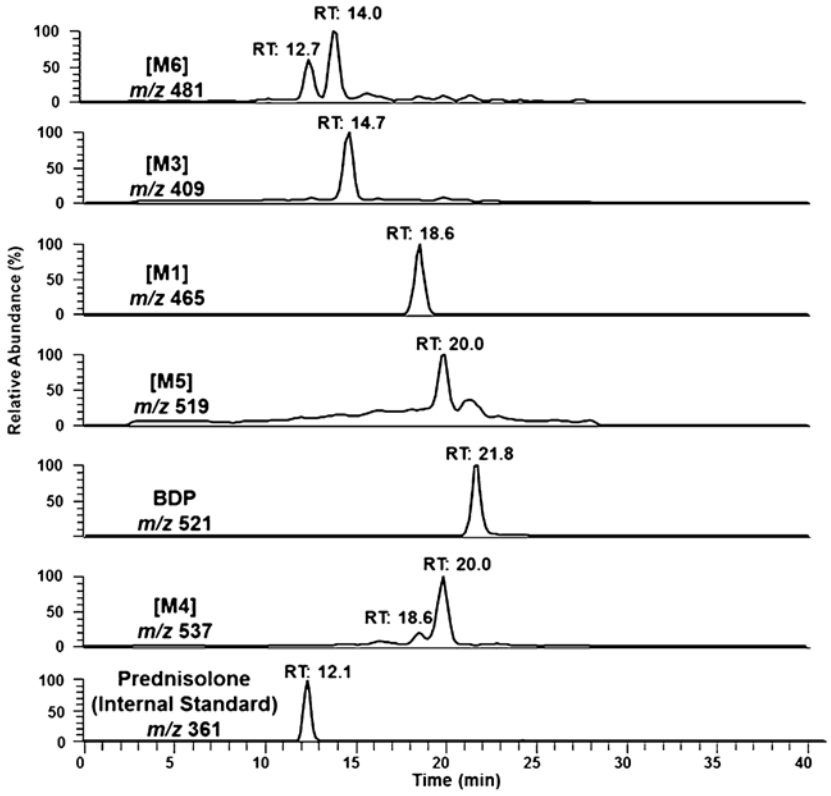

Fig. 3. Representative LC/MS selected-ion monitoring (SIM) chromatograms showing the analyte peaks corresponding to prednisolone (the internal standard), BDP, and BDP metabolites generated by CYP3A4 in vitro. Individual SIM traces are labeled with the corresponding metabolite identities, m/z filter, and analyte elution time.

in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (Life Technologies, Grand Island, NY). DPX2 cells (HepG2 background with human PXR stably overexpressed to drive the expression of a CYP3A4 reporter gene construct by PXR agonists) were provided by Dr. Judy Raucy (Puracyp Inc., Carlsbad, CA). DPX2 cells were cultured in Puracyp media (Puracyp Inc.) (Lamb et al., 2010). Both cell lines were plated in six-well plates and treated with BDP (10 μM) in Opti-MEM 1 reduced serum media (Life Technologies) at 70% confluence, with and without esterase inhibitors (paraoxon and eserine 1:1; 175 μM), for 24 hours. Cells treated with esterase inhibitors were pretreated for 2 hours, followed by addition of BDP for 22 hours. The cell culture medium was extracted using 2× volume (6 ml) methyl *tert*-butyl ether containing prednisolone (1 nM). Samples were centrifuged and the organic fraction was collected, dried under air at room temperature, reconstituted in 60 μl H_2O:methanol (1:1 v/v), clarified again by centrifugation, and the supernatant transferred to auto sampler vials for LC/MS/MS analysis. Treatments were also performed in the presence of ketoconazole (1 μM), a selective CYP3A inhibitor, to determine if the metabolites observed in cell culture media were due to CYP3A enzyme activity.

Quantification of CYP3A Enzyme Expression in A549 and DPX2 Cells. Cells were treated as described above using dimethyl sulfoxide as the negative control. TRIzol reagent (Life Technologies) was used to extract total RNA, as per the manufacturer protocol. Total RNA was used to synthesize cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio Rad, Hercules, CA), as per the manufacturer's protocol. The sequences of PCR primers for CYP3A4, 3A5, 3A7, and β2-macroglobulin (B2M) are provided in Table 1. Real-time quantitative PCR (qPCR) was performed using LightCycler 480 Probes Master (CYP3A5) or LightCycler 480 SYBR Green I Master Mix (CYP3A4, CYP3A7, and β2 macroglobulin) (Roche, Indianapolis, IN) on a Light-Cycler 480 Instrument. The PCR program for assays using the probe mix consisted of a 5-minute incubation at 95°C, followed by 45 cycles of 95°C for 10 seconds, 55°C for 30 seconds, then 72°C for 1 second. The PCR program for samples using SYBR Green I mix consisted of a 5-minute incubation at 95°C, followed by 40 cycles of 95°C for 10 seconds, 63°C for 5 seconds, then 72°C for 10 seconds. Experiments were performed on six biologic replicates (n = 6), mRNA copy number was determined from a standard curve, and the data normalized to the copy number for B2M.

Results

Metabolism of BDP In Vitro. The known esterase-dependent BDP metabolites [M1], [M2], and [M3] (Foe et al., 1998a,b, 2000a,b; Daley-Yates et al., 2001; Mutch et al., 2007) were detected in all incubations lacking esterase inhibitors; formation of [M1–M3] was inhibited when esterase inhibitors were used, with the caveat that trace quantities of [M1] were detected at the level observed in control samples where only BDP was added (i.e., the BDP source material contained trace quantities of [M1] or an [M1] like compound). Additionally, three NADPH- and cytochrome P450-dependent metabolites, [M4] (m/z 537 at 20 minutes), [M5] (m/z 519 at 20 minutes), and [M6] (m/z 481 at 12.7 and 14 minutes), were observed in incubations containing either human liver microsomes or recombinant CYP3A4 or CYP3A5 but not CYP3A7. The proposed metabolite identities are depicted in Fig. 4.

TABLE 1
qPCR primer sequences for the CYP3A genes and B2M

Gene	Primer Sequence
CYP3A5	Forward, 5′-CCTATCGTCAGGGTCTCTGGAA-3′
	Reverse, 5′-TGATGGCCAGCACAGGGA-3′
	Probe [6FAM]ATGTGGGGAACGTATGAA[BHQ1]
CYP3A4	Forward, 5′-GAAAGTCGCCTCGAAGATAC-3′
	Reverse, 5′-ACGAGCTCCAGATCGGACAG-3′
CYP3A7	Forward, 5′-TTCCGTAAGGGCTATTGGAC-3′
	Reverse, 5′-TCTGTGATAGCCAGCATAGG-3′
B2M	Forward, 5′ GATGAGTATGCCTGCCGTGTG-3′
	Reverse, 5′-CAATCCAAATGCGGCATCT-3′

Because authentic [M4], [M5], and [M6] were not available as standards, these metabolites were characterized by comparing the metabolite MS/MS spectra to those of BDP, [M1], [M2], and comparable metabolites produced by CYP3A enzymes using other structurally similar glucocorticoids (Moore et al., 2013). The MS/MS spectrum for [M4] (m/z 537 at 20 minutes; Fig. 3) was characterized by an MH+ ion at m/z 537 (i.e., +16 amu relative to BDP; Fig. 4, A and B). This mass shift and corresponding 16-amu shifts in several diagnostic fragment ions of BDP, most notably m/z 409, 427, and 445, which corresponded to the neutral loss of the D-ring substituents on C-17 and C-21, loss of water, and HCl, indicated hydroxylation of the core structure of BDP. The fragmentation pattern for [M4] excluded oxygenation on the propionate groups, suggesting that [M4] was most likely 6β-OH-BDP based on the fact that steroids and structurally similar glucocorticoids preferentially undergo 6β-hydroxylation by CYP3A enzymes and [M4] was a common metabolite for both CY3A4 and 3A7 (Jonsson et al., 1995; Teng et al., 2003; Peet et al., 2005; Pearce et al., 2006; Hughes et al., 2008).

The MS/MS spectrum for [M5] (Fig. 4C; m/z 519 at 20 minutes in Fig. 3) was characterized by a molecular ion at m/z 519 (versus m/z 521 for BDP; Fig. 4A), consistent with desaturation of BDP. Cytochrome P450 3A enzymes dehydrogenate the C-6/7 bond of multiple steroid molecules and other glucocorticoids (Teitelbaum et al., 1981; Edsbacker et al., 1987; Moore et al., 2013). Therefore, [M5] is presumed to be Δ^6-BDP. Consistent with this identification, the fragment ions m/z 501 (MH^+-H_2O), 409 (loss of the ester group on C-21), and 393 (loss of the ester group on C-17, H_2O, and HCl) were 2 amu less than the corresponding fragment ions for BDP (Fig. 4, A and C). Of note, [M5] was detected in cell culture incubations when [M4] was not present, indicating that [M5] was not the product of [M4] dehydration during LC/MS analysis (i.e., it is a cytochrome P450-dependent metabolite). Additionally, glutathione did not reduce [M5] formation as shown for the dehydrogenated metabolites of other glucocorticoids (Moore et al., 2013), indicating that [M5] was not electrophilic (unpublished data).

Two additional analyte peaks/metabolites, designated [M6] (m/z 481 at 12.7 and 14 minutes in Fig. 3) were also observed. The MS/MS spectra of the two [M6] chromatographic peaks were indistinguishable. The MS/MS spectrum for the [M6] metabolite peak eluting at 14 minutes is shown in Fig. 5 and is characterized by the addition of oxygen, presumably to the B-ring as described above for [M4], in addition to the removal of an ester group at the C-21 position. On the basis of the MS/MS data, the two [M6] component peaks are believed to arise from oxygen addition at two different sites on the steroid core structure, as discussed for [M4] and CYP3A4 below, and not from differential de-esterification at the C-17 and C-21 positions. The formation of [M6] could theoretically occur through two routes: oxygenation of the B-ring by cytochrome P450 enzymes followed by esterase-mediated cleavage at the C-21 position or the reverse

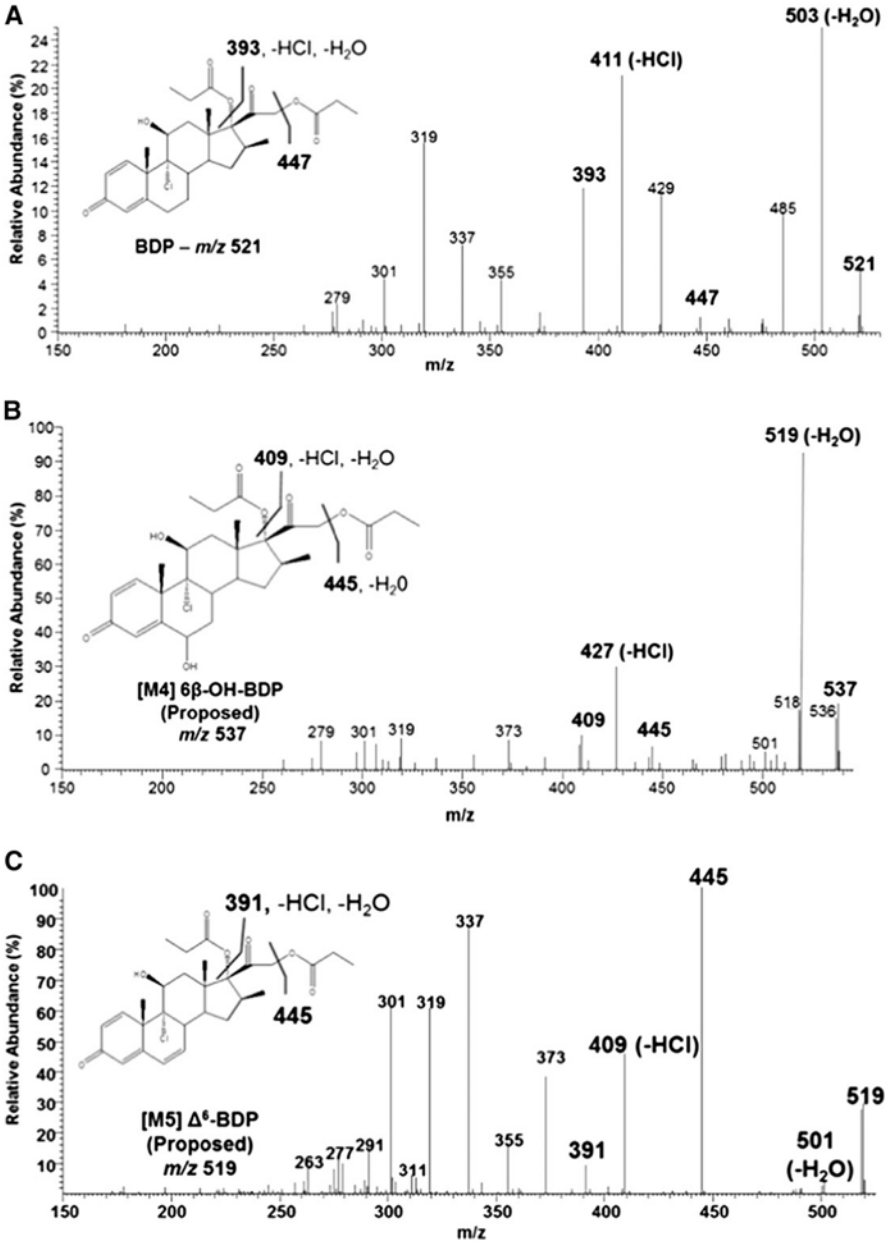

Fig. 4. (A) MS/MS spectra for BDP, (B) [M4], and (C) [M5]. Diagnostic fragment ions for the metabolites [M4] and [M5] versus the corresponding fragments for BDP are highlighted in bold text. For [M4], these ions were +16 amu and for [M5] they were −2 amu, relative to BDP. Insets show the proposed metabolite structure and neutral loss events leading to the formation of the diagnostic fragment ions highlighted for each metabolite.

sequence, as depicted in Fig. 6. Later studies showed that only CYP3A4 could produce [M6] in the presence of active esterases.

Differential Metabolism of BDP by CYP3A4, 3A5, and 3A7. Half-lives for the disappearance of BDP in incubations containing recombinant CYP3A4, 3A5, or 3A7 were determined using identical experimental conditions. CYP3A4 ($t_{1/2}$ = 55 ± 15 minutes) and CYP3A5 ($t_{1/2}$ = 43 ± 12 minutes) displayed similar kinetics for BDP decomposition, resulting in an approximate 25% decrease in BDP concentration over the course of a 20-minute incubation. CYP3A7 did not metabolize BDP, even at longer time periods.

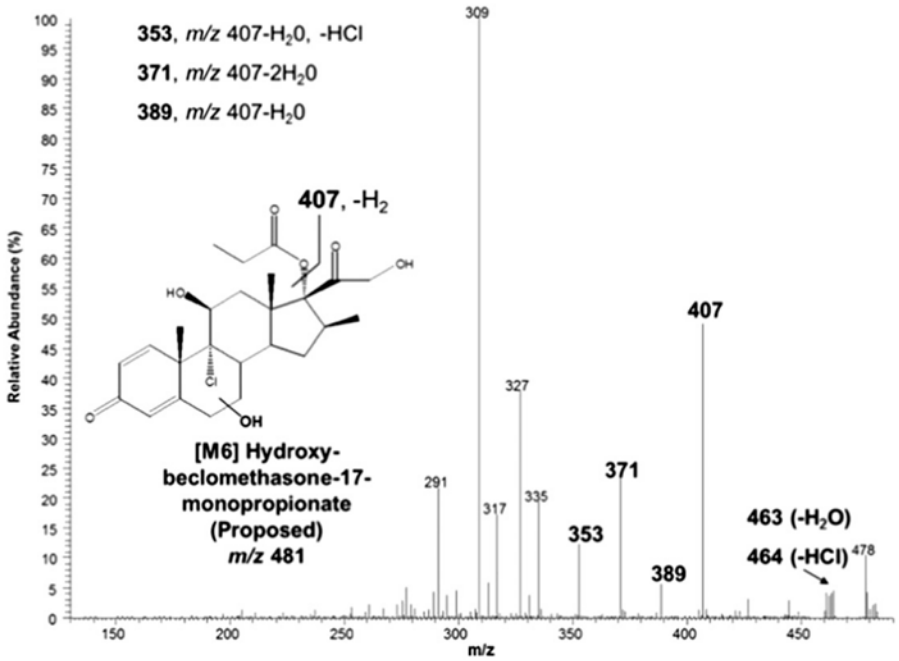

Fig. 5. MS/MS spectrum of the [M6] peak eluting at 14 minutes (Fig. 3), with the proposed structure and neutral loss events leading to the diagnostic fragment ions depicted in the inset.

CYP3A4 and CYP3A5 Produced [M4] and [M5] In Vitro. Incubations were performed to determine which CYP3A enzymes produced which BDP metabolites. Recombinant CYP3A5 produced both [M4] and [M5], whereas CYP3A4 produced [M4], [M5], and [M6] as well as an additional hydroxylated metabolite eluting at 18.6 minutes in the m/z 537 chromatogram of Fig. 3. The MS/MS spectrum of this minor CYP3A4-specific metabolite was identical to [M4] (Fig. 4B), indicating oxygenation of the core steroid structure similar to [M4]. It is postulated that this metabolite could represent a C-7 hydroxylated metabolite arising from the same metabolic intermediate as [M4] and [M5] or a metabolite oxygenated at either the C-9 or C-11 position, as described previously (Foe et al., 1998a,b). No de-esterified metabolites (i.e., [M1], [M2], or [M3]) were produced by the CYP3A enzymes.

Metabolism of BDP by CYP3A Enzymes in Cells. BDP metabolite formation was evaluated in A549 (Fig. 7A) and DPX2 (Fig. 7B) cells. [M1] and [M3] were produced by both cell lines when esterases were active, albeit to a greater extent in A549 cells. Consistent with the role of esterases in [M1] and [M3] production, including esterase inhibitors in the cell treatment solutions inhibited [M1] and [M3] formation, low levels of [M1] were attributed to [M1] (or a similar compound) occurring in the source BDP reagent. The production of the cytochrome P450-dependent metabolite [M5] was also observed in both A549 and DPX2 cells, but was not detected when A549 cells were pretreated and cotreated with the CYP3A inhibitor ketoconazole (unpublished data). These data confirm that [M5] was produced by CYP3A enzymes in A549 cells, presumably CYP3A5 based on Fig. 7C showing that only CYP3A5 mRNA was expressed. DPX2 cells, unlike A549 cells, also produced the cytochrome P450-generated metabolite [M4] (Fig. 7B), but both [M4] and [M5] were only observed when esterase activity was inhibited, presumably the result of preventing further metabolism of [M4] and [M5] to de-esterified products. Neither [M2] nor [M6] was detected in incubations from A549 or DPX2 cells.

Expression of CYP3A Enzymes in A549 and DPX2 Cells. qPCR was used to determine the expression of CYP3A4, 3A5, and 3A7 mRNA in A549 cells (Fig. 7C) and DPX2 cells (Fig. 7D). Only CYP3A5 mRNA was detected in A549 cells, which was significantly (~2-fold) induced by BDP treatment, presumably via [M1] binding to the glucocorticoid receptor (GR), as previously suggested (Dvorak and Pavek, 2010). In DPX2 cells, only mRNA for CYP3A5 and 3A7 were detectable under basal conditions, but 2- to 3-fold induction of mRNA for all three cytochrome P450 enzymes was observed following BDP treatment in the presence of esterase inhibitors (Fig. 7D), presumably the result of PXR activation by BDP, as previously described (Dvorak and Pavek, 2010).

Discussion

Inhaled glucocorticoids are the mainstay treatment of asthma. Although therapy is effective for the majority of patients, ~30% do not achieve adequate asthma control (Mjaanes et al., 2006). A possible explanation for ineffectiveness of treatment by glucocorticoids, referred to as steroid insensitivity, may be variations in the metabolic clearance capacity of therapeutically active glucocorticoids from lung cells involving CYP3A enzymes. Thus, the goal of this study was to evaluate whether the CYP3A family of enzymes, which are known to metabolize glucocorticoids,

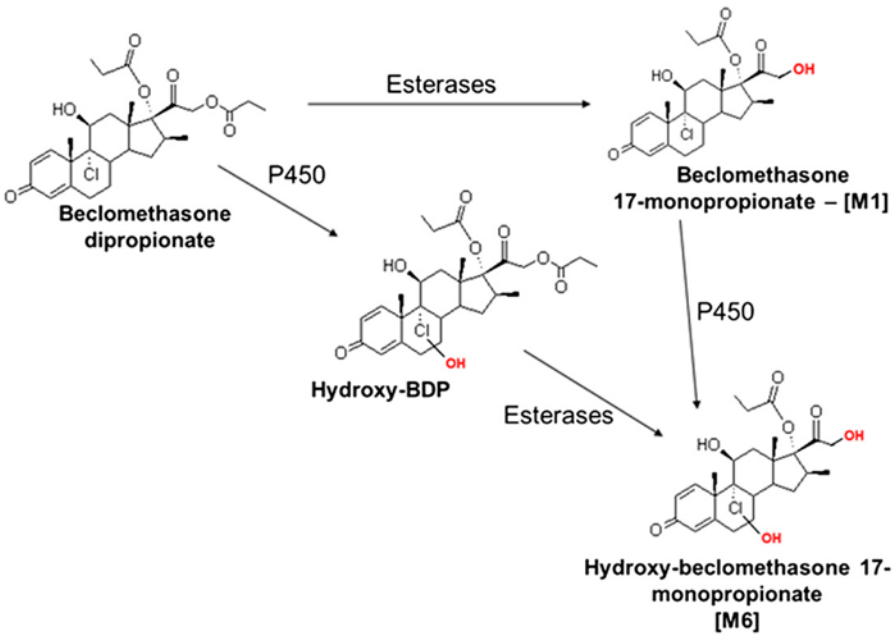

Fig. 6. Proposed metabolic scheme for the production of [M6] by esterase and CYP3A4 enzymes.

contributed to the pharmacological activation of BDP by hydrolyzing the ester at the C-21 position to form [M1] or if cytochrome P450 metabolism only produced pharmacologically less active or inactive metabolites such as [M2],[M3], and presumably [M4–6].

Characterization of BDP metabolism using human liver microsomes, recombinant CYP3A enzymes, lung, and liver cells demonstrated that cytochrome P450 enzymes were not involved in the production of the pharmacologically active metabolite [M1] but produced a number of previously uncharacterized and presumed inactive clearance metabolites [M4–6]. Further evaluation of BDP metabolism by individual CYP3A enzymes indicated that CYP3A4 and CYP3A5 metabolized BDP at similar overall rates but that BDP was not a substrate for CYP3A7 in vitro. Characterization of individual metabolites of BDP generated by CYP3A4 and CYP3A5 revealed the production of previously uncharacterized oxygenated [M4] and dehydrogenated [M5] metabolites, as well as [M6], a product of both CYP3A4-mediated oxygenation and esterase-mediated ester cleavage. Collectively, these results support the hypothetical concept that BDP efficacy may be impacted by the rate and extent to which BDP is metabolized to pharmacologically inactive metabolites in lung cells and/or elsewhere in the body by CYP3A enzymes.

In support of this idea, a recent publication by Stockmann et al. (2013) demonstrated that the expression of the *CYP3A4*22* allele, which codes for an inactive enzyme in the liver (Elens et al., 2011a,b, 2012, 2013; Kitzmiller et al., 2013), coupled with treatment of these patients with the CYP3A4 and 3A5 mechanism-based inhibitor fluticasone propionate (Murai et al., 2010), correlated with improved asthma control. As such, a decrease in CYP3A-mediated metabolism of fluticasone propionate (and possibly other glucocorticoids) in the lung and liver may increase the bioavailability of the active drug in the lung, leading to greater efficacy. Similarly, several genetic polymorphisms of CYP3A5 influence the expression levels of functional enzyme. The most common polymorphism is *CYP3A5*3*, which codes for an inactive form of CYP3A5 (Kuehl et al., 2001; Westlind-Johnsson et al., 2003). The majority of Caucasians are homozygotes for the *CYP3A5*3* allele and therefore do not express active CYP3A5 in the lung or elsewhere in the body. However, individuals that express the *CYP3A5*1* allele, express an active form of CYP3A5 (Kuehl et al., 2001). Following the logic above, such individuals may exhibit increased clearance capacity for BDP in lung cells and elsewhere in the body, potentially contributing to glucocorticoid insensitivity. However, this intriguing concept is speculative.

Results from his study may also suggest that developmental differences in CYP3A activity could influence BDP (and other glucocorticoid) activity. Specifically, because CYP3A7 is the dominant CYP3A enzyme expressed in newborn patients (Schuetz et al., 1994; Lacroix et al., 1997) and CYP3A7 does not efficiently metabolize BDP, infants could have decreased clearance of BDP in lung and liver cells relative to children and adults, potentially leading to greater exposure to active drug at lower doses. If this idea is confirmed by future studies, the ability to use lower doses to achieve therapeutic benefit could potentially reduce the risk for toxicity, such as adrenal crisis in patients receiving high doses of inhaled glucocorticoids (Newman, 2003).

Finally, the relationship between esterase and CYP3A-dependent metabolism and glucocorticoid receptor activity were explored using lung (A549) and liver (DPX2) cells (Fig. 7).

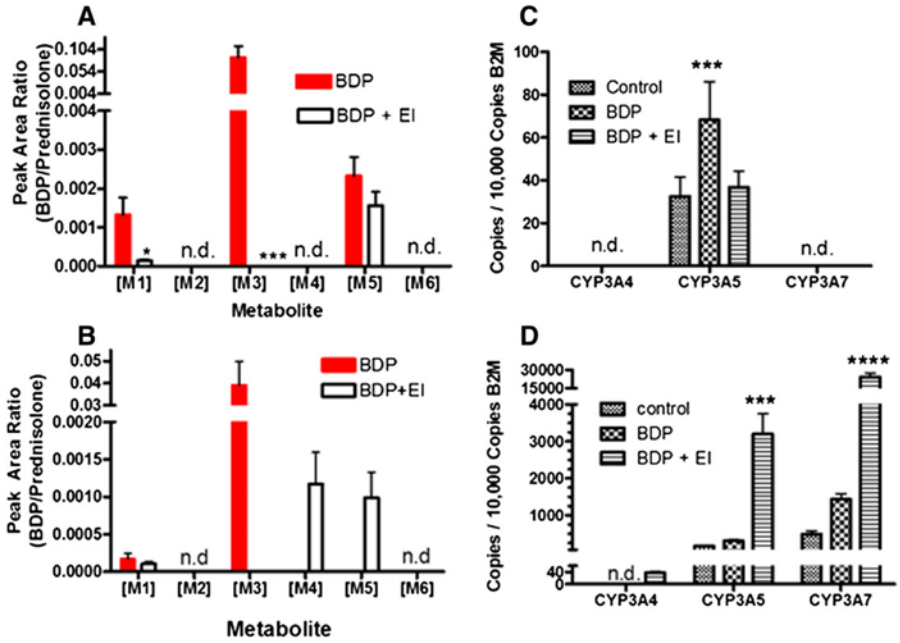

Fig. 7. (A) Quantification of BDP metabolites produced by A549 cells treated with BDP or BDP with esterase inhibitors (+EI). (B) Relative quantification of BDP metabolites produced by DPX2 cells treated with BDP or BDP with esterase inhibitors (+EI). Data are the mean and standard deviation from six replicates. n.d. Signifies that the metabolite was not detected. (C and D) CYP3A enzyme mRNA abundance, measured by qPCR in A549 (C) and DPX2 (D) cells. Data are represented as the number of mRNA copies per 10,000 copies of β2-macroglobulin (a "housekeeping" gene). Statistics used for A549 cell data analysis were one-way analysis of variance with Dunnett's post-hoc test. For DPX2 cell data analysis two-way ANOVA with Bonferronni post-hoc testing was used. Data are the mean and standard deviation from 6 replicates. n.d. Signifies that mRNA was not detected. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

In A549 and DPX2 cells, BDP was metabolized by esterases and CYP3A enzymes to yield [M1], [M3], [M4], and [M5], albeit to varying levels (Fig. 7, A and B). In A549 cells, the formation of [M1] from BDP was dependent upon esterases (Fig. 7A). Ketoconazole, a selective CYP3A enzyme inhibitor, blocked [M5] production in A549 cells but not [M1] production (data not shown), confirming that CYP3A5 did not contribute to the activation of BDP in lung cells. Exploring the induction of CYP3A enzymes in response to BDP showed that only CYP3A5 was induced in A549 cells when esterases were active (Fig. 7B). These data show that the production of [M1], likely acting through the glucocorticoid receptor, was responsible for the induction of CYP3A5. In DPX2 cells, however, CYP3A4, 3A5, and 3A7 mRNA expression was significantly induced by BDP, but only when esterase activity was inhibited (Fig. 7D). These results suggest that rapid esterase-mediated clearance of BDP in the absence of esterase inhibitors limits CYP3A4 mRNA induction in liver cells and that the induction of CYP3A enzyme mRNA was primarily mediated by PXR activation by BDP, not [M1], as documented in the literature (Dvorak and Pavek, 2010). These metabolic profiles and induction results suggest two different pathways regulate CYP3A gene expression in the lung and the liver.

The induction of CYP3A enzymes in response to glucocorticoid treatment has been extensively studied in the liver. At micromolar concentrations, induction of CYP3A enzymes occurs through PXR (which is not expressed in lung cells) (Dvorak and Pavek, 2010). At submicromolar concentrations, glucocorticoid receptor ligands promote glucocorticoid receptor homodimer assembly and translocation into the nucleus where the transcription of the constitutive androstane receptor is induced. The constitutive androstane receptor nuclear receptor then binds to the retinoid X receptor α, forming a constitutive androstane receptor:retinoid X receptor α heterodimer, which ultimately promotes the transcription of CYP3A genes (Dvorak and Pavek, 2010). This pathway, however, has not been fully evaluated in lung cells, and the results presented in Fig. 7 indicating a role for the glucocorticoid receptor in regulating CYP3A5 expression by BDP, and presumably other glucocorticoids, suggest that more studies are needed to determine not only how CYP3A5 is induced in the lung by glucocorticoids, but also to assess the pharmacological significance of increased CYP3A gene/enzyme expression in the lung, relative to the efficiency of glucocorticoids.

In summary, this study expands our knowledge of cytochrome P450-mediated metabolism of the glucocorticoid BDP, including the preliminary identification of several new cytochrome P450-specific metabolites: [M4], [M5], and [M6]. Although these studies do not link variations in CYP3A gene expression and/or function in lung cells, or elsewhere in the body, to variations in the disposition and efficacy of BDP in humans, these data support such a hypothesis. Further research into the ideas generated by this study may ultimately help advance our understanding of glucocorticoid resistance among asthmatics and improve selection of the most effective inhaled glucocorticoid for specific patients.

Acknowledgments

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Authorship Contributions

Participated in research design: Roberts, Moore, Reilly, Ward, Yost.

Conducted experiments: Roberts, Moore.

Performed data analysis: Roberts, Moore.

Wrote or contributed to the writing of the manuscript: Roberts, Moore, Reilly, Ward, Yost.

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CHAPTER 4

REGULATION OF CYP3A GENES BY GLUCOCORTICOIDS IN LUNG CELLS

Abstract

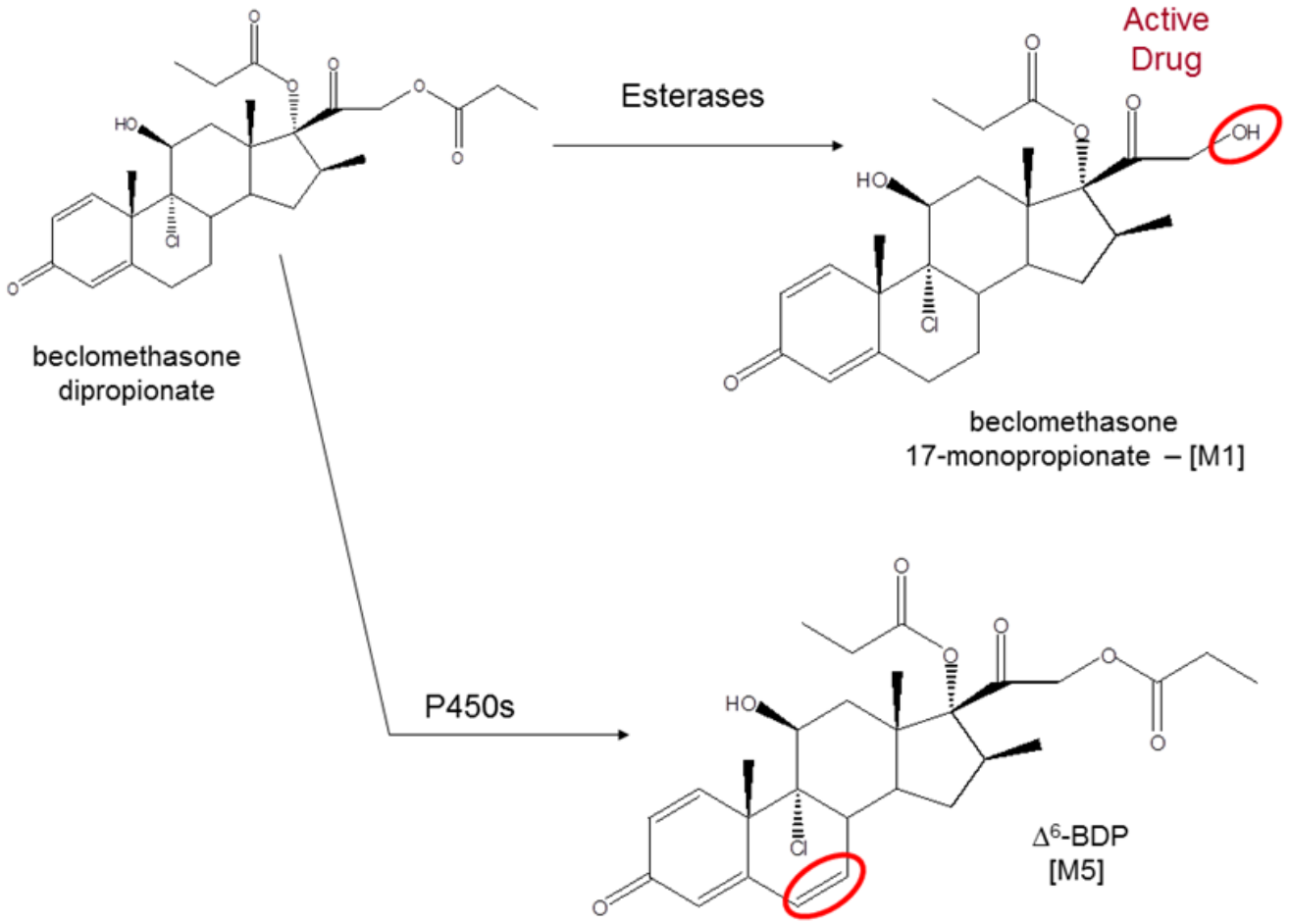
Inhaled glucocorticoids are the first line treatment for patients with persistent asthma. However, approximately 30% of patients do not respond to treatment due to glucocorticoid insensitivity, which may involve excess metabolic clearance of the glucocorticoids by CYP3A enzymes in the lung. CYP3A4, 3A5, and 3A7 enzymes metabolize glucocorticoids and glucocorticoids induce CYP3A5 in A549 cells. However, the mechanism by which CYP3A5 expression is regulated in A549 cells in response to glucocorticoids has not determined. In hepatocytes glucocorticoids bind to the glucocorticoid receptor, which induces the expression of the constitutive androstane receptor, which binds the retinoid X receptor α, leading to the induction of CYP3A4, 3A5, and 3A7. This pathway was evaluated as the mechanism for CYP3A5 mRNA induction by glucocorticoids in A549, BEAS-2B, NHBE, SAEC, lobar, and tracheal epithelial cells. In A549 cells, beclomethasone 17-monopropionate ([M1]) induced CYP3A5 mRNA through the glucocorticoid receptor. CYP3A5 mRNA induction by five different glucocorticoids was attenuated by inhibiting the glucocorticoid receptor using ketoconazole, and for beclomethasone dipropionate, using siRNA –mediated inhibition of

glucocorticoid receptor expression. The constitutive androstane receptor was not expressed in lung cells. SAEC cells, a primary lung cell line, expressed CYP3A5, but CYP3A5 mRNA was not induced by glucocorticoid treatment despite evaluating a multitude of cell culture conditions. None of the other lung cells expressed CYP3A4, 3A5 or 3A7 mRNA. These studies demonstrate that CYP3A5 message is induced in response to glucocorticoid treatment in A549 cells via the glucocorticoid receptor, but an additional undefined regulatory process may exist in primary lung cells.

Introduction

Inhaled glucocorticoids are the first line treatment for asthma (*1-3*). Glucocorticoids bind to the glucocorticoid receptor to reduce the expression of genes that produce a variety of pro-inflammatory mediators and mucus in the lung (*4-6*). The most commonly prescribed glucocorticoids are beclomethasone dipropionate (BDP), triamcinolone acetonide (TCL), budesonide (BUD), fluticasone propionate (FLT), and flunisolide (FLN) (*1*). BDP is a prodrug and requires removal of the C-21 propionate group to become pharmacologically active; the active drug is beclomethasone 17-monopropionate, referred to as [M1] (Scheme 4.1) (*7*). Pharmacological inactivation and clearance of glucocorticoids, such as BDP and its active metabolite [M1], is mediated, in part, by cytochrome P450 (CYP) enzymes (Scheme 4.1), mainly the CYP3A enzymes., CYP3A4, 3A5, and 3A7.

In humans, CYP3A4, 3A5, and 3A7 are involved in glucocorticoid metabolism (*8-11*). CYP3A4 is the most abundant CYP3A enzyme in the liver and intestines (*8, 12, 13*), CYP3A5 is preferentially expressed in the lung (*12, 14-16*), and CYP3A7 is expressed in



Scheme 4.1. Scheme for the production of [M1] (the active form of BDP) by esterase enzymes and [M5] by CYP3A enzymes.

fetal liver, but diminishes after birth when CYP3A4 becomes the dominant adult hepatic CYP3A enzyme (*17, 18*). Expression of CYP3A7 in fetal and adult respiratory tissue has also been reported (*16*).

Regulation of CYP3A enzymes in response to glucocorticoid treatment has been extensively characterized in the liver, but little is known about this phenomenon in the lung. In hepatocytes, CYP3A enzyme induction is mediated by the pregnane X receptor (PXR) (*19, 20*). However, PXR is not expressed in the lung (*21*). The glucocorticoid receptor (GR) and the constitutive androstane receptor (CAR) also regulate CYP3A induction by glucocorticoids in the liver (*22, 23*). Briefly, glucocorticoids bind GR in the cytosol, which forms a homodimer and translocates to the nucleus, leading to increased transcription of CAR. CAR forms a heterodimer with the retinoid X receptor alpha (RXRα), which binds to the CAR/RXR- response element and induces the expression of CYP3A enzymes (Figure 4.1). This pathway, however, has not been demonstrated in the lung.

The purpose of this study was three fold: 1) to evaluate changes in the expression of CYP3A mRNA in lung cells treated with glucocorticoids; 2) to determine if the GR/CAR/RXRα pathway was responsible for glucocorticoid-induced changes in CYP3A mRNA expression; and 3) to determine the role of metabolism in this phenomenon. The cell lines used in this study were BEAS-2B (immortalized bronchial epithelial cell line), NHBE (normal human bronchial/tracheal epithelial cells), lobar epithelial cells (secondary bronchus epithelial cells), primary cells recovered from tracheal washes of pediatric patients on mechanical ventilators, SAEC (small airway epithelial cells), and A549 (human lung adenocarcinoma) cells. It was hypothesized that CYP3A5 induction in A549

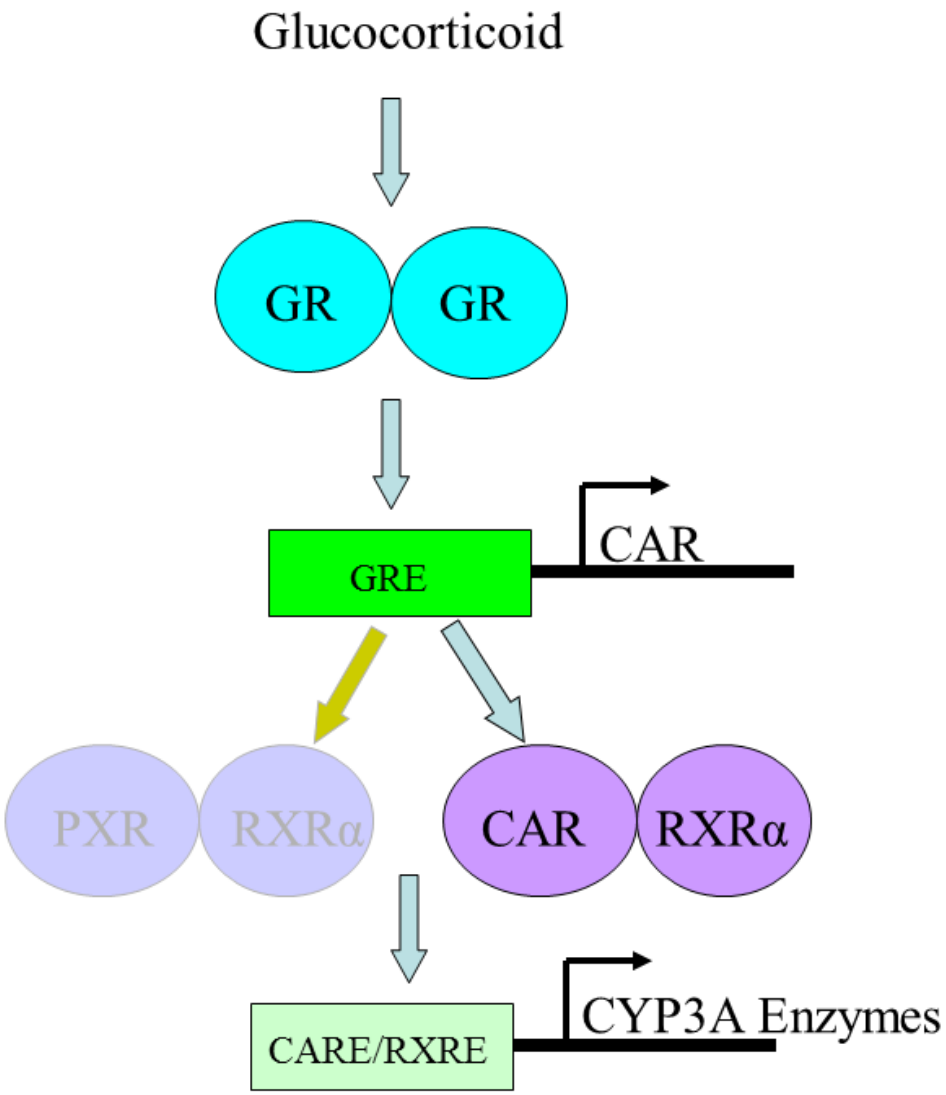


Figure 4.1. Proposed mechanism for the induction of CYP3A genes in lung cells. Active glucocorticoid will bind to the glucocorticoid receptor (GR), which forms a homodimer and translocates to the nucleus. The homodimer binds to the GR response element (GRE) and induces the expression of the constitutive androstane receptor (CAR). CAR forms a heterodimer with the retinoic X receptor (RXRα), which in turn induces the expression of the CYP3A enzymes via binding to the CAR, RXR response element (CARE/RXRE).

cells by BDP (*11*) and other glucocorticoids would occur via a mechanism involving GR/CAR/RXRα, as previously documented in hepatocytes.

Materials and Methods

Chemicals, Reagents, and Treatments

Beclomethasone dipropionate (BDP), triamcinolone acetonide (TCL), fluticasone propionate (FLT), flunisolide (FLN), budesonide (BUD), prednisolone, ammonium acetate, eserine, and methanol were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Paraoxon was purchased from Chem Service (West Chester, PA).

Cell Culture

A549 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 5% fetal bovine serum (Life Technologies, Grand Island, NY). SAEC cells (LONZA, Walkersville, MD; patient numbers 11662, 14453, 14457) were cultured in small airway epithelial growth medium, supplemented with the SAGM bullet kit. Cells were cultured with and without hydrocortisone by adding or not adding the hydrocortisone component from the SAGM bullet kit. NHBE cells (LONZA; patient numbers 15268, 5S03795) were grown in bronchial epithelial cell growth medium (BEGM Bullet kit) (LONZA). BEAS-2B cells (American Type Culture Collection) were cultured in LHC-9 medium (Life Technologies). Lobar cells (patient number 01334) were cultured in BronchiaLife Basal Medium supplemented with the BronchiaLife B/T supplement kit (Lifeline Cell Technology, Walkersville, MD). All cells except A549 cells were plated in 12-well plates

pre-coated with LHC basal medium (Life Technologies). Tracheal epithelial cells were recovered from tracheal washes from mechanically ventilated pediatric patients in the neonatal intensive care unit and pediatric intensive care unit at Primary Children's Medical Center at the University of Utah, with IRB approval (00026839). Briefly, cells were separated from sputum by centrifugation at 900 xg for 30 min in 14 mL of DMEM/F12 media. Cells were plated in a 12-well plate pre-coated with 2% gelatin (Life Technologies) and cultured in DMEM/F12 media + 10% fetal bovine serum (FBS) (Life Technologies). All cells were cultured in an atmosphere of 5% CO_2:95% air at 37ºC.

Cell Treatments

Cell treatments were prepared in treatment media with a final concentration of DMSO less than 1%. Cells were treated at ~70% confluence (*11*). A549 cells were treated in OPTIMEM (Life Technologies) and SAEC cells were treated in growth media with and without hydrocortisone and with and without heat inactivated and/or charcoal-stripped FBS. All other cell lines were treated in their respective growth medium, also heat inactivated to eliminate esterase activity from the FBS which would metabolize BDP before it could diffuse into the cells. Cytotoxicity assays were performed using the Dojindo Cell counting kit-8 (Dojindo Laboratories, Rockville, MD) to determine glucocorticoid, esterase inhibitor, and ketoconazole concentrations exhibiting <20% cytotoxicity in A549 cells. All other cell lines were treated with the same concentrations as determined with A549 cells. Glucocorticoid treatments were as follows: BDP (10 μM), TCL (1 μM), BUD (10 μM), FLT (1 μM), and FLN (100 nM). Pre-treatments in various experiments included ketoconazole (50 μM, 10 μM, and 1 μM, to inhibit GR), esterase

inhibitors (1:1 mixture of eserine and paraoxon, each at 175 μM, to inhibit [M1] formation), and 1-aminobenzotriazole (1-ABT; 200 μM, to inhibit P450-mediated metabolism) for 2 h prior to a 22 h glucocorticoid cotreatment. Controls were treated with an equivalent concentration of DMSO. All A549 cell treatments were carried out in 6-well plates for 24 h (n=6). All other cell lines were cultured in precoated 12-well plates and treated for 24 h (n=3).

Analysis of BDP Metabolites

After treatment, BDP and BDP metabolites were extracted from the collected media by adding 2x volume (6 mL for A549, 4 mL for all other cell lines) methyl *tert*-butyl ether containing 1 nM prednisolone (internal standard for quantification) and shaking for 25 min. Samples were clarified by centrifugation, the organic fraction was collected, dried under air, reconstituted in 100 μL 1:1 H_2O:MeOH, clarified again by centrifugation, and transferred to autosampler vials for analysis by liquid chromatography-mass spectrometry (LC/MS/MS). LC/MS/MS was conducted on a Thermo LCQ Advantage Max ion trap instrument equipped with a Finnigan Surveyor LC pump, Surveyor Autosampler and universal Ion Max source operated with Thermo Xcalibur software version 2.0 (Thermo Fisher Scientific, Waltham, MA) as previously described (*11*).

Quantitative Reverse Transcription-PCR

Total RNA was isolated from cells using TRIzol reagent (Life Technologies). cDNA was synthesized using iScript Reverse Transcription Supermix for qPCR (BIO

RAD, Hercules, CA). qPCR was performed using either LightCycler 480 Probes Master mix (CYP3A5) or LightCycler 480 SYBR Green I Master Mix (all other genes) (Roche, Indianapolis, IN) with a Light-Cycler 480 System. The PCR program for probe mix consisted of a 5 min incubation at 95ºC, followed by 45 cycles of 95ºC for 10s, 55ºC for 30s, then 72ºC for 1s. The PCR program for SYBR Green I mix consisted of a 5 min incubation at 95ºC, followed by 40 cycles of 95ºC for 10s, 63ºC for 5s for CYP3A4, CYP3A7 and β2-microglobulin. For GR and CAR, annealing was performed at 65ºC for 5s and extension at 72ºC for 10s. mRNA copy number was determined from standard curves for each gene and was normalized using β2-microglobulin. Primer sequences for the various genes are listed in Table 4.1 (*24*).

siRNA-Mediated Protein Knockdown

Pre-annealed, short interfering "Smart Pool" siRNAs specific to human GR were purchased from Dharmacon (Waltham, MA). siRNA directed against GFP (*25*) was used as a negative control with the following sequences: 5′-CUGGAGUUGUCCCAAUUCCT T-3′ and 5′-AGAAUUGGGACAACUCCAGTT-3′ (the 2-nucleotide overhanging of 2′-deoxythymidine is indicated as TT and denoted by underlines). Control siRNA was synthesized at the University of Utah oligonucleotide synthesis core and annealed by combining 40 μM of each strand and incubating in annealing buffer (100 mM potassium acetate, 30 mM HEPES KOH, 2 mM magnesium acetate adjusted to pH 7.4) for 1 min at 90ºC followed by 1 h at 37ºC, in a final volume of 0.5 mL. A549 cells were plated into 6-well plates containing 20 nM siRNA per well, previously complexed with Lipofectamine 2000 using a ratio of 3:2 lipid to siRNA in 100 μL of OPTIMEM (Life Technologies).

Table 4.1. Primer sequences for qPCR assays.

CYP3A5	F- 5' CCTATCGTCAGGGTCTCTGGAA 3' R- 5' TGATGGCCAGCACAGGGA 3' Probe [6FAM]ATGTGGGGAACGTATGAA[BHQ1]
CYP3A4	F- 5' GAAAGTCGCCTCGAAGATAC 3' R- 5' ACGAGCTCCAGATCGGACAG 3'
CYP3A7	F- 5' TTCCGTAAGGGCTATTGGAC 3' R- 5' TCTGTGATAGCCAGCATAGG 3'
GR	F- 5' CCAACGGTGGCAATGTGAAA 3' R- 5' CCGCCAGAGGAGAAAGCAAA 3'
CAR	F- 5' CCGTGTGGGGTTCCAGGTAG 3' R- 5' CAGCCAGCAGGCCTACGAAC 3'
B2M	F- 5' GATGAGTATGCCTGCCGTGTG 3' R- 5' CAATCCAAATGCGGCATCT 3'

The cells were grown for 48, 72, and 96 h to determine the time at which maximum decreases in GR mRNA occurred (72 h). In subsequent experiments, cells were treated with DMSO, 10 μM BDP, or 10 μM BDP + 175 μM esterase inhibitors (1:1 eserine:paraoxon) for 24 h to determine the effects of attenuated GR expression on the induction of CYP3A5 in A549 cells.

Results

Inhibition of [M1] Formation Prevented CYP3A5 mRNA Induction by BDP in A549 Cells

Media from A549 cells treated with BDP (10 μM) for 24 h was extracted and analyzed for metabolites of BDP produced by CYP3A enzymes. The only CYP3A-mediated metabolite detected was [M5] (Scheme 4.1 and Figure 4.2A) (*11*). For the remainder of the studies, [M1], the active metabolite, was used as a marker for esterase activity and [M5] was used as a marker for CYP3A5 activity. BDP treatment significantly induced the expression of CYP3A5 mRNA (~2-fold) compared to the DMSO control (Figure 4.2B). CYP3A4 and CYP3A7 mRNA was not detected in A549 cells, as previously documented (*11*, *16*). Inhibiting the production of [M1] using esterase inhibitors also blocked the induction of CYP3A5 mRNA (Figures 4.2A and 4.2B); esterase inhibitor (EI) treatment alone had no effect on CYP3A5 expression. 1-ABT, a mechanism-based inactivator of P450 enzymes, also inhibited esterase activity (i.e., [M1] formation) (Figure 4.2A), and as a result, prevented the induction of CYP3A5 mRNA (Figure 4.2B).

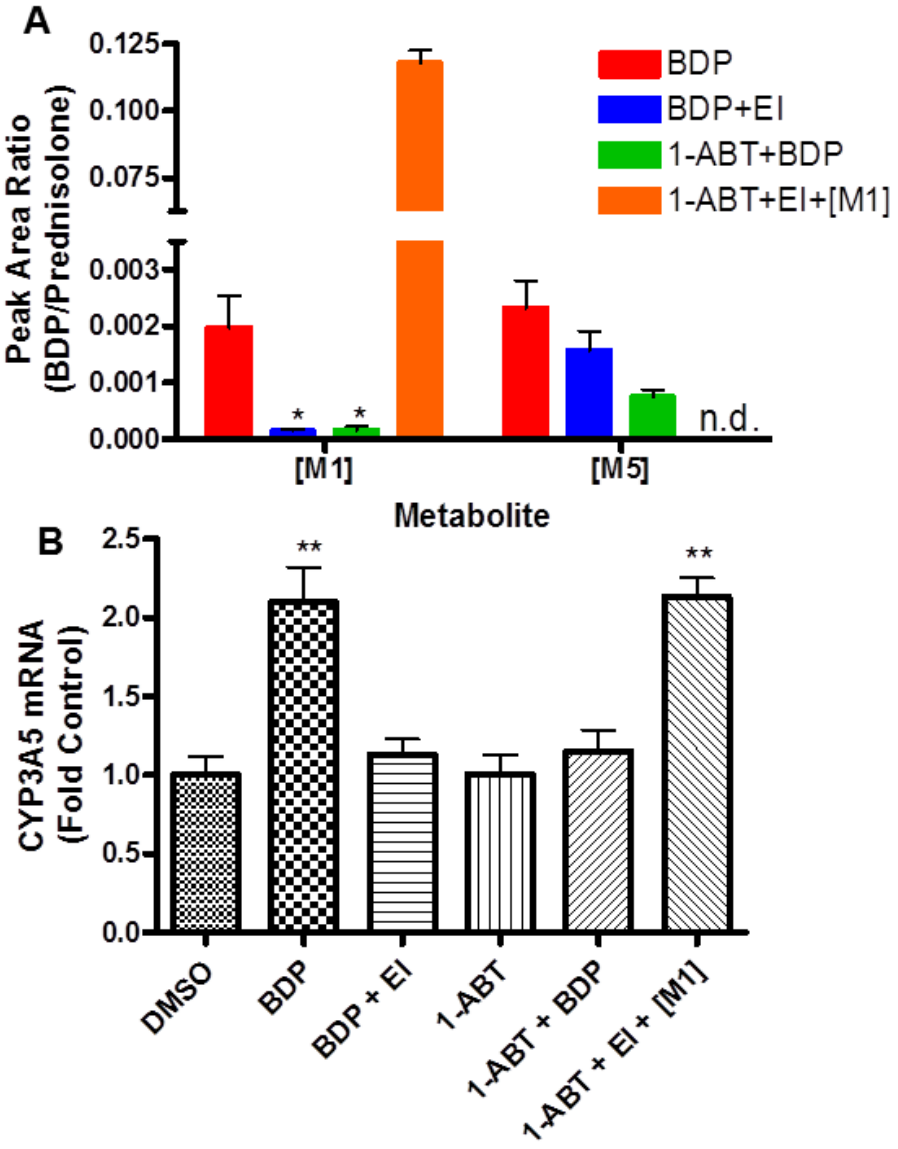


Figure 4.2. Metabolite formation and CYP3A5 mRNA induction in A549 cells. (A) Relative quantities of [M1] and [M5] measured by LC/MS/MS in A549 cell culture media following BDP treatment alone, BDP + esterase inhibitors (EI), 1-ABT + BDP, and 1-ABT + EI + [M1]. **(B)** CYP3A5 mRNA detected in A549 cells following DMSO control, BDP treatment alone, BDP + (EI), 1-ABT alone, 1-ABT + BDP, and 1-ABT + EI +[M1]. Values are expressed as fold over DMSO controls. Statistics used for data analysis were one-way ANOVA with Dunnett's post-hoc test. Data are the mean and standard deviation from n=6 replicates. * $p<0.05$, ** $p<0.01$.

[M1] was Sufficient to Induce CYP3A5 mRNA in A549 Cells

Cells were treated with [M1] in the absence and presence of 1-ABT and esterase inhibitors. [M1] treatment was sufficient to induce CYP3A5 mRNA (~2-fold), independent of esterases (Figure 4.2B), indicating that CYP3A5 mRNA induction in A549 cells was mediated by [M1], presumably involving GR.

GR, but not CAR, Regulated the Induction of CYP3A5 mRNA in A549 Cells

GR and CAR mRNA were quantified in A549 cells. A significant increase in GR message (~2.4-fold) was observed following 24 h treatment with BDP (Table 4.2). CAR mRNA was not detected in A549 cells prior to or following glucocorticoid treatment (Table 4.2), consistent with previous studies (*15*), suggesting that GR, not CAR, was responsible for the induction of CYP3A5 message in A549 cells.

Inhibition of GR with Ketoconazole Attenuated CYP3A5 mRNA Induction by Glucocorticoids in A549 Cells

Ketoconazole is a competitive antagonist of GR (*24*). Ketoconazole alone had no significant effect on CYP3A5 mRNA expression (Figure 4.3A). As the concentration of ketoconazole was decreased, dose-dependent increases in the expression of CYP3A5 mRNA were observed for BDP, TCL, FLT, BUD, and FLN (Figure 4.3A-E): BDP caused a ~2-fold induction, BUD caused a ~4-fold induction, TCL caused a ~5.5-fold induction, FLT caused a ~3.5-fold induction, and FLN caused a ~5.5-fold induction, relative to their respective controls. These data support the conclusion that the induction of CYP3A5

Table 4.2. Comparison of GR, CAR, and CYP3A5 mRNA expression in lung cell cultures.

Cell Type	GR Expression	GR Induction by GC Treatment	CAR Expression	CAR Induction by GC Treatment	CYP3A5 Message	CYP3A5 Induction by GC treatment
BEAS-2B	+	N.D.	N.D.	N.D.	N.D.	N.D.
NHBE	+	N.D.	N.D.	N.D.	N.D.	N.D.
Patient Tracheal Washes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lobar	+	N.D.	N.D.	N.D.	N.D.	N.D.
A549	+	2.4 $\pm$ 0.35 **	N.D.	N.D.	+	2.1 $\pm$ 0.55 **
SAEC	+	N.D.	N.D.	N.D.	1 out of 3 patients	N.D.

Data are represented as fold over DMSO control. Statistics used for data analysis were one-way ANOVA with Dunnett's post-hoc test. ** $p < 0.01$, N.D. = not detected, GC = glucocorticoid

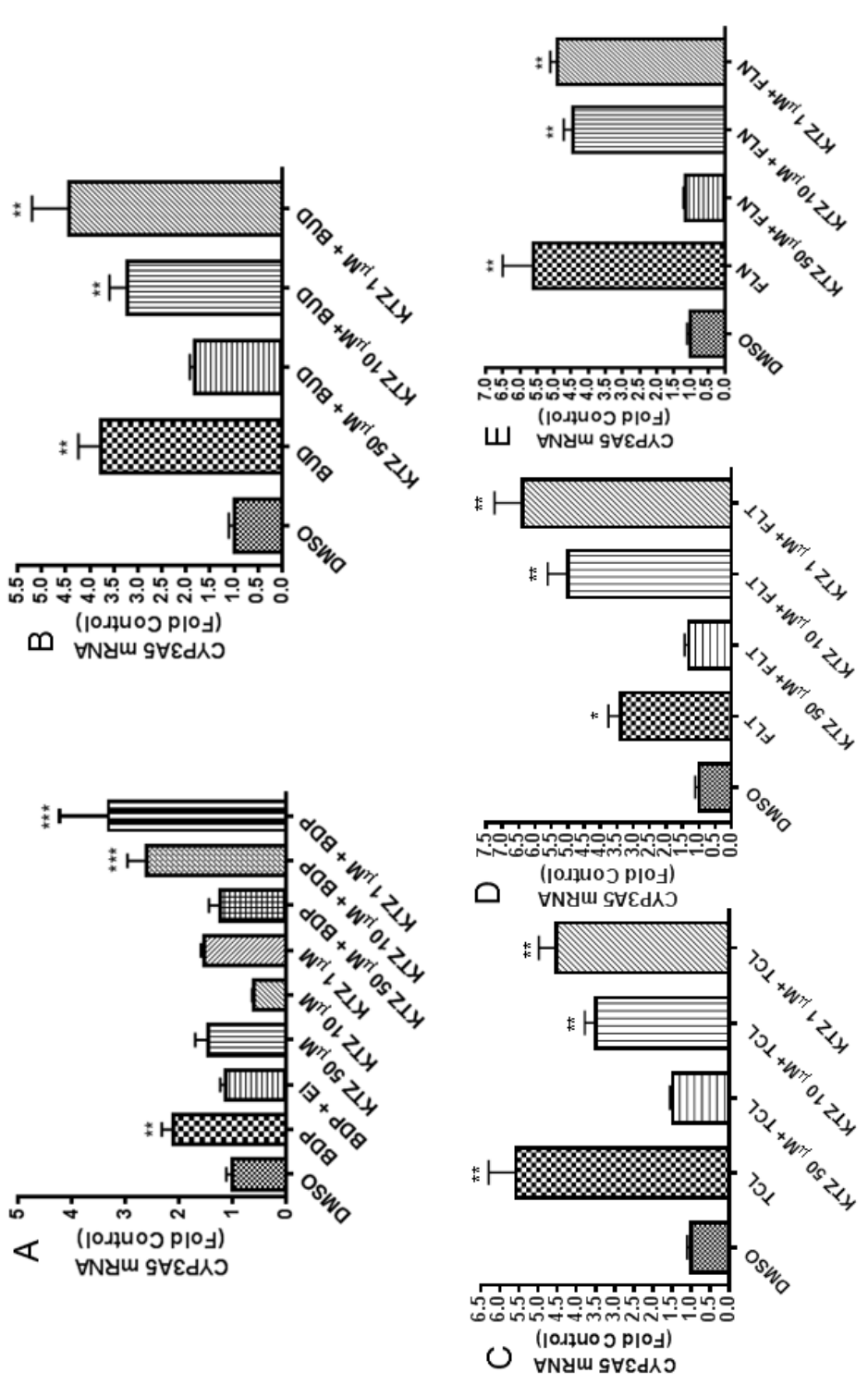


Figure 4.3. CYP3A5 message detected in A549 cells treated with (A) BDP, (B) BUD, (C) TCL, (D) FLT, and (E) FLN, with and without ketoconazole (KTZ), a competitive antagonist for the glucocorticoid receptor. Results are presented as fold over DMSO controls. Statistics used for data analysis were one-way ANOVA with Dunnett's post-hoc test. Data are the mean and standard deviation from n=6 replicates. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

mRNA in A549 cells was mediated by GR.

siRNA-mediated Knockdown of GR also Attenuated CYP3A5 mRNA Induction by BDP in A549 Cells

Cells were transfected with siRNA and grown for 48, 72, and 96 h to determine the time of maximum GR mRNA suppression (Figure 4.4A). Maximum suppression occurred as early as 48 h, but the 72 h time point was chosen for further experiments to ensure efficient GR protein depletion. An approximate 2-fold induction of CYP3A5 mRNA was observed in A549 cells following treatment with BDP in control cells transfected with "nonsense" siRNA directed against GFP. Consistent with previous results (Figures 4.2A and 4.2B), CYP3A5 mRNA induction was prevented by esterase inhibitors (Figure 4.4B). Cells transfected with siRNA targeted for GR mRNA showed no change in CYP3A5 mRNA with BDP treatment, further confirming the role of GR in directly regulating the induction of CYP3A5 mRNA in A549 cells treated with BDP and presumably the other glucocorticoids used in Figure 4.3.

CYP3A5 was not Expressed or Induced by Glucocorticoid Treatment in Tracheal/Bronchial Epithelial Cells

Neither CYP3A5 mRNA expression nor induction of CYP3A5 mRNA following BDP or other glucocorticoid treatment was observed in NHBE, BEAS-2B, lobar, and freshly isolated tracheal wash samples (Table 4.2).

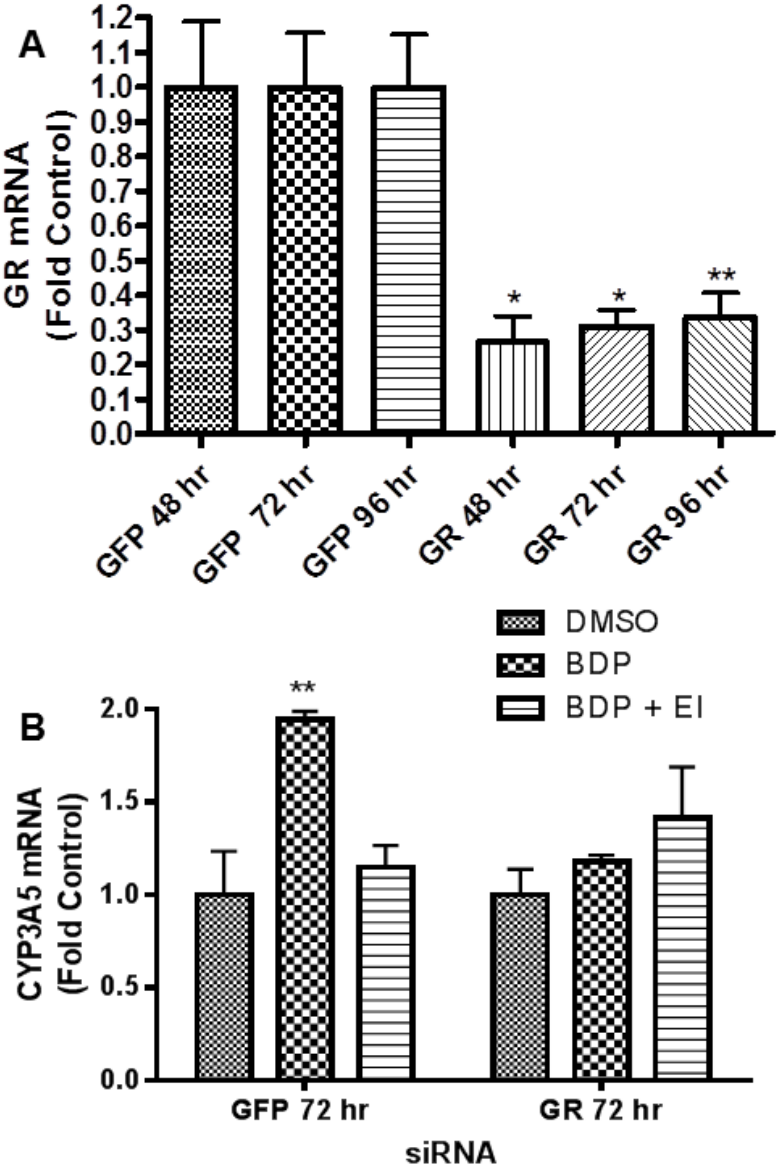


Figure 4.4. GR mRNA knockdown in A549 cells. (A) siRNA-mediated GR knockdown in A549 cells at 48, 72, 96 h compared to "nonsense" GFP siRNA (negative control), expressed as fold over designated GFP control for each time point. **(B)** Cells were exposed to siRNA for 72 h then treated with DMSO, BDP, or BDP + EI. Statistics used for data analysis were one-way ANOVA with Dunnett's post-hoc test. Data are the mean and standard deviation from 3 replicates. * $p < 0.05$, ** $p < 0.01$.

SAEC Cells Expressed CYP3A5, but mRNA for CYP3A5 was not Induced by Glucocorticoid Treatment

SAEC cells from three separate patients were evaluated for CYP3A5 mRNA expression and induction in response to glucocorticoid treatment. Initial experiments demonstrated that mRNA for CYP3A, but not CYP3A4 or 3A7, was expressed in one of the three SAEC samples (patient #11662), but that expression levels were not altered by glucocorticoid treatment. It was hypothesized that the high concentration of hydrocortisone (500 µM) in the SAEC growth media prevented the induction of CYP3A5 mRNA by substantially lower concentrations of the glucocorticoids used in the treatments. Elimination of hydrocortisone from the media decreased the basal expression of CYP3A5 mRNA (Figure 4.5). However, no change in mRNA abundance was observed over a 24 h treatment period with BDP. Furthermore, neither increasing the treatment concentration of BDP to 50 µM, nor treatment with [M1] at 150 µM led to an increase in CYP3A5 mRNA in SAEC cells. It was subsequently hypothesized that phthalates or other substances in the FBS might alter GR function and CYP3A5 mRNA induction by glucocorticoids (*26*). However, neither heat inactivation nor charcoal-stripping of the FBS in media with and without hydrocortisone led to CYP3A5 mRNA induction. The various manipulations to SAEC culture conditions and results for CYP3A5 induction are summarized in Table 4.3.

Discussion

Inhaled glucocorticoids are used to control undesirable symptoms in asthmatic patients. However, about 30% of the population does not benefit from this first-line

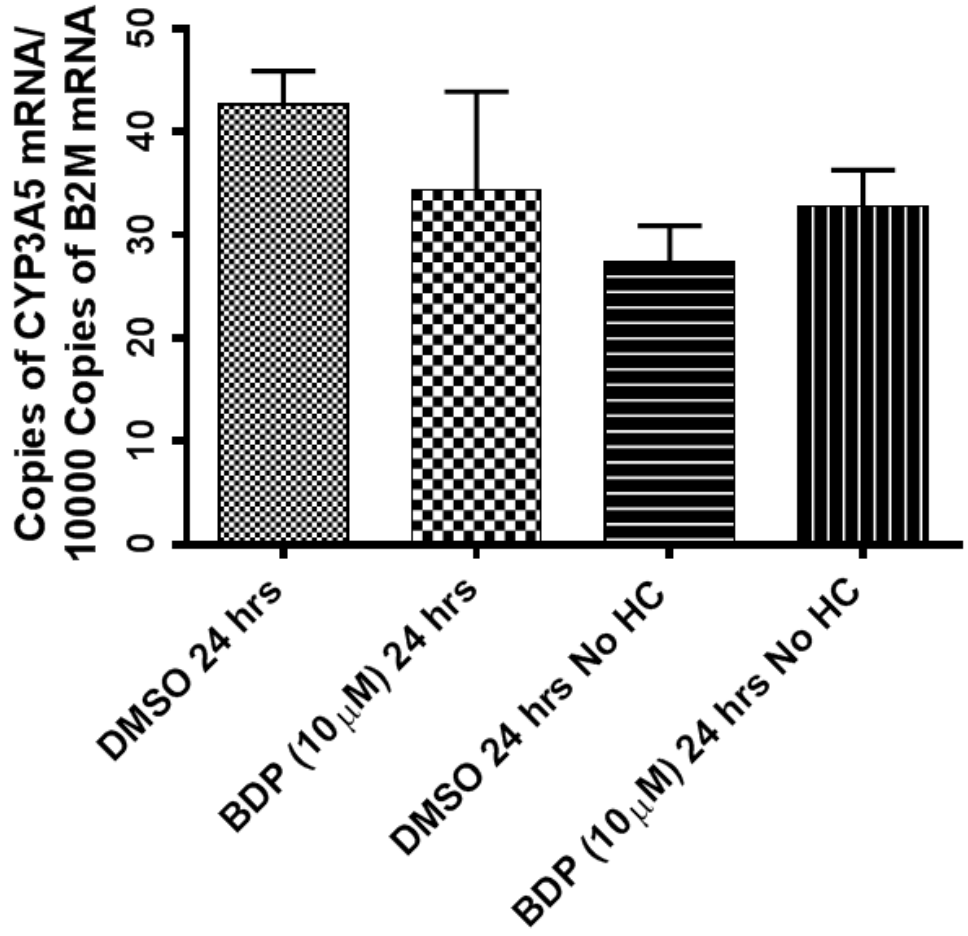


Figure 4.5. SAEC cells treated with BDP (10 μM; 24 h) or DMSO matching controls with and without hydrocortisone (HC). There was no significant difference between treatments or matching controls using one-way ANOVA with $p<0.05$ (n=3).

Table 4.3. Modifications made to SAEC culture media.

Basal Culture Conditions	Experimental Modifications	Result
Cultured in growth media	-Heat inactivated media for treatment -10 μM BDP for 24 h	-Basal CYP3A5 mRNA expression observed in 1 out of 3 patients -No change in CYP3A5 mRNA
Cultured in growth media without hydrocortisone	-Heat inactivated media for treatment -10 μM BDP for 24 h	-Lowered basal level of CYP3A5 mRNA -No change in CYP3A5 mRNA
Cultured in growth media without hydrocortisone	-Heat inactivated media for treatment -50 μM BDP or 105 μM [M1] for 24 h	-No change in CYP3A5 mRNA
Cultured in growth media without hydrocortisone	-Treated in heat inactivated and charcoal stripped FBS -50 μM BDP or 105 μM [M1] for 24 h	-No change in CYP3A5 mRNA

treatment (*6*). Prior work demonstrated that the five most commonly prescribed glucocorticoids used in the treatment of asthma are metabolized by CYP3A enzymes, specifically CYP3A4, CYP3A5, and CYP3A7 (*10, 11*). Therefore, it has been proposed that unusually high rates of metabolism of glucocorticoids in lung cells by these enzymes might contribute to the decrease and/or lack of efficacy in some individuals. However, it is not understood how the expression of CYP3A enzymes is regulated in the lung in response to glucocorticoid treatment, despite extensive knowledge of this phenomenon in hepatocytes and the liver (*22*).

Using A549 cells, it was demonstrated that CYP3A5 mRNA was induced by glucocorticoid treatment (Figure 4.2B and 4.3A-E); neither CYP3A4 nor CYP3A7 mRNA were detected in A549 cells. Subsequent studies using a competitive antagonist of GR (ketoconazole) and siRNA selective for GR mRNA, demonstrated that inhibition of GR function prevented the induction of CYP3A5 mRNA by BDP and other glucocorticoids in A549 cells (Figure 4.3A-E and 4.4B). It was also demonstrated that CAR mRNA was not expressed by lung cells, consistent with previous RT-PCR data (*15*), and therefore could not be involved in the regulation of CYP3A5 expression by glucocorticoids as occurs in hepatocytes. It was concluded that CYP3A5 expression was directly regulated by GR. Schuetz *et al.* (*27*) previously described two "half sites" of GR (TGTTCT) separated by 160 bp in the promoter region of CYP3A5 in HepG2 cells and in human and rat hepatocytes. It was demonstrated that dexamethasone induced the expression of CYP3A5 by the GR homodimer binding to these two joined "half-sites" which could be blocked by RU-486, a GR antagonist. It is plausible these same sites are involved in the regulation of CYP3A5 in lung cells by BDP and other glucocorticoids.

Regardless of the exact mechanism of regulation, the current results illustrate that glucocorticoids have the capacity to induce the expression of CYP3A5 in A549 cells. These data, in conjunction with prior metabolism studies of glucocorticoids by this laboratory, support the hypothesis that treating patients with glucocorticoids could increase levels of CYP3A5 in the lung, and therefore increase pulmonary glucocorticoid metabolism, ultimately increasing clearance, and potentially decreasing the concentration of active drug in lung cells. Though most of the population expresses the inactive form of CYP3A5 (*CYP3A5*3*) (*13*, *28*) those expressing *CYP3A5*1*, the active form of CYP3A5 (*13*), could exhibit increased clearance of the drug, and therefore could account for at least some of the 30% of patients who do not respond to inhaled glucocorticoid therapy.

In order to further support the hypothetical scenario above, the induction of CYP3A enzymes by glucocorticoids in various lung cells was studied. CYP3A5 mRNA expression was quantified in primary lung cells, which presumably more closely model epithelial cells of the human respiratory tract and lung. NHBE, lobar, and cells recovered from tracheal washes of mechanically ventilated children were evaluated for CYP3A enzyme expression and induction by glucocorticoids. Results in Table 4.2 show that CYP3A mRNA was not expressed in cells of the conducting airways in response to glucocorticoid treatment, indicating that these epithelial cells likely do not play a role in CYP3A-dependent metabolism of glucocorticoids in the lung. In contrast, SAEC cells, representing cells of the distal bronchioles, alveolar ducts, and alveoli, did express CYP3A5 (Table 4.2). However, there was no change in CYP3A5 message when these cells were treated with glucocorticoids. A thorough examination of potential confounding issues associated with cell culture revealed a high concentration of hydrocortisone (500

μM) in the growth media. Because cells were treated with only 10 μM BDP, it would stand to reason that no change in CYP3A5 mRNA would occur because CYP3A5 expression would already be maximized as a result of hydrocortisone activating the GR pathway. Experiments conducted in A549 cells showed that culturing cells in 500 μM hydrocortisone increased the basal expression of CYP3A5 mRNA by 2-fold, masking the induction routinely observed using 10 μM BDP for 24 h. When A549 cells were subsequently cultured in media without hydrocortisone for a 48 h, providing sufficient time for a "wash out" of the hydrocortisone, the basal expression of CYP3A5 mRNA was reduced, and ~2-fold induction of CYP3A5 mRNA occurred with the 10 μM BDP, 24 h treatment. Therefore, hydrocortisone was omitted from the SAEC growth media. Subsequent experiments in SAEC cells showed no change in CYP3A5 mRNA in response to glucocorticoid treatment (Figure 4.5), albeit removal of hydrocortisone from the media caused a slight decrease in the basal level of CYP3A5 mRNA expression, suggesting GR plays a role in the regulation of CYP3A5. It is feasible that because cells had been exposed to such high concentrations of hydrocortisone during their isolation and expansion, that 10 μM of BDP was not sufficient to induce CYP3A5 mRNA, even after culturing the cells in the absence of hydrocortisone for multiple division cycles. Therefore, the concentration of BDP was increased to 50 μM and an additional treatment group using 150 μM [M1] was added. Again no increases in CYP3A5 mRNA were observed. Heat-inactivated and charcoal-stripped FBS were also utilized to remove potential interfering compounds from FBS, and still no change was observed. To our knowledge, no one has observed a change in CYP3A mRNA expression in any primary human lung cell cultures. However, Cyp3a11, 3a13, and 3a16 mRNA and protein

induction have been documented in mouse lung following dexamethasone treatment (*29*). As such, additional studies using animal models and relevant samples from human patients need to be evaluated in order to conclusively confirm or reject the hypothesis that CYP3A genes are regulated in response to glucocorticoid treatment in human lungs, since current *in vitro* models are unexplainably limited in value for such studies.

In summary, the data presented herein demonstrate that, in A549 cells, glucocorticoid binding to the glucocorticoid receptor regulates the expression of CYP3A5, and therefore, corroborates the hypothesis that increased metabolism of glucocorticoids may occur in some patients. However, further research is needed to determine if changes in CYP3A5 expression occur in the human respiratory tract similar to A549 cells, the precise mechanism by which this process occurs, and whether changes in the local metabolism of glucocorticoids by CYP3A5 ultimately impact glucocorticoid efficiency.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The studies described in this dissertation focused on two research objectives: first to characterize the metabolism of commonly prescribed inhaled glucocorticoids by CYP3A4, CYP3A5, and CYP3A7 enzymes; and second, to evaluate how CYP3A enzyme expression may be altered in lung cells by glucocorticoid treatment. Using recombinant *in vitro* systems, CYP3A4 and CYP3A5 were shown to be the principle enzymes involved in the metabolic decomposition of beclomethasone dipropionate (BDP), triamcinolone acetonide (TCL), fluticasone propionate (FLT), and budesonide (BUD). CYP3A7 did not metabolize BDP or flunisolide (FLN), but did metabolize TCL, FLT, and BUD, albeit typically at lower rates than those observed for CYP3A4 and 3A5. CYP3A4 was the only enzyme that metabolized FLN. All CYP3A-mediated metabolism resulted in the formation of metabolites that were presumed to be pharmacologically inactive (*1, 2*). Common P450 metabolites produced for FLN, TCL, BUD, and BDP were the result of 6β-hydroxylation and Δ^6-dehydrogenation. Immortalized and primary lung cell culture systems were used to evaluate CYP3A metabolism of glucocorticoids as it related to the induction of CYP3A gene transcription through GR and the constitutive androstane receptor (CAR). Studies performed using A549 cells demonstrated that only GR participated in the induction of CYP3A5 by glucocorticoids, a mechanism that was unique

from liver cells. BEAS-2B, NHBE, and lobar cells, as well as cells collected from tracheal washes of pediatric patients did not express any of the CYP3A enzymes. Selected patient samples of SAEC cells did express CYP3A5, but induction of CYP3A5 mRNA by glucocorticoid treatment was not observed. Furthermore, manipulations to the SAEC cell culture system did not lead to the induction of CYP3A5 by glucocorticoid treatment, as was observed in A549 cells. The metabolism data and the gene induction data from this research project provide possible explanations as to why there exists a broad range of glucocorticoid efficacy in patients with asthma.

Glucocorticoids are first-line therapeutics for treating asthma, but roughly 30% of patients are not responsive to treatment (*3*). A possible explanation for this "steroid insensitivity" may be that excess metabolism of glucocorticoids to clearance metabolites by CYP3A enzymes could decrease the bioavailability of the pharmacologically active drug systemically and/or in lung cells. The results described in this dissertation support this hypothesis by demonstrating that CYP3A enzymes metabolize glucocorticoids to metabolites that are predicted from published structure activity studies (*1, 2*) to be pharmacologically less active or inactive. For FLN, TCL, and BUD, the major metabolites produced by CYP3A enzymes were hydroxylated and dehydrogenated products resulting from metabolism at the 6 position of the B ring. Metabolism of FLT and TCL by CYP3A enzymes produced D ring metabolites arising from ester cleavage. For BDP, CYP3A5 produced a hydroxylated metabolite, [M4], and a dehydrogenated metabolite, [M5], likely occurring at the C6 position of the B ring, similar to the other glucocorticoids. CYP3A4 also generated these metabolites at similar rates as CYP3A5, but in addition produced [M6], a hydroxylated and de-esterified metabolite, arising from

the combined action of esterases and CYP3A4. Because CYP3A5 metabolism leads to the pharmacological inactivation of four glucocorticoids, it stands to reason that increased expression of CYP3A5 enzyme in the lung could also decrease glucocorticoid efficacy. The CYP3A5 enzyme has two major polymorphisms: *CYP3A5*1* and *CYP3A5*3*. The majority of the Caucasian population expresses *CYP3A5*3*, which codes for an inactive CYP3A5 enzyme (*4, 5*), and therefore would not metabolize glucocorticoids regardless of the level of enzyme expressed. However, about 20% of the Caucasian population, and approximately 90% of the African American population, express *CYP3A5*1*, which codes for an active enzyme. Such individuals are characterized as "extensive metabolizers" (*6*). This population, in particular, could exhibit increased metabolism of glucocorticoids in the lung, particularly if CYP3A5 is induced by the glucocorticoid treatment, potentially causing a decreased benefit from glucocorticoid treatment. This intriguing hypothesis requires further investigation to be confirmed or refuted and should be carefully evaluated in future studies of factors that influence glucocorticoid efficacy.

In addition to exploring how CYP3A enzymes metabolized glucocorticoids, it was important also to determine if these enzymes were induced in the lung in response to glucocorticoid treatment. Previous research has established that in the liver, treatment with submicromolar concentrations of glucocorticoid leads to the induction of GR. GR then forms a homodimer and translocates into the nucleus where it induces transcription of CAR. CAR protein then forms a heterodimer with the retinoid X receptor (RXRα) which increases the transcription of CYP3A genes (*7*). This pathway, however, had not been explored in the lung. A549 cells were used as a general lung cell model to evaluate whether this pathway was also responsible for CYP3A gene expression in lung cells.

Experiments demonstrated that treatment with BDP induced *CYP3A5*1* ~2-fold after 24 h. Blocking the production of [M1], the active metabolite of BDP, using esterase inhibitors, attenuated the induction of CYP3A5. Inhibiting both cytochrome P450 enzymes and esterases, using 1-ABT (a mechanism-based inhibitor of CYP enzymes) and a esterase inhibitor cocktail (eserine and paraoxon), and treating with [M1] led to the induction of CYP3A5 mRNA, demonstrating that [M1] was both required and sufficient to induce CYP3A5 mRNA expression and that GR was involved in this pathway. This conclusion was further verified using siRNA targeted against GR mRNA: maximal depletion of GR mRNA correlated with inhibited induction of CYP3A5 mRNA by BDP. CAR mRNA, however, was not detected in A549 cells, prior to or following treatment with glucocorticoids, suggesting that CAR did not play a role in the induction of CYP3A5 mRNA in A549 cells, which had been assumed based on studies using liver cells.

Primary lung cells were also used to confirm the mechanism for CYP3A5 induction in A549 cells. Primary cells representative of the tracheal and bronchial epithelium did not express CYP3A enzymes. These data suggest that CYP3A enzymes likely do not contribute to the metabolism of BDP in the conducting airways. SAEC cells, which are isolated from the distal bronchioles, alveolar ducts, and alveoli, did express CYP3A5, but not CYP3A4 or CYP3A7. Surprisingly, CYP3A5 mRNA was not induced in SAEC cells by glucocorticoid treatment. Various manipulations were made to the SAEC culture conditions to evaluate the GR→CYP3A5 regulatory pathway characterized in A549 cells. However, even with extensive manipulation of cell culture media supplementation, treatment concentrations, and treatment durations, CYP3A5 mRNA was not inducible in SAEC cells. Further investigation of literature related to this topic

showed that all of the research completed to date had been performed in A549 cells and no work had been published using primary cell culture systems. Because the results of the primary cells were inconclusive, it was not possible to determine if the pathway of CYP3A5 mRNA induction demonstrated in A549 cells also functioned in the human lung. Completing a human study to evaluate this pathway would not be possible due to ethical issues with extracting human lung samples out of healthy individuals. It may be a suitable substitute to culture mouse bronchiolar and alveolar tissue and/or cells and perform the same experiments described with A549 cells to determine if glucocorticoid treatment will induce Cyp3a mRNA through GR in mouse lung. This experiment could be taken a step further by utilizing a mouse model, BALB/c mice (a common asthma model), and administering two doses of glucocorticoids via tracheal installation, 12 h apart, for 24 h. The lungs would then be extracted, homogenized, and assayed for Cyp3a mRNA, specifically Cyp3a11, Cyp3a16, Cyp3a41, and Cyp3a13, which are homologous to human Cyp3a enzymes (*8, 9*). If Cyp3a mRNA increased in mice following treatment as compared to control mice, it would agree with the work completed in A549 cells, and would further support the hypothesis that glucocorticoid treatment increases CYP3A5 expression in human lung. Such results would also justify additional mechanistic studies to determine how glucocorticoids induce CYP3A in the intact lung system, including studies to link the effects of metabolic capacity on glucocorticoid efficacy.

The cumulative results presented in this dissertation unquestionably demonstrate a role for GR in the induction of CYP3A5 mRNA in A549 cells. However, it remains unclear if there are other regulatory elements involved, which limit CYP3A5 induction in primary lung cells. Studies conducted by Schuetz *et al.* identified two “half-sites”

(TGTTCT) of the glucocorticoid response element separated by 160 bp in the CYP3A5 promoter region in HepG2 cells (immortalized hepatic cells) and primary human and mouse hepatocytes (*10*). It was demonstrated that mutating the GR homodimer inhibited the induction of CYP3A5 message with dexamethasone treatment. It was also established that mutating either of these DNA "half sites" of the CYP3A5 promoter region inhibited the binding of the GR homodimer and therefore blocked the induction of CYP3A5 with dexamethasone treatment. Thus, it is plausible that a similar role for these half-sites occurs in A549 cells. Future experiments in A549 cells could be conducted by mutating one or both of these half-sites in the CYP3A5 promoter to confirm their role in CYP3A5 expression and induction by glucocorticoids in lungs cells. Specifically, if these mutations block the induction of CYP3A5 after glucocorticoid treatment, then a direct interaction between GR and CYP3A5 occurs. If the mutations do not block CYP3A5 induction, then another regulatory element and/or elements is/are involved, and more thorough studies of promoter region binding elements would need to be completed and evaluated in the primary lung cell models, particularly of negative regulatory factors exist. The known mechanism of action for glucocorticoids is to bind to GR, which ultimately decreases inflammation and mucus production in the lung by increasing the transcription of annexin 1, interleukin 10, and inhibitor of nuclear factor kappa B (*3, 11-14*). The glucocorticoid receptor can also modulate expression of proinflammatory genes by binding to transcription factors such as nuclear factor kappa B and activator protein-1 and decrease their expression (*15*). These actions are considered to be direct genomic actions of glucocorticoids (*16*). A major finding of this work was that binding of glucocorticoids to GR, or genomic action, induces the expression of CYP3A5 mRNA in A549 cells.

Presuming a similar phenomenon also occurs in the human lung, it is possible that continued use of inhaled glucocorticoids could promote higher levels of CYP3A5. For patients that express the active form of CYP3A5, this could increase metabolism of glucocorticoids in lung cells, and therefore, potentially reduce the efficacy of glucocorticoids over time (see Figure 5.1 for a schematic representation of this concept). A possible way to combat this cycle would be to include a selective CYP3A inhibitor that could be coadministered by inhalation with the glucocorticoid. An inhibitor that could be used is the antifungal drug, ketoconazole, which is a well-known inhibitor of CYP3A enzymes. Experiments conducted in A549 cells with ketoconazole at 1 μM and glucocorticoids at 10 μM (BDP and BUD), 1 μM (TCL and FLT), and 100 nM (FLN) showed that CYP3A5 was sufficiently inhibited, because no CYP3A-generated metabolites were produced. Additionally, ketoconazole itself did not inhibit GR at this concentration, because induction of CYP3A5 was not blocked, and therefore, the glucocorticoid would still be able to target GR and inhibit inflammation. This same idea could be used in patients or mouse models. However, the ratio of ketoconazole to glucocorticoid would need to be carefully controlled. Specifically, dosing would require that the CYP3A inhibitor, ketoconazole or a similar agent, be at a high enough concentration to effectively inhibit CYP3A enzyme activity, but low enough so that the glucocorticoid could outcompete for GR binding, since ketoconazole is also a GR antagonist. Another alternative could be to utilize fluticasone in patients expressing *CYP3A5*1*. Murai *et al.* showed that FLT is a potent mechanism-based inactivator of CYP3A5, with some inactivation also occurring with CYP3A4 (*17*). Thus, patients receiving FLT treatment may receive the benefit of reduced glucocorticoid clearance by

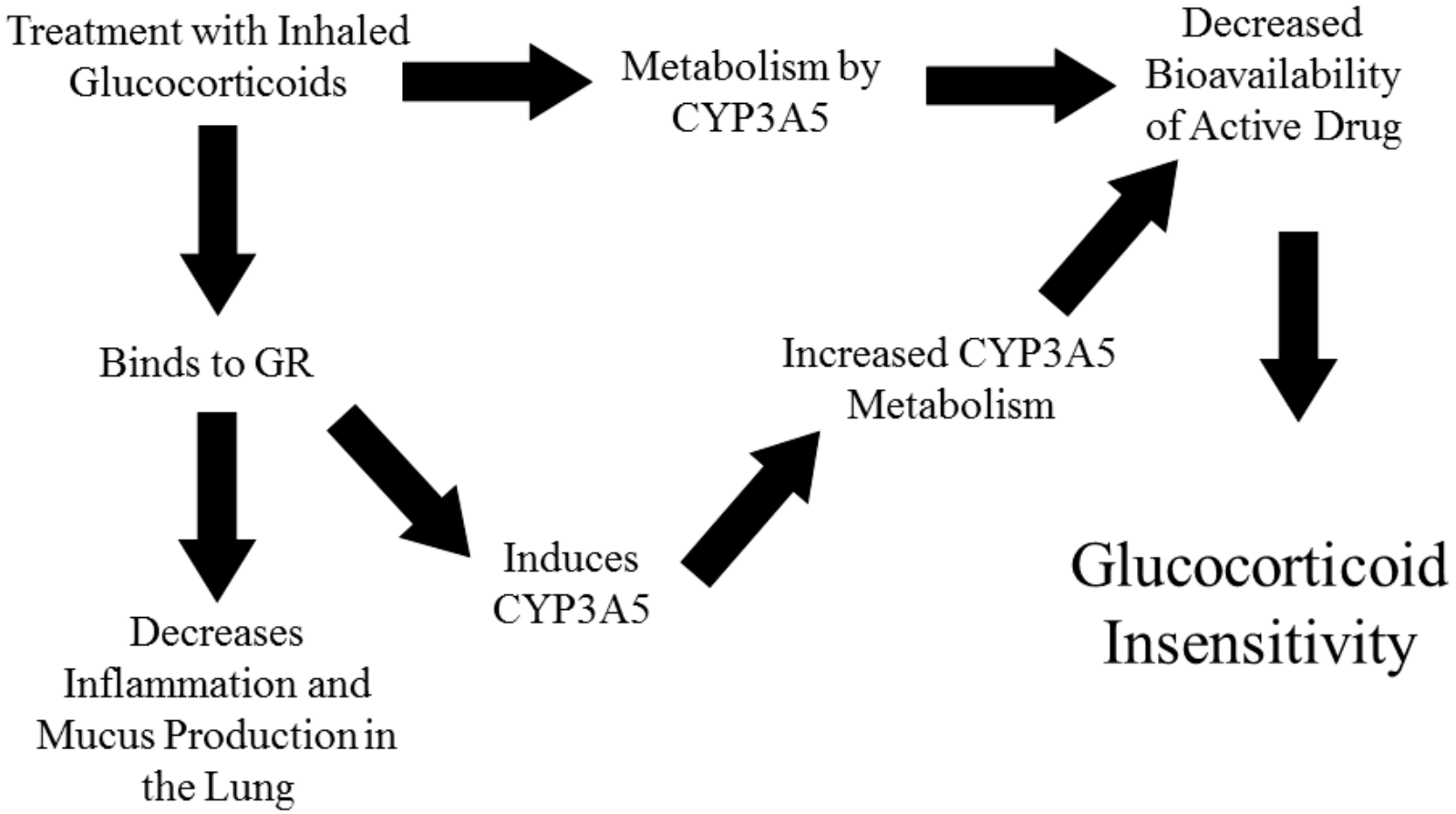

Figure 5.1. Schematic representation of the hypothesis that continued use of glucocorticoids will perpetuate glucocorticoid insensitivity. Glucocorticoids bind to GR to decrease inflammation and mucus production in the lung. Binding to GR may also induce the expression of CYP3A5, which will increase CYP3A5-mediated metabolism of glucocorticoids, decrease bioavailability of active drug in lung cells, and contribute to glucocorticoid insensitivity. Continued treatment with inhaled glucocorticoids may perpetuate this cycle.

CYP3A enzymes in lung cells, in addition to the intended effect through GR using a single drug. This intriguing concept is supported by the results published by Stockmann *et al.* where it was demonstrated that patients expressing CYP3A4*22, which is associated with decreased hepatic CYP3A4 activity, and receiving FLT, showed greater control of asthma symptoms than individuals with the *CYP3A4*22* genotype who were receiving other inhaled glucocorticoids, (with a limited number of subjects receiving drugs other than FLT) and compared to patients with the active forms of CYP3A4 (*18*). It has also been demonstrated that patients who switch to FLT from other glucocorticoids are able to manage their asthma symptoms using a lower dose of FLT (*19*). Therefore, patients who are not showing benefit from glucocorticoid therapy should first be switched to FLT and possibly treated with another selective CYP3A enzyme inhibitor, to potentially better treat their asthma symptoms. As such, future studies should aim to confirm the basis of the relationship between FLT treatment and metabolic clearance by CYP3A enzymes as a possible way to improve how steroid insensitive patients are cared for.

In addition to genomic effects of glucocorticoids on GR, there are "off-target" effects, or nongenomic actions that occur with glucocorticoid treatment. Nongenomic actions have a rapid onset, about 90 seconds, and are short in duration (*16*). These effects can be divided into three subcategories, as described by Stahn and Buttgereit (*20*); nonspecific interactions of glucocorticoids with plasma membranes, interaction with membrane bound GR, and nongenomic effects through binding to cytosolic GR. Briefly, these nongenomic effects most frequently interfere with mineral and adenosine triphosphate transport across plasma membranes, resulting in immune cell suppression

(*21, 22*), inhibit Lck/Fyn kinases, which are downstream from T-cell receptors, and suppress major pathways important in T-cell activation (*23, 24*), or block arachidonic acid production, which has been shown to decrease bronchoconstriction and mucus production (*25, 26*). While these experiments have been shown *in vitro*, they have not been demonstrated *in vivo*, and more than likely do not play a role in glucocorticoid insensitivity due to rapid onset of the effects and short duration (*16*).

Polymorphisms of GR have also been demonstrated and could possibly play a role in glucocorticoid insensitivity in asthma patients. One GR polymorphism, ER22/23EK, occurs in 3% of the population and affects exon 2, replacing a lysine with an arginine at position 23 (*27*). ER22/23EK is associated with lower transcriptional activity by GR in reporter assays (*28*) and has been seen in patients classified as glucocorticoid insensitive in other disease states, but not in asthma (*29*). More genotyping studies need to be completed in asthma patients that respond to glucocorticoid therapy, and those who do not, in order to determine if this polymorphism is playing a role in glucocorticoid insensitivity in the context of asthma.

Esterases play an important role in the bioactivation of BDP and could also play a role in glucocorticoid insensitivity. Esterases can be divided into three classes: A esterases, B esterases and C esterases (*30, 31*). Those most pertinent to the hydrolysis of esterases in the lung are B esterases, which compromise carboxylesterases, cholinesterases, and acetycholinesterases, and are serine-dependent enzymes that participate in the bioactivation of xenobiotics (*32-34*). This class of enzymes has been shown to cleave ester groups on a similar glucocorticoid (ciclesonide) to BDP (*35*) and may contribute to activation of BDP in the lung. It is possible that differences in

expression of esterases in the lung, either leading to decreased bioactivation of BDP or increased metabolism of BDP, could contribute to glucocorticoid insensitivity, independent of CYP3A5 metabolism. However, glucocorticoid insensitivity occurs with all glucocorticoids and most of the glucocorticoids used to treat asthma do not contain ester side groups. Therefore, esterases could only play a role in glucocorticoid insensitivity related to certain drugs, not all drugs, as is observed. As such, a role for esterases in glucocorticoid insensitivity is expected to be minimal, if any role at all.

In summary, the work described in this dissertation supports the over-arching hypothesis that individuals who show lower benefit from inhaled glucocorticoid treatment may also exhibit increased glucocorticoid metabolism in the lung. Further work described herein should be completed to fully evaluate this interesting possibility in human populations, since the elucidation of key factors that affect glucocorticoid efficacy could significantly improve the treatment of patients who currently exhibit poor control of asthma symptoms with their current inhaled glucocorticoid therapy.

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