INTERACTIONS BETWEEN DRUGS OF ABUSE

AND HIV PROTEASE INHIBITORS

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INTERACTIONS BETWEEN DRUGS OF ABUSE AND HIV

PROTEASE INHIBITORS

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ABSTRACT

Drug abuse is an escalating problem prevalent in both large metropolitan and rural places and is a major cause of mortality and morbidity all over the world. Drugs of abuse such as morphine and nicotine are consumed by people for prolonged periods of times to improve their physical and mental condition as well as to get relief from pain and other medical conditions. This prolonged intake often overlaps with the clinical regimen of several chronic neuropsychological, cardiovascular, pulmonary, infectious and neoplastic diseases. One in four patients living with human immunodeficiency virus (HIV) infection reported use of drugs of abuse. Achieving target intracellular concentrations during long term therapy of several diseases can be challenging due to number of factors such as poor adherence, drug resistance and drug-drug interactions. One important mechanism of multidrug resistance involves the up-regulation of multidrug resistance transporter, p-glycoprotein (p-gp), member of ATP binding cassette (ABC) superfamily that effluxes most of the therapeutic drugs and reduce their intracellular accumulation. Drug-drug interactions can result from inhibition and induction of the cytochrome P450 enzymes

and/or efflux transporters. Drugs of abuse have the ability to potentiate or attenuate the effects of co-administered therapeutic drugs that can lead to toxic effects or a reduction in the therapeutic activity of the co-administered drugs. This thesis investigates the chronic effect of morphine and nicotine on the expression and functional activity of efflux transporters (MDR1, MRP2, and BCRP) and metabolizing enzymes (CYP3A4). Induction of Pregnane-X-Receptor (PXR) was found to regulate the induction of MDR1 and CYP3A4 gene expression. Interactions between drugs of abuse and therapeutic drugs provide crucial insights into the failure of clinical regimen in patients suffering from HIV, cancer and other infections. Results from this thesis elucidate the mechanism behind the interactions between morphine and nicotine and HIV protease inhibitors. Studies were performed primarily through the use of *in vitro* models; e.g. LS180 and Caco-2 (for intestine) and HepG2 (for liver).

In the second set of my studies, expression and functional activity of efflux transporters in Calu-3, human airway epithelial cell line was investigated. Expression and functionality of efflux and influx transporters in the airways is poorly identified and characterized. Results from this project allow understanding of the drug absorption in airways. As part of the study, molecular and functional activity of breast cancer resistance protein was identified for the first time. Folic acid receptor-alpha and proton coupled folic acid transporter expression was identified at molecular and protein level across human bronchial epithelial cell line, Calu-3. Since nicotine is smoked through lungs, effect of nicotine on the expression and functional activity of efflux transporters

and metabolizing enzymes was determined. Nicotine was found to induce MDR1, BCRP expression and CYP3A4/A5 metabolism in Calu-3 cells. Male Sprague Dawley rats were treated with nicotine to investigate the effect of nicotine on CYP3A4 mediated rat lung metabolism. Cortisol was used as a model substrate to evaluate CYP3A4 mediated metabolism. Cortisol metabolism enhanced in nicotine treated rats than control rats signifying the enhanced CYP3A4/A5 metabolism. Furthermore, cortisol metabolism enhanced in microsomes obtained from smokers when compared to microsomes obtained from non-smokers.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of School of Graduate Studies have examined the dissertation titled "Interactions Between Drugs of Abuse and HIV Protease Inhibitors" presented by Durga Kalyani Paturi, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS

ATP   : Adenosine Triphosphate

BCRP   : Breast cancer resistance protein

cDNA   : Complementary Deoxy-Ribonucleic Acid

CAR   : Constitutive Androstane Receptor

CYP3A4   : Cytochrome P450-3A4

DNA   : Deoxy-Ribonucleic Acid

dNTP   : Deoxy Nucleotide Triphosphate

DPBS   : Dulbecco's Phosphate Buffered Saline

FBS   : Fetal Bovine Serum

DMEM   : Dulbecco's Modified Eagle Medium

EDTA   : Ethylene Diamine Tetraacetic Acid

GAPDH   : Glyceraldehyde Phosphate Dehydrogenase

HEPES   : 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

MDR   : Multidrug Resistance

MEM   : Minimum Essential Medium

$MgCl_2$: Magnesium Chloride

MMLV   : Moloney Murine Leukemia Virus

mRNA   : Messenger Ribo Nucleic Acid

NaCl   : Sodium Chloride

NaF   : Sodium Flouride

Na$_3$VO$_4$: Sodium Orthovanadate

NEAA : Non Essential Amino Acids

NADPH : Nicotinamide Adenine Dinucleotide Phosphate-Oxidase

PCR : Polymerase Chain Reaction

P-gp : P-glycoprotein

PXR : Pregnane- X-Receptor

RNA : Ribonucleic Acid

RXR : Retinoid-X-Receptor

S.D. : Standard Deviation

SJW : St. John's Wort

Taq : Thermus Aquaticus

UV : Ultra Violet

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Dedicated to my family and myself

CHAPTER-1

INTRODUCTION

Overview

Drug abuse is an escalating problem prevalent in both large metropolitan and rural places and is a major cause of mortality and morbidity all over the world. Drugs are abused by people for prolonged periods of time to improve their physical and mental condition as well as to get relief from pain and other medical conditions. This prolonged intake often overlaps with the clinical regimen of several chronic neuropsychological, cardiovascular, pulmonary, infectious and neoplastic diseases. Global human immunodeficiency virus (HIV) epidemic continues to grow in many countries and abuse of tobacco, alcohol and illicit drugs poses a risk for HIV clinical regimens. Global estimates in 2013 indicate that approximately 34 million people were estimated to be living with HIV infection and one in four people living with HIV reported use of drugs of abuse. HIV protease inhibitors are the frontline drugs in the treatment of HIV infections and are routinely administered with non-nucleoside reverse transcriptase inhibitors and nucleoside reverse transcription inhibitors. Achieving target plasma concentrations during long term therapy can be challenging due to number of factors such as poor adherence, viral resistance and drug interactions. Also, patients receive therapy for prophylaxis of opportunistic infections such as pneumocystis pneumonia, tuberculosis infection, mycobacterium avium complex disease, bacterial respiratory disease, bacterial enteric infections, oropharyngeal and esophageal candidiasis and other diseases that are more

severe due to immunosuppression. These drug combinations result in drug-drug interactions and these primarily result from inhibition and induction of the cytochrome P450 enzymes and/or efflux transporters. HIV protease inhibitors are known to be substrates and inhibitors for efflux transporters such as MDR1, MRP2 and metabolizing enzymes such as CYP3A4. Kim et al. demonstrated that plasma concentrations of indinavir, nelfinavir, and saquinavir were elevated 2 to 5 fold in MDR knockout mice relative to wild-type mice after oral administration. Fitzsimmons et al reported that CYP3A4 mediated metabolism contributes to poor bioavailability of HIV protease inhibitors. Results from these studies indicate that P-gp and CYP3A4 can affect the disposition of HIV protease inhibitors resulting in lower efficacy.

Long term exposure to drugs of abuse can modulate the expression of efflux transporters and metabolizing enzymes and thereby alter the efficacy of the HIV therapy resulting in clinically significant drug interactions. These interactions may cause HIV patients to require a large dosage of drugs and therapeutic drug monitoring of these drugs is conducted to prevent sub-therapeutic or toxic concentrations. Short term exposure of mouse fibroblast NIH-3T3 cells with morphine induced p-glycoprotein expression. Further studies are needed to investigate the effect of chronic exposure of these drugs of abuse on the expression of efflux transporters and metabolizing enzymes. A classic example to this hypothesis involves the effect of long term treatment of rifampicin on HIV protease inhibitor ritonavir and saquinavir. HIV patients who are receiving long term therapy of Rifampicin will need higher doses of HIV protease inhibitors to maintain

adequate plasma concentrations. Efflux transporters such as MRP2 and BCRP also play a major role in the disposition of several therapeutic drugs.

Pulmonary infections are the main cause of morbidity in patients with HIV. HIV is frequently detected in lungs and alveolar macrophages have also been shown to be reservoirs for HIV disease. HIV infected persons are at increased risk of manifesting pulmonary conditions such as chronic obstructive pulmonary disease (COPD), lung cancer, and pulmonary arterial hypertension. Pulmonary delivery is an efficient non-invasive route of delivery for the treatment of pulmonary and systemic diseases. Our understanding of pulmonary influx, efflux mechanisms and metabolism is very limited. Furthermore, recent studies have shown that HIV protease inhibitors are being studied extensively for their anticancer properties against lung cancer and pulmonary opportunistic infections. Expression and modulation of activity of efflux transporters, metabolizing enzymes and nuclear receptors is cell type specific. Therefore, role of efflux transporters and metabolizing enzymes in lungs needs to be investigated.

Statement of Problem

Resistance to chemotherapy remains a major challenge for physicians in the clinical management of life threatening infections such as HIV. Development of drug resistant HIV viruses after multiple drug administration ranging from months to years is frequently witnessed among HIV patients[1]. One of the main cause by which cells acquire drug resistance is the induction of efflux transporters and metabolizing enzymes. Increase of drug resistant viral load combined with therapeutic failure result in several deaths. Furthermore, co-administered drugs that can induce efflux transporters and metabolizing enzymes pose an additional risk for induction as well as therapeutic failure [2].

Drug abuse is one of the main public health problems in the world with an estimated 200 million people abusing drugs illegally and an estimated 1 million people live with HIV in United States[3]. Drugs of abuse such as nicotine and morphine can cause inhibition or induction of these efflux transporters and/or metabolizing enzymes. Inhibition of efflux transporters and metabolizing enzymes lead to toxicity whereas induction results in therapeutic failure. Thus, drug interactions may compromise the safety and effectiveness of therapeutic medications[4].

Failure to predict the induction potential of these drugs of abuse may lead to therapeutic failure of the administered drugs intended for HIV therapy. This thesis aims to investigate the mechanism of drug-drug interactions between nicotine and morphine and HIV protease inhibitors. Chronic effects of nicotine and morphine on the expression and functional activity of efflux transporters (MDR1, BCRP, MRP2) and metabolizing

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enzymes (CYP3A4) were determined. Induction mediated drug interactions are cell type specific and depend upon the expression and functional activity of efflux transporters, metabolizing enzymes and nuclear receptors[5]. Therefore, we aim to study the inductive effects of nicotine and morphine in in vitro model cell lines LS180, Calu-3 and HepG2 and investigate the expression and functional activity of efflux transporters.

Hypothesis

According to National Institute of Drug Abuse, approximately, 69.6 million people of ages 12 years or older reported current use of tobacco. Cigarette smoking kills an estimated 440,000 U.S citizens each year[6]. Nicotine in tobacco is highly addictive. Clinicians often ask their patients if they are smokers or nonsmokers. It was observed clinically that pharmacokinetic profile of inhaled insulin is significantly affected owing to its higher concentrations in smokers than in nonsmokers. Smokers might need higher doses of medication than nonsmokers. Medications that interacts with smoking often needs dosage adjustment to achieve the therapeutic levels[7]. Furthermore, cigarette smoking is contraindicated along with hormonal contraceptives and inhaled corticosteroids because of the increased risk of adverse cardiovascular effects. Morphine is the opioid of choice for moderate to severe cancer pain and is a substrate of p-glycoprotein. Morphine has been shown to induce p-glycoprotein expression in rat brain. Studies utilizing p-glycoprotein knock out mouse models have shown that p-glycoprotein restricts the uptake of morphine into the brain. This study concluded that modulation of p-

glycoprotein expression at the blood brain barrier by morphine has an impact on the central analgesic activity of morphine[8]. Increased uptake of morphine has been demonstrated in rats with the concomitant administration of GF120918, a known P-gp and BCRP inhibitor[9]. Also, rats treated with morphine for 5 days resulted in a 2 fold induction of p-glycoprotein expression in brain.

It has been demonstrated that MDR1 mRNA can be induced by short term exposure of some cytotoxic agents[10]. Such induction of MDR1 mRNA and further upregulation of p-glycoprotein levels leads to development of drug resistance. Drug delivery via inhalation is an alternative route of systemic administration. Also, drugs are delivered directly to lungs to treat life threatening diseases such as lung cancer. Lung is an important sanctuary organ for HIV replication and proliferation. During HIV infection, low viral RNA always exists in sanctuary sites such as lungs and brain. Lungs are one of the major targets during HIV infection and advanced complications of AIDS[11]. Opportunistic infections along with serious pulmonary complications are often observed in 50% of the HIV patients[12]. Moreover, p-glycoprotein expressed at the sanctuary sites limit the concentrations of therapeutic agents.

Oral Delivery

HIV protease inhibitors have been successfully used to control disease progression in HIV-1 patients. Clinical outcome of the therapy depends on the higher intracellular concentrations of HIV protease inhibitors in cells infected with HIV[13]. However, clinical

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outcome of antiretroviral therapy often fails due to complex factors such as poor adherence, virological resistance, drug interactions and cellular resistance[14]. HIV protease inhibitors are known substrates of efflux transporters such as p-glycoprotein, MRP2[15] and metabolizing enzymes such as CYP3A4[16] at the intestinal barrier. Drugs of abuse such as morphine and nicotine may alter the expression and activity of these efflux transporters and metabolizing enzymes. Clinically significant interactions have been reported with the chronic administration of HIV protease inhibitors with drugs that are substrates for CYP3A4. For example, chronic administration of ritonavir increases the oral clearance of methadone, phenytoin and ethinyl estradiol[17]. Furthermore, PXR has been shown to play a major role in the induction of efflux transporters and metabolizing enzymes. Drugs which activate PXR levels may induce CYP3A4 expression and activity[18]. Three mechanisms involved in drug-drug interactions are depicted in figure-2.1.
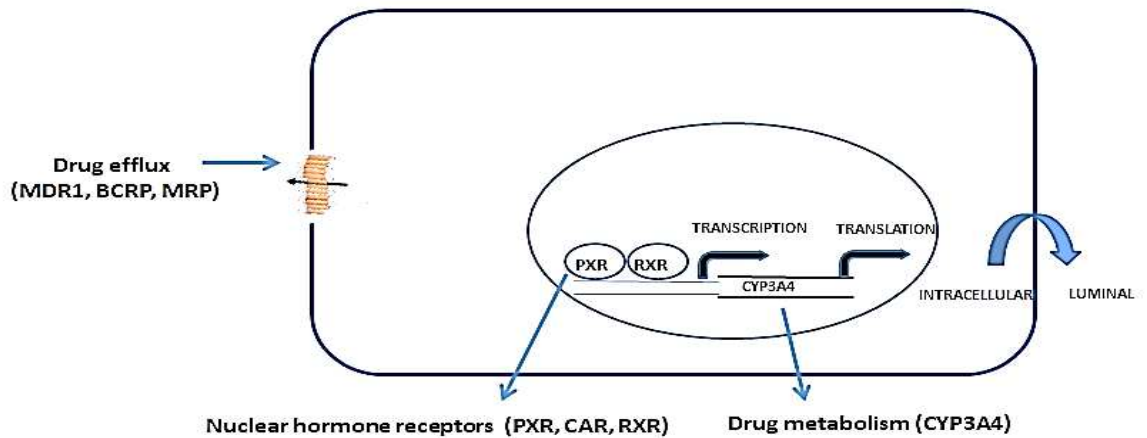
Figure 2.1 Mechanism of drug-drug interactions

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Pulmonary Delivery

Pulmonary delivery provides a noninvasive alternative mode of delivery to subcutaneous as well as intravenous route. FDA approved several drugs for treating respiratory and pulmonary conditions such as allergy, asthma, bronchitis, cystic fibrosis, emphysema, lung disease and others[19]. Inhaled drugs market was estimated to reach to $ 44 billion by 2016 and the pulmonary market is considered to be the fourth largest therapeutic area in pharmaceutical sales. Lungs offer number of physiological benefits such as larger surface area, thin barrier and high vascularization) for drug delivery when compared to other routes. Drugs are inhaled through the lungs and enter the blood stream the alveolar epithelium. Drug transporter proteins are important determinants in drug disposition and response with subsequent effect on the pharmacokinetics of drugs. Also, metabolizing enzymes and influx transporters play an important role in the modulation of the permeability of the drugs across alveolar epithelium. P-glycoprotein and MRP1 expression in human lung suggests the protective role of these transporters against exogenous toxic substances entering from the air and blood stream. Scheffer et al detected higher expression of p-glycoprotein in lungs of mice[20]. P-glycoprotein kinetics was measured by measuring lipophilic amine dye rhodamine 6G in intact lung. This study showed higher rhodamine 6G accumulation in the presence of p-gp inhibitors Verapamil and GF120918[21]. Another multidrug resistance transporter MRP1 is highly expressed at the basolateral side of the human bronchial epithelial cells. Lehmann et al detected high MRP1 expression in normal human lung[22]. Functional activity of MRP1 was also detected

in Calu-3 cells in human bronchial epithelial cell line[23]. BCRP protein levels are expressed in epithelial cell layers as well as endothelial blood capillaries suggesting its role in the protection of lungs against toxic compounds from the air and blood stream respectively[24]. Higher BCRP expression was found to correlate with poor clinical outcome of platinum based chemotherapy for advanced non-small cell lung cancer cells[25]. These studies depict the importance of efflux transporters in the distribution of pulmonary drugs to the site of action. Therefore, delivery of pulmonary drugs to the site of action may depend on the activity and expression of the efflux transporters and metabolizing enzymes. Little is known about the functional activity and modulation of these efflux transporters and metabolizing enzymes.

Majority of the inhaled toxicants pass through the respiratory tract, exposing pulmonary epithelium to higher concentrations than liver cells. Higher concentrations can contribute to significant metabolism in lungs. In the same way, higher concentrations of tobacco smoke can modulate the metabolism of CYP enzymes. Several CYP enzymes are expressed in the lungs of mammals, but studies on their modulation are very limited. Thus, studies answering the lung specific activation of efflux transporters and metabolizing enzymes needs to be performed. Finding results for this study is more difficult in humans, therefore *in vitro* lung airway epithelial cell models were utilized for these studies.

Objectives

With this background the objectives of this dissertation are:

➢ **To investigate the chronic effect of nicotine and morphine on expression and functional activity of efflux transporters and metabolizing enzymes and to study their contribution to the intracellular accumulation of HIV protease inhibitors.**

➢ **To characterize the expression and functional activity of folic acid carriers in human bronchial epithelial cell line, Calu-3**

➢ **To study the role of efflux transporters in human bronchial epithelial cell line, Calu-3**

➢ **To characterize the molecular and functional activity of breast cancer resistance protein in human bronchial epithelial cells, Calu-3**

➢ **To investigate the effect of chronic exposure to nicotine on the levels of efflux transporters and metabolic enzymes in Calu-3 and rat lungs**
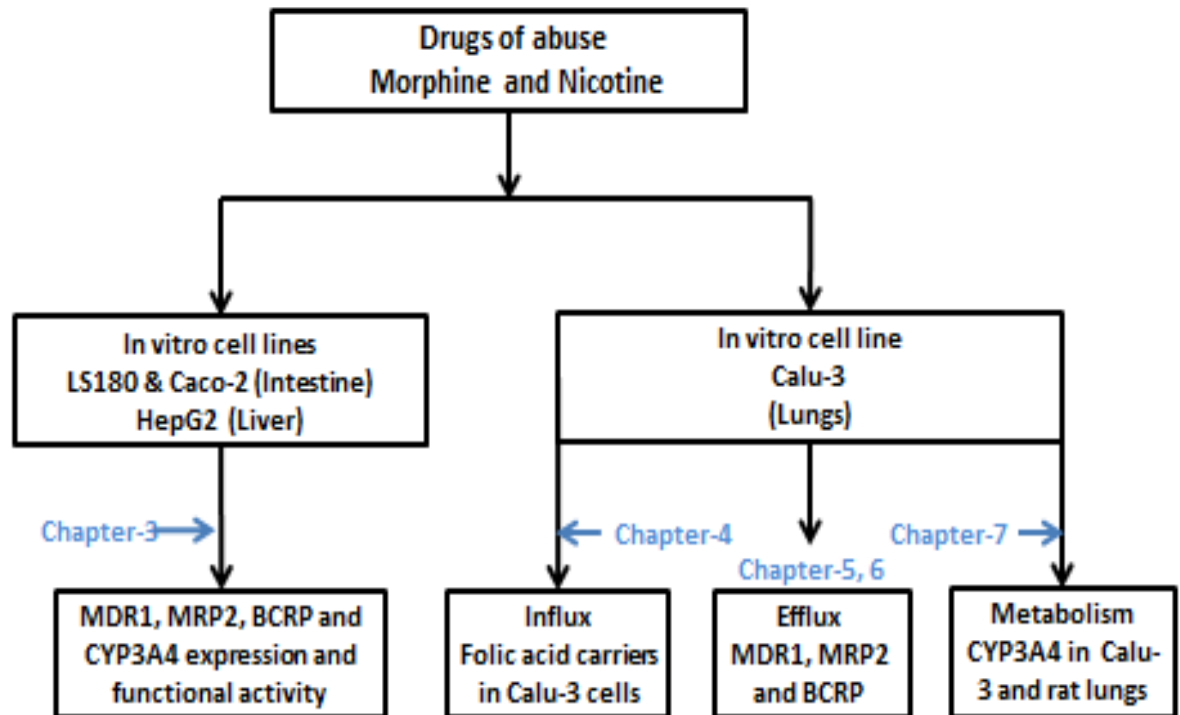
Figure-2.2 Flow chart depicting the objectives of this thesis

CHAPTER-2

LITERATURE REVIEW

Adverse drug reactions are one of the leading causes of death in the United States. Pharmacokinetic drug-drug interactions are responsible for 22% of adverse drug interactions[26]. According to National Institute of Health Sciences, drug interaction is defined by "The pharmacologic or clinical response to the administration of a drug combination different from that anticipated from the known effects of the two agents when given alone". Pharmacological desired and undesired effects of a drug occur from concentrations at its site of action. Concentration of a drug at its site of action depends upon the systemic concentrations which are again governed by absorption, metabolism, distribution and elimination. Variations in systemic concentrations can either cause treatment failure of life threatening infections or adverse toxicities. Interactions are of particular concern for drugs with narrow therapeutic index[27].

Drug Interactions

Drug-drug interactions can be classified into pharmacokinetic, pharmacodynamic and pharmaceutical interactions. Pharmacokinetic interactions affect absorption, distribution, metabolism and excretion of a target drug (figure-2.3). Pharmacokinetic interactions are again divided into three subclasses: drug absorption, protein binding and drug metabolism interactions.
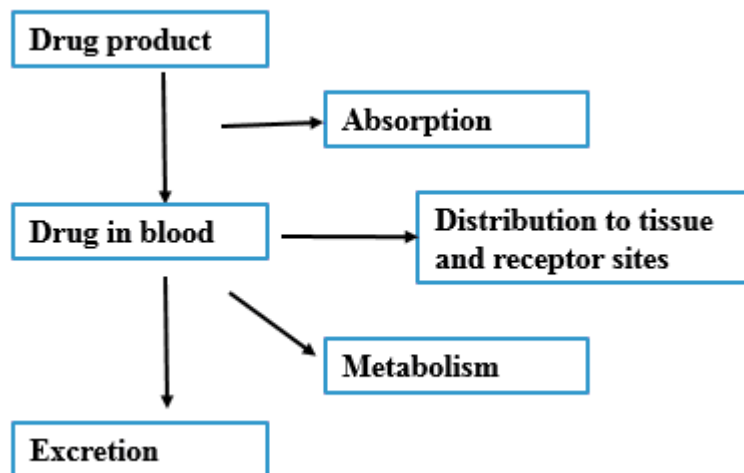
Figure 2.3 Simplified depiction of drug-drug interactions

Inhibition Interactions

Majority of drug interactions are attributed to alteration in pharmacokinetics of therapeutic drug of interest by concomitant drugs which are inhibitors of p-glycoprotein and \or CYP3A4[28]. One of the classical examples is the drug interactions of cardiac glycosides which have a narrow therapeutic index. For example, verapamil, when administered at a dose of 160 mg, led to a 40% increase in the plasma concentration of digoxin, whereas verapamil 240 mg increased the plasma concentration of digoxin by 60% to 80%. These data suggest a dose-dependent inhibition of P-gp when verapamil and digoxin are administered concomitantly[29]. When both the co-administered drugs are substrates of an efflux transporter and\or metabolizing enzymes, drug interactions are warranted. In addition, inhibition of multiple CYP enzymes and one or more transporters

can have a significant effect on therapy. Sedative effect of benzodiazepines can be increased or decreased upon co-administration with ethanol or caffeine respectively[30].

Drugs get absorbed across the intestinal cellular membranes into the blood stream. Physico-chemical properties of the drug, formulation characteristics, food and gastric emptying affect the process of absorption[31]. Factors that alter the absorption have a profound effect on bioavailability of the drugs. These factors could be especially significant if the therapeutic drugs are prodrugs that require metabolic activation to produce functional activity. For example, anticancer drugs are metabolically activated through first pass effect in the intestine and\or liver before they reach systemic circulation[32]. Decreased absorption of chlorambucil was reported in leukemia patients when the drug was administered in fed state rather than fasting state[33]. Fluoroquinolone antibiotics were prone to chelation with cations such as calcium and magnesium, thereby affecting the absorption[34].

Enzyme Inhibition

Majority of the drugs are metabolized to active or inactive metabolites in intestine and liver. Inhibition of efflux transporters and metabolizing enzymes can result in enhanced plasma concentrations. Ritonavir, a potent CYP3A4 inhibitor is commonly used as a pharmacological boost to enhance the bioavailability of other protease inhibitors such as lopinavir and saquinavir[35]. Grape juice has been shown to enhance the bioavailability of many therapeutic drugs such as calcium channel antagonists, benzodiazepines, HMG-CoA reductase inhibitors and cyclosporine. Chemical

constituents present in grape fruit juice such as naringin, naringenin, quercetin and kaempferol are shown to have inhibitory effect on MDR1 and CYP3A4[36].
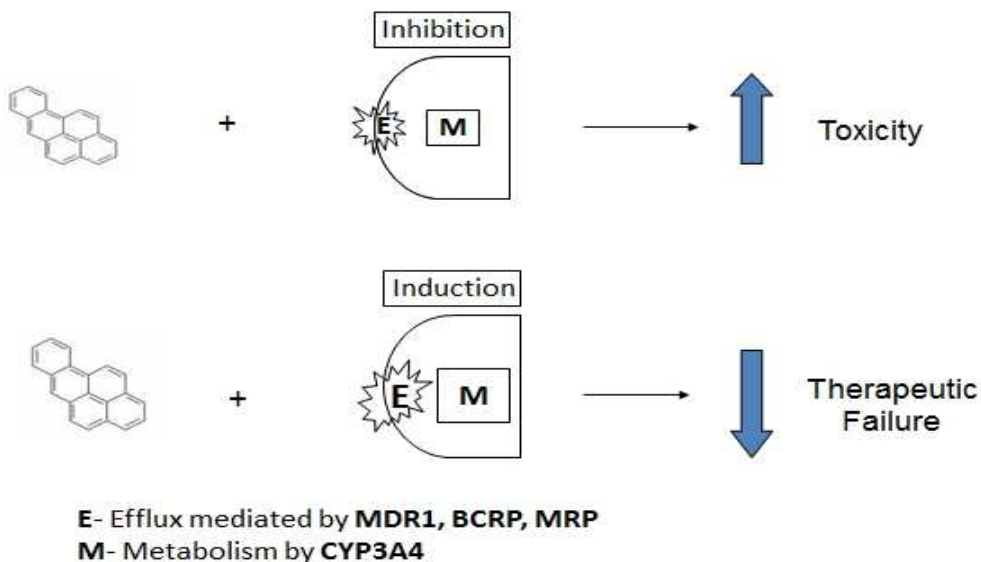
Figure 2.4 Depiction of inhibition and induction drug-drug interactions

Induction Interactions

Most of the clinically relevant drug-drug interactions are often mediated by the inhibition and induction of CYP3A4 (figure-2.4). CYP3A4 is the major route of elimination of majority of therapeutic drugs. Induction of mRNA levels of CYP enzymes result in higher protein levels followed by enhanced intestinal and hepatic enzyme activity. Increased metabolism can affect the drug bioavailability and systemic disposition leading to reduced efficacy. Thus, new therapeutic drug entities are screened for their metabolism data and their potential to induce CYP3A4 has become increasingly

important as part of preclinical studies. Rifampicin is a strong inducer of CYP3A4 and therefore, its co-administration with other prescription drugs can result in their sub-therapeutic plasma levels and thereby can alter the clinical outcomes. Induction of efflux transporters such as MDR1, MRP2 and BCRP also play an important role on the oral bioavailability of therapeutic drugs. For example, MDR1 gene expression was induced in human tumors after in vivo exposure to cytotoxic drug doxorubicin. Number of studies in cultured cell lines has indicated that change in steady state mRNA levels was found to depend on two pathways, rate of synthesis and rate of degradation. Promoters of efflux transporters and metabolizing enzymes can be activated by heat shock, heavy metals, differentiating enzymes and several therapeutic drugs. Also, activation of nuclear receptor PXR can regulate the expression of MDR1 and CYP3A4.

Efflux Transporters

Overexpression of efflux transporters was found to be primary mechanism involved in the development of multi drug resistance[37]. Efflux transporters are energy dependent membrane proteins that actively efflux the drug out of the cells. ATP binding cassette proteins are a superfamily of proteins that mediate multi drug resistance through energy driven pumps. P-glycoprotein, multi drug resistance protein (MRP 1-6) and breast cancer resistance protein (BCRP) belonging to ABC superfamily have been identified and characterized. Even though, these efflux transporters belong to the same superfamily, they have different with respect to their structure and amino acid sequence[38](figure-2.5). Drugs may cross the cell membrane by two methods: passive diffusion and carrier

transport. By passive diffusion, drugs cross the cellular membrane down a concentration gradient. Rate of permeation depends upon the concentration gradient across the membrane and solubility of the drug. Highly lipid soluble drugs can diffuse more easily across the membrane than highly polar drugs. Carrier mediated transport is divided into facilitated diffusion and active transport. By active transport, drugs cross the membrane against a concentration gradient with energy expenditure. In contrast, facilitated diffusion does not require energy for drug transfer[39]. Studies have identified that most of the therapeutic drugs are substrates, inhibitors and inducers of these efflux transporters (Table-1). They are localized and expressed in both normal and malignant cells and are involved in the transport of many substances including excretion of toxins from liver, kidneys and intestine. In addition, they limit the permeability of xenobiotics across blood-placental barrier, blood-brain barrier and testis.

P-glycoprotein: MDR1 gene, also called as ABCB1 gene was originally identified through its ability to confer multidrug resistance in tumor cells. MDR1 encodes p-glycoprotein, a 170 KDa. plasma membrane protein and was first identified by Juliano and Ling in Chinese hamster ovary cells. P-glycoprotein is a member of adenosine triphosphate binding cassette (ABC) superfamily of energy dependent efflux pumps[40]. P-gp is expressed not only in tumor tissues but also in normal tissues such as intestine, lungs, liver, kidney and brain. P-gp actively effluxes a wide range of structurally and functionally unrelated substrates such as steroids, anticancer drugs, HIV protease inhibitors, antiemetics, antibiotics, histamine receptor antagonists, calcium channel

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blockers and immunosuppressants[41]. P-glycoprotein has different roles in different tissues.
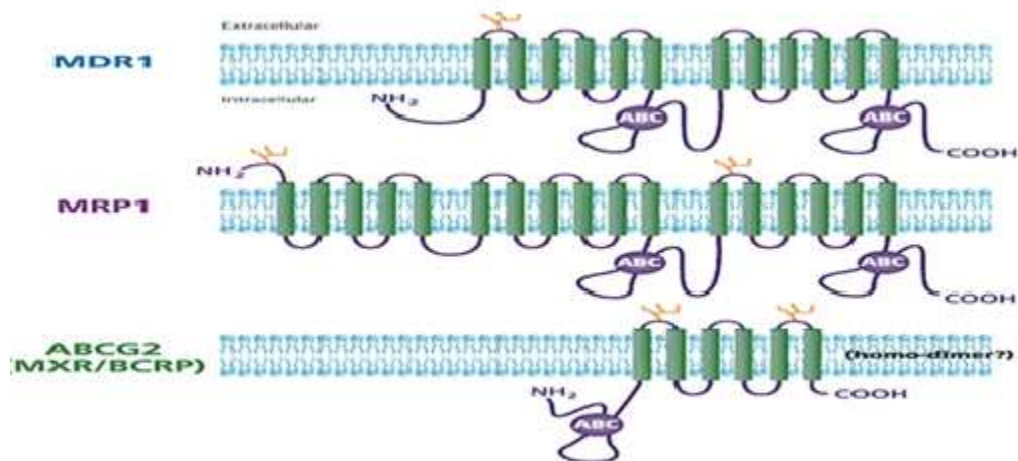
Figure 2.5 Structure of efflux transporters

P-glycoprotein has a protective role in eliminating xenobiotics and toxic substances by actively effluxing them across several barriers like blood brain barrier and blood placental barrier[42]. In liver, it plays an important role in drug elimination by effluxing the drugs to bile. In vivo studies in mice have shown a significant change in pharmacokinetics of drugs when given simultaneously with specific p-gp inhibitors[43]. Also, MDR1 knockout mice had increased the cellular concentrations in tissues such as brain and liver[44].

MRP2: Cole et al (1992) discovered a second type of efflux proteins MRPs in cancer cells other than MDR1[45]. MRP, an ATP dependent efflux transporter belongs to ABC superfamily of proteins. MRP, a 190 KDa membrane protein specifically transport

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glutathione and glucuronide conjugated drugs. MRP family consists of 9 members and unlike p-gp and BCRP, MRPs are expressed in a polarized manner on either apical or basolateral side of the membrane[46].

Breast cancer resistance protein: Breast Cancer Resistance Protein (BCRP) was identified in a breast cancer derived cell line that showed drug resistance even in the presence verapamil (a potent P-gp inhibitor)[47]. Although this protein is not over expressed not only in breast cancer, but it is termed BCRP as it was first isolated from a breast cancer cell line. The same efflux pump was also identified in a mitoxantrone-resistant human colon carcinoma cell line S1-M1–80 and hence this protein also received the name MXR[48]. BCRP/MXR is the second member of the G subfamily of ABC transporters. Under the recommendation of Human Gene Nomenclature Committee BCRP has been renamed to ABCG2. It is also referred to as ABCP (p stands for placenta), to signify its high levels of expression in that tissue. BCRP, MXR and ABCP are homologous proteins differing only in one or two amino acid sequence. BCRP structurally diverges from the other prominent ABC transporters. BCRP is a so called half transporter having only six transmembrane helices and only one nucleotide binding domain[49]. With the help of low resolution crystallography it has been shown that functional BCRP has a homodimeric structure[50]. BCRP is a high efficiency efflux pump and has broad substrate specificity. A significant amount of expression has also been observed in normal human tissues. BCRP expression is significantly expressed in placenta, intestine and liver.

MDR1	BCRP	MRP2	CYP3A4
Carbamazepine Phenobarbital Phenytoin Quercetin Hyperforin Rifampicin Dexamethasone HIV protease inhibitors Clotrimazole Prazosin Doxorubicin Imatinib	Carbamazepine Corticosteroids Rifampicin Doxorubicin Topotecan Cisplatin Imatinib	Carbamazepine Clecoxib Phenobarbital Fluoroquinolones Aminoglycosides Rifampicin Doxorubicin	Carbamazepine Corticosteroids Efavirenz Phenobarbital Phenytoin Rifampicin Hyperforin Doxorubicin HIV protease inhibitors

Table 1. Substrates of efflux transporters and metabolizing enzymes

Drug Metabolism

Drug metabolism may have a profound effect on the pharmacokinetics of the drugs. Drug metabolism can be divided into two types: Phase I metabolism which involves the pharmacological activation or inactivation of the drug by oxidation, reduction, hydrolysis and cyclization reactions. Phase II metabolism involves conjugation of phase I products to highly polar endogenous substances[51] (figure-2.6). CYPs are superfamily of enzymes found in the endoplasmic reticulum responsible for biotransformation of a wide range of xenobiotics. Cytochrome P450 enzymes are

responsible for pharmacological activation and detoxification of anticancer drugs such as doxorubicin, etoposide and ifosfamide[52].

1-Influx   2-Efflux   3-Phase-I Metabolism   4-Phase-II Metabolism
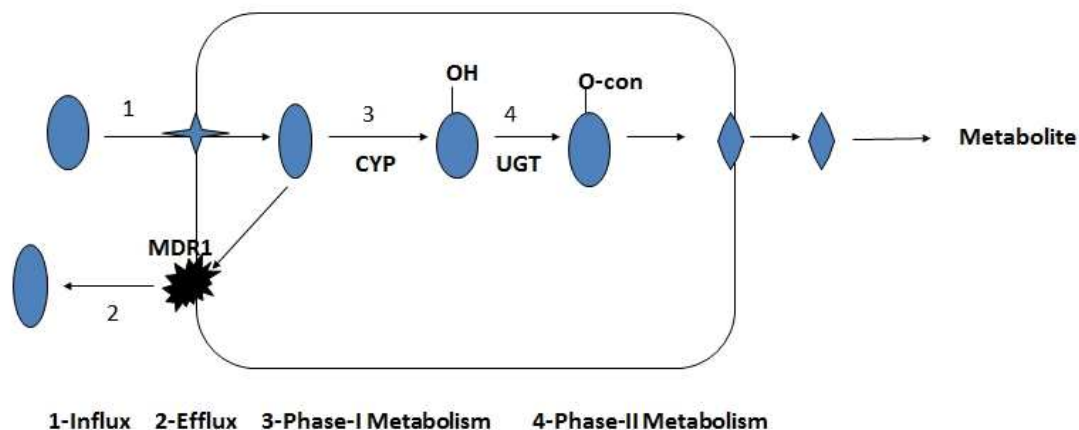
Figure 2.6   Phase I and phase II metabolism

CYP3A enzymes are the most abundant cytochrome P450 dependent mixed function oxidase enzymes involved in the metabolism of wide variety of endogenous compounds and diverse xenobiotics[53]. CYP3A4 is widely distributed in human tissue, with about 29% expressed in human liver. CYP3A4 and CYP3A5 accounts for 30-40% of the total CYP450 present in the liver and small intestine[54]. There is a significant interindividual variability in the expression and activity of CYP3A4 due to environmental, genetic and physiological factors. Also, the nuclear receptor pathways such as PXR and CAR regulate the expression and functional activity of CYP3A4[55].

HIV Protease Inhibitors

AIDS (Acquired immune deficiency syndrome) is a serious pandemic disease of the human immune system caused by human immunodeficiency virus (HIV). An estimated 33.4 million people are living with HIV by the year 2009 and more than 25 million people have died of AIDS since 1981. HIV, a lentivirus is a member of retroviral family that primarily infects helper T cells, macrophages and dendritic cells[56]. This infection lowers the $CD4^+T$ cell count and thereby weakens the immune system leading to life threatening opportunistic infections. Several antiretroviral agents have been used either as a single agent or in combination for the treatment of HIV infections[57]. HIV infects T cells that carry CD4 antigen on their surface. After the fusion of virus to host cellular membrane, the virus enters the cell and its RNA gets reverse transcribed to DNA by the enzyme reverse transcriptase. The Viral DNA then integrates itself into host cell DNA by the enzyme integrase. HIV protease cleaves the translated viral polyproteins into individual mature proteins. The virions formed by the assembly of viral RNA and proteins are then released to infect another healthy cell[58]. Treatment with HIV protease inhibitors significantly lowered the plasma viral RNA levels and the virus replication. Studies have shown that metabolism of HIV protease inhibitors is mediated by CYP3A enzymes. Since HIV protease inhibitors are proven to be substrates and inhibitors of efflux proteins and metabolizing enzymes, drugs of abuse taken simultaneously can lead to enhanced plasma concentrations resulting in toxic effects. On contrary, chronic intake

of drugs of abuse lead to HIV treatment failure due to decline in their plasma concentrations.
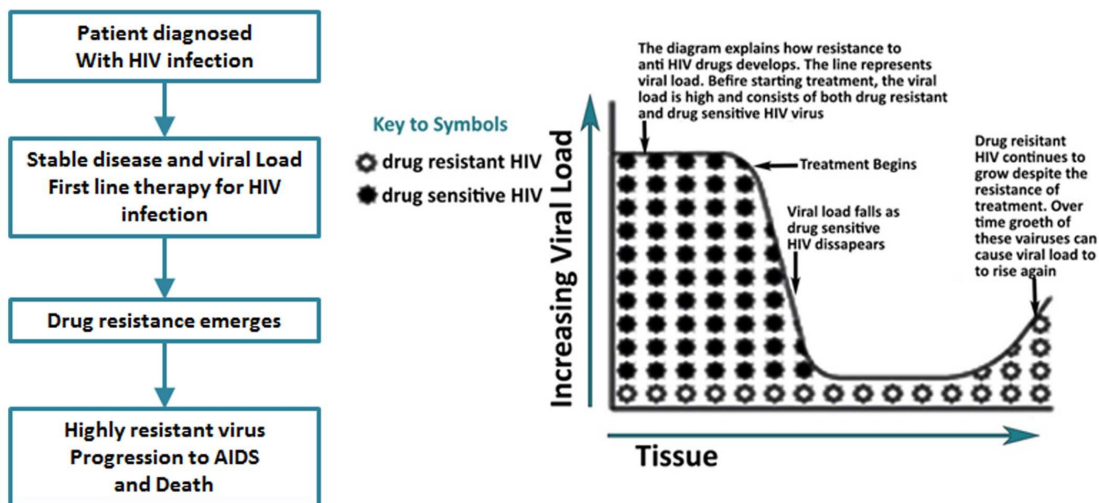
Figure 2.7   HIV virus resistance

HIV protease inhibitors inhibit the cleavage of gag-pol protein substrate leading to the release of structurally defective and functionally inactive virus particles (Figure- 2.7). They are also active against HIV-1 and HIV-2 viral strains[59]. HIV protease inhibitors have low oral bioavailability mainly due to limited absorption and first pass metabolism by CYP3A4 metabolizing enzymes. The main aim of therapy is to maintain plasma concentrations of these drugs above their minimum effective dose so that they can target the viral sanctuary sites and halt the replication. Several factors influence the bioavailability of these drugs[60]. Membrane efflux proteins, influx transporters, metabolizing enzymes and plasma proteins alter the absorption and disposition of these drugs. Ketoconazole has been shown to increase the AUC and C_{max} of saquinavir by 3

fold and bioavailability by 67% respectively[61]. Drugs such as astemizole, terfenadine and cisapride are contraindicated to be given concomitantly because of the toxic effects associated with enhanced blood saquinavir concentrations caused by CYP inhibition[62]. On the other hand, rifampicin, a known CYP3A4 inducer reduces plasma concentrations of saquinavir by 80% and therefore it is contraindicated with saquinavir[63]. A 70 fold decrease in viral production in cells overexpressing p-gp and a 50 fold increase in cells overexpressing MRP1 compared with control is observed[64]. Nuclear receptors such as PXR, RXR and CAR play an important role in the induction of efflux proteins and metabolizing enzymes[65].

Drugs of Abuse

When drugs are taken in an inconsistent manner for a non-therapeutic effect, it is considered as drug abuse. Drug abuse has been termed as the primary preventable health problem in United States[66]. Drug abuse is a chronic relapsing disorder accounting for several deaths and cost up to $400 billion every year[67]. Intake of drugs of abuse changes the mental or physical functioning of a person and lead to addiction[68]. Drug abuse lead to two different conditions: tolerance and dependence. In case of tolerance, a person takes drugs in larger doses upon time to produce the same effect. Dependence refers to physical or psychological dependence of a person to a specific drug. Whenever the intake of abused drug is stopped, it leads to development of withdrawal symptoms such as tremors and vomiting (physical dependence) and depression (psychological dependence). Addictive drugs show short term symptoms such as euphoria, drowsiness, increased

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motor and speech activity. Long term addiction lead to physical deterioration, psychiatric problems, accidents and intellectual problems[69]. Narcotic analgesics, stimulants, depressants, hallucinogens, cannabis and volatile solvents are some of the frequently abused drugs. According to National Institute of Drug Abuse (NIDA), morphine is classified under narcotic analgesics whereas nicotine is classified under stimulants. Figure-2.8 shows the structure of morphine and nicotine, two of the widely abused drugs.

Morphine

Morphine, a narcotic drug is a phenanthrene alkaloid obtained from opium poppy. Morphine elicits its pharmacological actions by acting on opioid μ-receptor[70]. Morphine is a scheduled II narcotic drug that possess an addiction forming ability[71]. Morphine can be injected, swallowed and smoked. Morphine is also used for pain relief used in different forms like tablets, capsules and injections. Morphine is metabolized primarily through glucuronidation pathway by conjugating with uridine diphosphate glucuronosyl transferase enzymes. Morphine is metabolized to morphine-6-glucuronide, which is a potent analgesic[72].

Figure 2.8   Structure of nicotine and morphine

Nicotine

According to national institute of drug abuse research report, tobacco use kills at least half a million Americans each year[73]. Nicotine is the main component present in tobacco that is responsible for addiction. Nicotine produces its effects by binding to acetylcholine receptors. Stimulation of the acetylcholine receptors triggers the release of dopamine responsible for the stimulant and dependence properties of nicotine[74]. When tobacco is smoked, it enters the blood stream through the lungs. But, when it is chewed or sniffed, it crosses the mucosal membranes and enters the blood stream. Long term addiction of nicotine leads to chronic lung disease, cardiovascular disease, stroke and cancer. With the enhanced knowledge at cellular and molecular level, several possible mechanisms of drug dependence have been elucidated. Interaction of these drugs at the receptor level and the intracellular pathways associated with these drugs has been studied[75].

<div align="center">

Importance of Drug-Drug Interactions

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The primary goal of antiretroviral therapy is to maintain suppressive levels of HIV protease inhibitors against the development of virus resistance. Drug levels are continuously monitored throughout the dosing period of HIV protease inhibitors to avoid viral replication and emergence of mutant viruses in sanctuary sites. For example, use of anti-tuberculosis drug rifampin decrease the serum concentrations of nevirapine[76] by 20-55%. Serum concentrations of indinavir, lopinavir and atazanavir were decreased by >90% when administered with ritonavir boosted dose in presence of rifampin. Therefore,

dosage adjustments were recommended necessitating high dose ritonavir boost as well as other HIV protease inhibitors[77]. As a result, hepatotoxicity was observed in HIV patients complicating the therapy. In view of these above situations, close therapeutic monitoring was recommended. Previous studies have shown that patients taking multiple drug combinations have their C_{min} or C_{trough} plasma concentrations below their IC_{50} during their dosing interval. Bioavailability of saquinavir was significantly increased during the coadministration of ritonavir. This is mainly due to the limited absorption and higher intestinal and hepatic metabolism mediated by CYP3A enzymes[78]. Knockout mice studies suggested that p-glycoprotein mediated efflux play a significant role on the oral bioavailability of saquinavir[79]. Due to the overlapping and broad substrate specificity of p-glycoprotein and CYP3A4, drug-drug interactions involve both the enzyme and transport systems. Both CYP3A4 and p-gp are present at higher concentration in the villus enterocytes of small intestine, the primary site of absorption of orally administered drugs. (Figure-2.9)

Models for Studying Induction

Enzyme induction refers to the up-regulation in the rate of enzyme synthesis from basal to a maximal level following the exposure to the drug. Enzyme induction is associated with the decrease in drug efficacy and in other cases, if the enzyme metabolite is toxic, enzyme induction results in cytotoxicity[80].
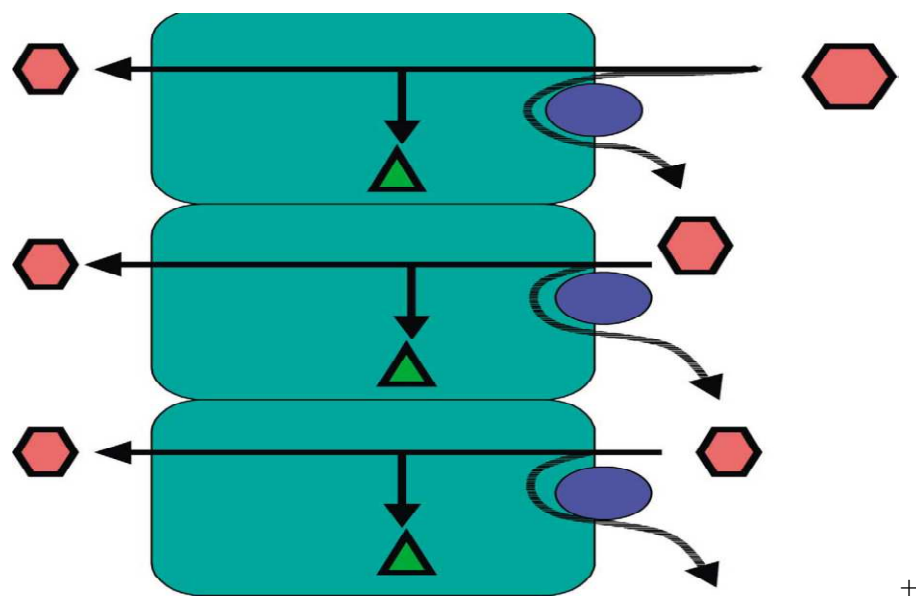
Figure 2.9 Synergistic effects of p-glycoprotein and CYP3A4

In vitro cell based models such as liver microsomes, primary hepatocytes, cryopreserved hepatocytes, reporter cell lines, recombinant cell lines, immortalized cells, precision cut liver slices have been developed to study human drug metabolism and induction of drug metabolizing enzymes in the liver[81]. Freshly isolated human hepatocytes are the primary *in vitro* model cell based systems used for studying drug metabolism. However, their utilization has been limited in industrial setting, due to unavailability. For this reason, cryopreserved human hepatocytes are most commonly employed to study the enzyme induction potential of new chemical entities. However, higher cost of these systems is the main disadvantage. For these studies, hepatocytes are cultured on multiwell plates and incubated with the drug of choice for the 72 hours. After the exposure, dose response activity and mRNA induction will be measured by adding a CYP specific probe. Along with these studies, immunoreactive protein and cell toxicity assays were also performed

to assess the transcriptional effect of the drug of choice on the hepatocytes. Subsequently, *in vivo* metabolic clearance of the drug can be predicted from the *in vitro* data[82].

Nuclear Receptors

Most common mechanism of CYP induction is transcriptional gene activation. Transcription factors such as PXR, CAR, RXR and AhR mediate the activation of drug metabolizing enzymes[83]. In general, nuclear receptors are associated with co-receptor complexes resulting in basal transcriptional levels. When a ligand enters the cytoplasm, it binds to the ligand binding domain of the nuclear receptor leading to conformational changes. Released co-receptors dimerize with other nuclear receptor partners resulting in chromatin modeling and subsequent transcriptional activation[84](figure-2.10).
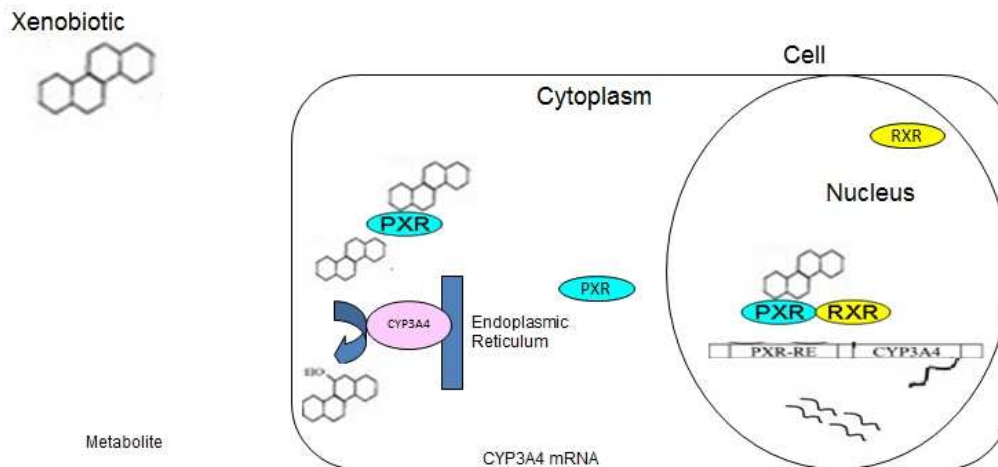
Figure 2.10 PXR mediated CYP3A4 metabolism

The induction potential of drugs of abuse on HIV protease inhibitors was investigated by conducting *in vitro* experiments in LS180 cells, Caco-2 and HepG-2 cells. The results of *in vitro* studies elucidate the mechanism of drug-drug interactions. Morphine and morphine-6-glucuronide were proven to be substrates for p-glycoprotein. There are no reports on induction mechanism pathways of nicotine and morphine on the inhibition and induction of MDR1 and CYP3A4. Nicotine is a substrate for CYP2D6 and the effect on cellular permeability of HIV protease inhibitors[85].

Regulation of Gene Expression

Efflux transporters mRNA can be induced by several factors such as heat shock, UV radiation, and chemotherapeutic agents. Despite the increase in mRNA levels, there is no increase in protein expression suggesting the translational control of gene expression[86]. Prolonged cellular exposure to cytotoxic agents has been reported to induce MDR1 gene expression through gene amplification as well as increased mRNA stability. Studies investigating the relationship between mRNA, protein levels and functional activity of efflux transporters indicated contrasting reports. Taipalensuu et al used digoxin as a specific marker for MDR1 dependent drug efflux in Caco-2 cells and indicated that MDR1 mRNA transcript upregulation correlates with increase in MDR1 protein expression as well as functional activity[87].

In Vitro Cell Models

In vitro cell models have led the way to design more efficient experiments to screen and to study the mechanisms of new chemical entities. Many pharmaceutical companies employ immortalized cell lines to study human drug metabolism and active transport of drugs. These *in vitro* cell lines have been able to predict efflux transporter and enzyme inhibition and induction to avoid potential clinical drug-drug interactions[88]. However, low and variable expression of efflux transporters and metabolizing enzymes pose an important challenge in the selection of in vitro model. Caco-2 and HepG2 cell lines have been employed to study intestinal drug absorption and hepatic drug absorption respectively[89]. Caco-2 cells were shown to express efflux transporters such as p-gp, MRP2 and BCRP. HepG2 cell lines express various liver specific metabolizing enzymes and transporters and are the most studied human hepatoma cell line[90]. Even though HepG2 is well characterized, this cell line shows undetectable and variable expression and activity of CYP[91]. Inducing agents such as 3-methylcholanthrene and rifampicin has been used to express higher levels of CYP[92]. LS-180, a microvillus expressing human colon carcinoma cell line is an excellent model cell line to study induction of CYP450 and efflux transporters[92]. Gupta et al has shown that LS-180 cell has been shown to enhance PXR mediated CYP3A4 expression when compared to HepG2[93](figure-2.11). Cell culture models of respiratory epithelium examine the mechanisms of drug delivery across alveolar and bronchial epithelium. Calu-3 cell line, derived from human bronchial

epithelial cell line has been extensively studied for drug delivery due to its ability to form

tight monolayers[94].
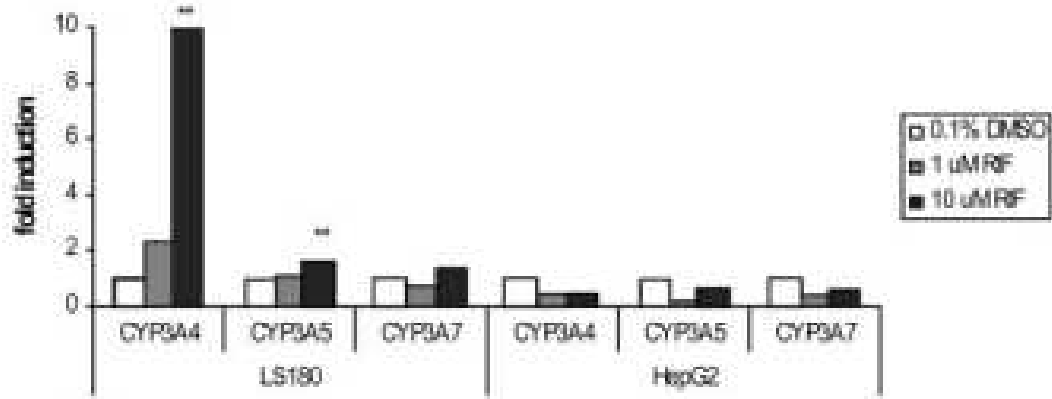
Figure 2.11 Induction of CYP450 metabolizing enzymes mRNA expression in LS180 cells

Calu-3 cells express p-glycoprotein and other metabolizing enzymes such as CYP1A1

and CYP2B6. Other respiratory cell lines such as A549, an alveolar epithelial cell line

lack the ability to generate high TEER in culture[93].

TO INVESTIGATE THE CHRONIC EFFECT OF NICOTINE AND MORPHINE ON EXPRESSION AND FUNCTIONAL ACTIVITY OF EFFLUX TRANSPORTERS AND METABOLIZING ENZYMES AND TO STUDY THEIR CONTRIBUTION TO THE INTRACELLULAR ACCUMULATION OF HIV PROTEASE INHIBITORS

Rationale

Development of multidrug resistance is a major obstacle to the treatment of life threatening diseases such as AIDS and cancer. One important mechanism of multidrug resistance involves the upregulation of multidrug resistance transporter, p-gp, that efflux the therapeutic drugs and decline their intracellular accumulation. Cigarette smoking alters the therapeutic response of respiratory drugs through several mechanisms. Induction of metabolic enzymes and efflux transporters is one of the mechanisms associated with poor response to chemotherapy. Smoking has been shown to induce CYP3A4 in in vitro studies. Hamilton et al reported that erlotinib, a drug primarily metabolized by CYP3A4 has a 2.8 fold lower systemic exposure in smokers than nonsmokers. Opiates are hallucinogens that are abused by many adults and teenagers around the world. Morphine is one of the most potent alkaloids of opium and is one of the main reasons for the extremely addictive nature of opiates. Morphine is used to treat moderate to severe pain and is a substrate of p-glycoprotein. Chronic exposure has been shown to enhance p-glycoprotein expression in rat brain. This upregulation of p-gp enhanced morphine efflux from the rat brain. Brain p-gp expression increased by 2 fold

in morphine treated rats when compared to saline treated rats. HIV protease inhibitors are generally administered for prolonged time periods to eliminate the viral sanctuaries in brain, lungs and lymph glands. Since nicotine and morphine has been abused for longer time periods, there is a need to evaluate the long term effect of nicotine and morphine on the induction of the efflux transporters and CYP3A4 proteins and functional activity.

Introduction

Drug-drug interactions involving efflux transporters and drug metabolizing enzymes are divided in two main categories: inhibition resulting in enhanced toxicity and induction resulting in loss of efficacy. P-glycoprotein, product of human MDR1 gene, transports numerous neutral and cationic compounds and effluxes them out of the cell using ATP[94]. P-glycoprotein is expressed in normal tissues such as gastrointestinal track, liver, kidney, testis and brain, where it prevents the accumulation of toxic substances (Figure-3.1). P-gp is overexpressed in cancer cells and confers resistance to a variety of compounds such as vinca alkaloids, anthracyclines, colchicines and paclitaxel[95]. CYP3A4 is involved in the metabolism of diverse chemotherapeutic compounds and accounts for approximately 30% of hepatic CYP and more than 70% of intestinal activity. CYP3A4 is the main contributor to presystemic elimination following oral administration. Previous *in vitro* studies suggests that 40-50% of the drugs administered in humans are metabolized by CYP3A4[96].

Both p-gp and CYP3A4 act synergistically to increase presystemic metabolism. P-glycoprotein mediated efflux increases the intestinal residence time and efflux of the metabolites of the drug results in more extensive metabolism by CYP3A4. In addition, overlapping specificity of the two proteins, similarities in gene regulation and tissue distribution of p-gp and CYP3A4 and the close spatial proximity of CYP3A4 to the apical membrane where p-gp is expressed supports the synergistic relationship between CYP3A4 and p-glycoprotein[97]. CYP3A4 mediated metabolism and p-gp mediated efflux are the two main barriers to drug absorption at intestine. Due to the overlapping and broad substrate specificity of p-glycoprotein and CYP3A4, drug-drug interactions involve both the enzyme and transport systems. Both CYP3A4 and p-gp are present at higher concentration in the villus enterocytes of small intestine, the primary site of absorption of orally administered drugs[98].

Many clinically significant drug-drug interactions have been reported involving their role. Inhibition of p-gp and CYP3A4 can cause an elevation in plasma/tissue drug concentrations can lead to toxicity, especially for drugs possessing narrow therapeutic index. Induction of p-gp and CYP3A4 can lead to subtherapeutic concentrations of the affected drug leading to therapeutic failure[99]. Many drugs such as Rifampicin, phenobarbital, carbamazepine are known to cause induction of CYP450 isoforms resulting in decrease of systemic exposure of coadministered drugs metabolized by CYP450. Herbal agents such as St. John's wort enhance hepatic and intestinal CYP3A4 activity through the activation of pregnane-x-receptor[100]. PXR is a member of nuclear

receptor superfamily of ligand regulated transcription factors. PXR is a key xenobiotic receptor that regulates the expression of genes encoding drug metabolizing enzymes and transporters. Studies have demonstrated that PXR is widely expressed in cancerous tissues as well as normal tissues such as liver and small intestine. PXR mediated induction of efflux transporters leading to decrease in the intracellular accumulation of p-gp substrates[101]. Enhanced p-gp expression has been associated with poor treatment response in various malignancies[102].
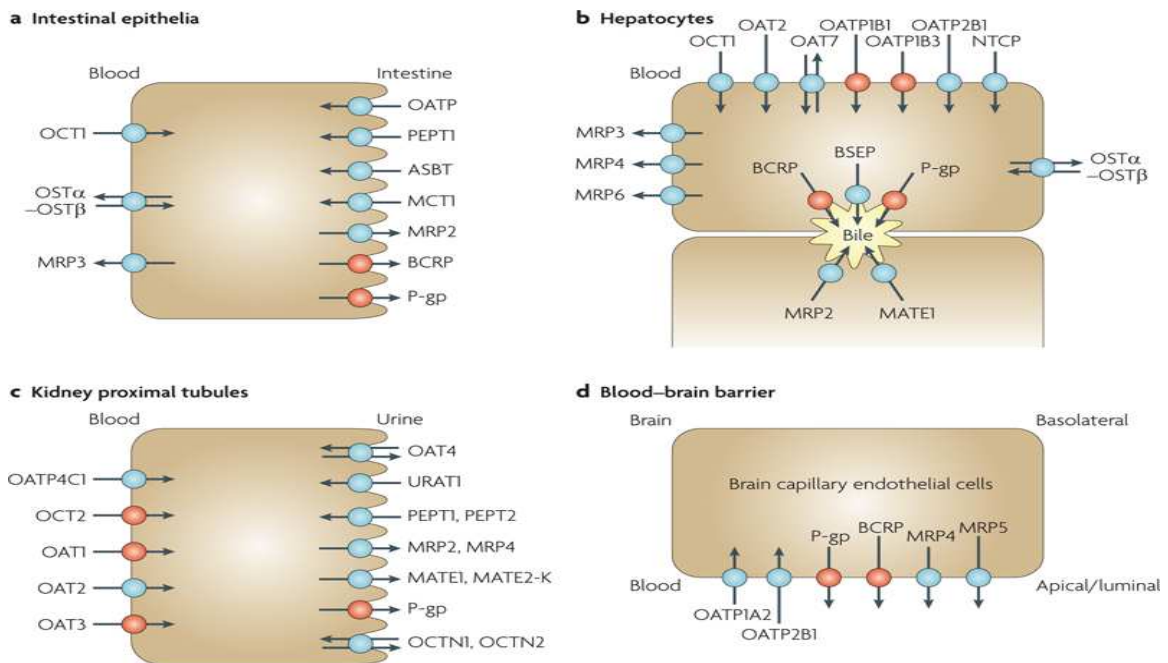
Figure 3.1 Efflux transporters localization across various tissues

Morphine is the opioid of choice for the treatment of moderate to severe pain, and is a substrate of p-glycoprotein. Morphine is one of the most potent alkaloids of opium and is one of the main reasons for the extremely addictive nature of opiates. After heroin,

morphine has the greatest dependence liability of the narcotic analgesics in common use and for heroin to be effective, it must be converted in the body to morphine[103]. Previous studies have demonstrated that brain p-gp levels were increased significantly in morphine tolerant rats. However, short term exposure of morphine did not result in any significant differences in p-gp expression and activity[104]. Nicotine is a major constituent of tobacco and it has been recently demonstrated that nicotine is an activator of PXR which regulates the gene expression of both p-gp and CYP3A4[105].

HIV protease inhibitors are known to have low oral bioavailability predominantly due to extensive hepatic metabolism as well as intestinal metabolism. Clinically important drug interaction has been proved between HIV protease inhibitors and herbal drugs such as St. John's wort and grape fruit juice[106]. Study of mechanism of drug-drug interactions between morphine and nicotine and HIV protease inhibitors can improve therapeutic strategies and individualization of therapy in drugs abusing HIV population. Studies using *in vitro* model cell lines are useful in identifying potential interactions and possibly permitting extrapolation of *in vitro* findings to *in vivo* scenarios. Opioid drugs are generally converted to morphine. Morphine is commonly used to study the effects in vitro and in vivo. Several drugs of abuse like morphine, nicotine, cocaine, alcohol are now identified as critical factors affecting drug disposition and drug interactions. Cigarette smoking is the greatest risk factor for lung cancer and cigarette smoking exposes the airways to at least 4000 chemicals[107]. Tobacco smoke may cause significant pharmacokinetic or pharmacodynamic interactions and plasma concentration–time

profiles[108]. According to American cancer society, cigarette smoking accounts for at least 30% of all cancer deaths. Nicotine is a poisonous alkaloid found in substantial amounts in all forms of tobacco and is one of the heavily used addictive drug in US[109]. Nicotine is a weak base with a pKa of 8.0 with most of its absorption occurring in unionized state. After absorption, Nicotine distributes rapidly throughout various tissues, including brain, liver, kidney and intestine. About 1-2 mg of nicotine is absorbed systemically during smoking[110].

In view of this, there is a need to study the long term effects of these drugs. Furthermore, their role in the induction of other efflux transporters such as BCRP has to be elucidated. Our current study explores the long term effects of morphine and nicotine by utilizing model cell lines LS180. Selective induction of *in vitro* CYP3A4 and p-gp expression by morphine and nicotine was observed in LS180 cells. Our results demonstrate that morphine and nicotine differentially can induce p-gp and CYP3A4. The results can offer mechanistic explanation in chronic nicotine and morphine users.

Materials and Methods

Cell Culture and Treatment Conditions

LS180 cells (human colon adenocarcinoma cell line) and Caco-2 cells (human colonic carcinoma cell line), HepG-2 were used for the studies. Cell lines were maintained according to previously published protocols from our lab. Cells were cultured in Dubelcco's Modified Eagle Medium supplemented with 10% heat inactivated fetal

bovine serum, MEM non-essential amino acids, HEPES, sodium bicarbonate, penicillin (100 µg/ml) and streptomycin (100 µg/ml). For the treatment, confluent cells were exposed to nicotine (2.5 µM, 10 µM), morphine (3 µM and 10 µM) and rifampicin (25 µM) for 72 hours. Fresh medium containing the above mentioned concentrations of nicotine, morphine and rifampicin were added to the culture medium every day for the length of the treatment time. Final concentration of DMSO did not exceed 0.5% for rifampicin treated cells. Cells were treated with selected concentrations of nicotine and morphine and experiments were designed as shown in figure-3.2.
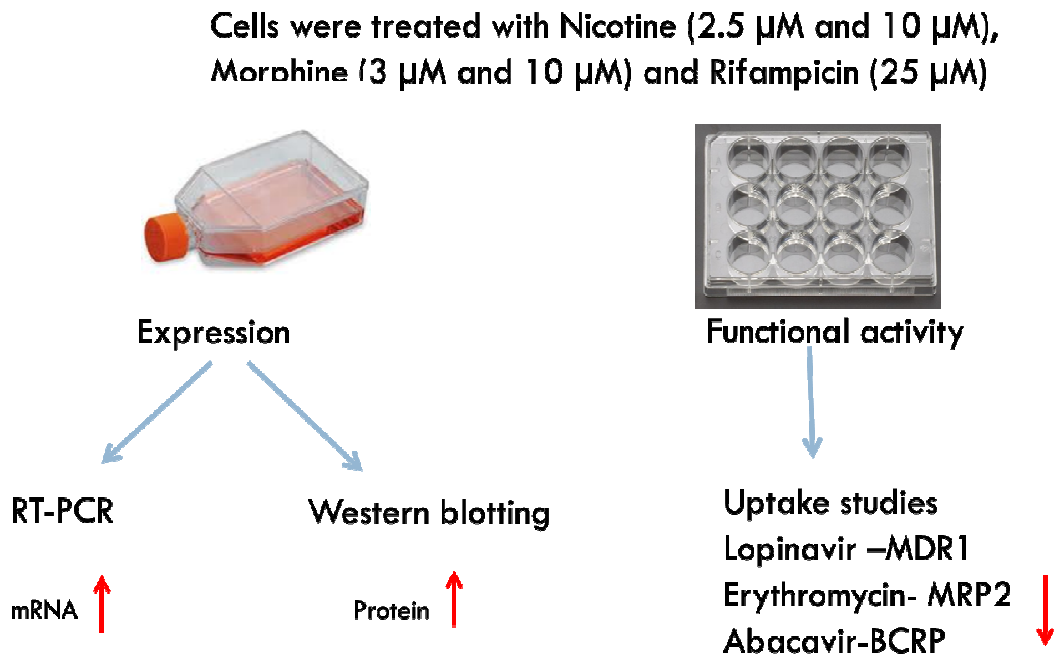
Figure 3.2 Experimental design to study the expression and functional activity of efflux transporters

Real Time PCR Studies

   RNA was extracted from the control and nicotine treated lung tissues using Trizol reagent (Invitrogen). All samples were normalized to 1 µg of total RNA. 1 µg of total RNA was mixed with 1.25 µl oligo dT primer at 70ºc for 10 minutes and then reverse transcribed to cDNA using MMLV-reverse transcriptase enzyme. Real time PCR was according to a standard protocol published by Roche. Real time PCR primers were designed using oligo perfect designer. All the primers were designed such that the amplicons generated were between 100-200 bp long to increase the efficiency of simultaneous amplification of target and reference genes. Briefly, the PCR mixture has a volume of 20 µl. SYBR green kit from Roche has 2X concentration of PCR master mix and PCR grade water. To this master mix, 250 nM each of forward and reverse primers of gene of interest were added. 5 µg/µl cDNA sample was added to this master mix to prepare 20 µl of PCR sample. The samples were then carefully centrifuged at 3000 g for 2 minutes. Samples were analyzed and fluorescence was quantified.

Table-2   RT-PCR primers for MDR1 and CYP3A4

Gene		Oligonucleotide sequence (5´-3´)	Location	Product Size (bps)
MDR1	Forward	CAGAGGGGATGGTCAGTGTT	1758	109
	Reverse	CGTGGTGGCAAACAATACAG	1867	
CYP3A4	Forward	ACCGTGACCCAAAGTACTGG	1309	118
	Reverse	GTTTCTGGGTCCACTTCCAA	1427	
GAPDH	Forward	CAATGACCCCTTCATTGACC	201	105
	Reverse	GACAAGCTTCCCGTTCTCAG	306	

Western Blot

Whole cell protein was extracted with reagent containing 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 1% Triton X - 100 and protease inhibitor cocktail at pH 7.4. Confluent cells were washed thrice with PBS and harvested using a cell scraper in 5 mL of PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes and the pellet was resuspended in freshly prepared lysis buffer for 15 minutes on ice. The extracted protein was then obtained by centrifugation and stored at -80°C. Protein content was determined using Bradford method. Polyacrylamide gel electrophoresis (PAGE) was run with 25 and 50 µg of each protein at 120 V, 250 mAmp. Transfer was carried out on polyvinylidene fluoride (PVDF) membrane at 25 V for 1 h 30 min, on ice. Immediately after transfer, the blot was blocked for 3 hours in freshly prepared blocking buffer (2.5 % non-fat dry milk and 0.25 % bovine serum albumin prepared in TBST pH-8). After a light wash for 10 sec, the blots were exposed to primary antibodies overnight (dilution - 1:500 for p-glycoprotein and CYP3A4). Blots were then exposed for 2 hours to secondary antibodies obtained from Santa Cruz biotechnology (1: 5000). The blots were finally washed three times with TBST and developed using SuperSignal West Pico chemiluminescence substrate. The blots were exposed for 30 sec after which the image was taken in Gel Doc Imager.

Uptake Studies

    After treatment with morphine and nicotine, cells were rinsed three times with DPBS (pH 7.4, 129 mM NaCl, 2.5mM KCl, 7.4mM Na_2HP0_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$ and 5.3 mM glucose) and equilibrated for 15 minutes with the buffer. [^{3}H]lopinavir (0.5 µCi/ml), [^{3}H]abacavir (0.5 µCi/ml) and [^{14}C]erythromycin (0.1 µCi/ml) were used as model substrates for MDR1, BCRP and MRP2 to determine the cellular accumulation. Uptake studies were performed by incubating the cells with fixed amount of 0.5 µCi/ml of the radioactive substrate for 30 minutes. Following incubation, the reaction was stopped by addition of ice cold stop solution (210 mM KCl, 2 mM HEPES; pH 7.4). After three washings with stop solution, cells were lysed by keeping them overnight in 1 ml of 0.1% Triton-X solution in 0.3% NaOH. Following overnight incubation, 500 µl of the cell lysate from each well was transferred to scintillation vials containing 5 ml of scintillation cocktail (Fisher Scientific, Fair lawn, NJ). Samples were analyzed by liquid scintillation counter (Beckman instruments Inc., CA, USA) and uptake was normalized to the protein content in each well. Amount of protein in the cell lysate was measured by the Bio-Rad protein estimation kit with bovine serum albumin as standard.

VIVID Assay

    Functional activity of metabolizing enzyme (CYP3A4) in HepG2 cells was assayed using VIVID™ CYP3A4 Red Substrate (Invitrogen), which was prepared as a 20mM stock solution in acetonitrile. Briefly, HepG2 cells were plated in 96 wells and

treated as described earlier. After 5 days of treatment, the cells were lysed and CYP3A4 activity assay was assayed according to the manufacturer's protocol. The rate of fluorescent metabolite production from the metabolism of a Vivid® CYP450 substrate was monitored kinetically at 37°C over a period of 30 minutes using a 96 well plate reader (SpectraFluor Plus, Maennedorf, Switzerland) at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. The readings were normalized to protein count as mentioned earlier.

Calcein-AM Assay

Culture medium was first removed from the 96 well plates and washed with DPBS buffer at pH-7.4. Cells were treated with morphine, nicotine and rifampicin for 7 days. Following treatment, intracellular accumulation of calcein-AM was determined for the remaining studies. Cells were incubated with varying calcein-AM 1 µM for 15 minutes. After 15 min incubation, reaction was arrested with stop solution (210 mM KCl and 2 mM HEPES; pH 7.4) and lysed with 1 ml of lysis buffer (0.1% Triton-X solution in 0.3% NaOH). Amount of intracellular calcein was measured with a fluorescence spectrophotometer. Excitation and emission filters were set at 370 and 450nm and the intracellular mean fluorescence was normalized to protein content.

Cytotoxicity Studies

LS180 cells and Caco-2 cells were exposed to nicotine, morphine for seven days. Following the treatment, cells were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide] reagent according to the previously published protocol.

Data Analysis

Data for the uptake studies was analyzed by student's t-test. The criteria of significance between the means (± standard deviation) were $p < 0.05$. Each experiment was performed in triplicate.

Results and Discussion

Expression of efflux transporters and metabolizing enzymes play a significant role in modulation of chemotherapeutic drugs across various cellular barriers. Inhibition and induction of these transporters have been reported to be one of the major cause of variability in intracellular drug accumulation. An altered expression of p-glycoprotein and CYP3A4 can have important clinical outcomes[111]. Inhibition can result in enhanced plasma concentrations leading to toxicity whereas induction can result in reduced plasma concentrations leading to therapeutic failure[112]. While most of the studies have investigated the *in vitro* p-glycoprotein and CYP3A4 inhibition studies, fewer studies have focused on their induction mechanisms. In this study, the effect of chronic exposure of morphine and nicotine on the expression and activity of p-glycoprotein, MRP2, BCRP and CYP3A4 *in vitro* was investigated. LS180, a human colon adenocarcinoma cell line was employed as a model cell line for *in vitro* studies. Previously, it was shown that

prolonged exposure to morphine can enhance the expression of p-glycoprotein in rat brain. Induction of p-glycoprotein led to reduced accumulation of morphine to rat brain[113]. However, the ability of morphine to induce MRP2 and BCRP was not investigated. Nicotine's effects on p-gp and CYP3A4 are also of clinical importance because it is a known activator of PXR. Eventhough, nicotine has been shown to induce CYP2D6, its effects on CYP3A4 and other efflux transporters MRP2, BCRP were never studied.

Quantification of MDR1 mRNA Expression in LS180 Cells
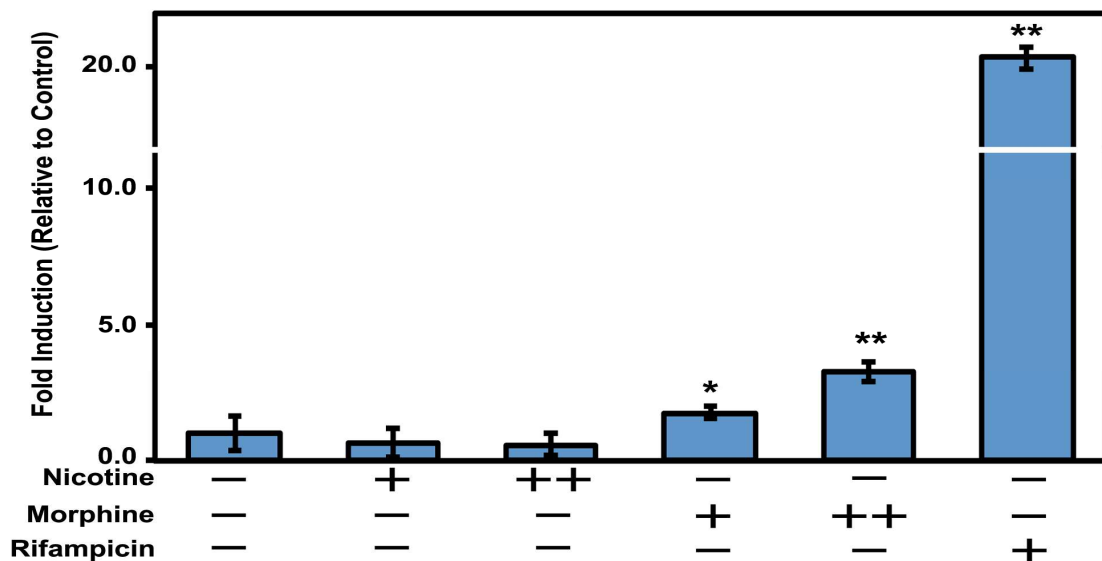
Figure 3.3 Quantification of MDR1 mRNA in LS180 cells (+ indicates 2.5 µM and ++ 10 µM for nicotine; + indicates 3 µM and ++ 10 µM for morphine; + indicates 25 µM for rifampicin)   (* indicates significant difference compared to control; $p<0.05, n = 3 \pm S.D$)

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Expression of MDR1 was detected after treating LS180 and Caco-2 cells for 72 hours and 10 days respectively. For functional uptake studies, LS180 and Caco-2 cells were treated for 7 and 15 days respectively. Following treatment, RNA was extracted and subjected to reverse transcriptase and gene expression was analyzed by real time PCR. Differential induction in MDR1 mRNA levels was observed following chronic morphine and nicotine.

Induction of MDR1 mRNA in Caco-2 Cells
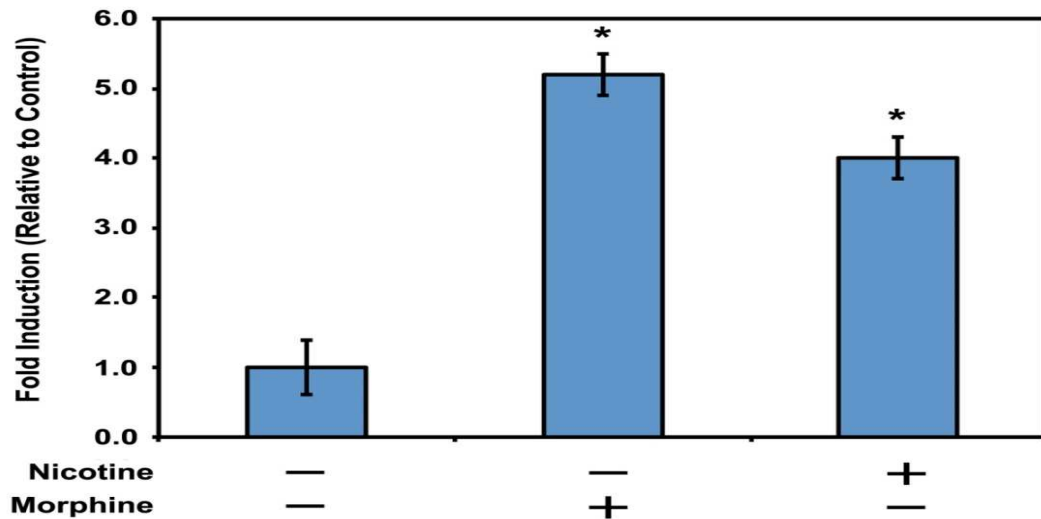
Figure 3.4 Quantification of MDR1 mRNA in Caco-2 cells (+ indicates 2.5 μM and ++ 10 μM for nicotine; + indicates 3 μM and ++ 10 μM for morphine; + indicates 25 μM for rifampicin)   (* indicates significant difference compared to control; $p < 0.05, n = 3 \pm$ S.D)

Chronic treatment with nicotine for 72 hours did not alter MDR1 mRNA levels compared to control. Morphine (3 µM and 10 µM) enhanced MDR1 mRNA by 1.8 and 3.2 fold respectively whereas rifampicin (25 µM), a positive inducer enhanced the MDR1 mRNA by 20.4 fold. GAPDH was used as a house keeping gene. Figure-3.3 shows the quantification of MDR1 mRNA following treatment. Since Caco-2 cells were treated for 10 days for gene expression studies, RT-PCR was also performed in Caco-2 cells to quantify MDR1 mRNA levels. Following treatment, morphine (3 µM) enhanced MDR1 mRNA levels by 5.12 fold and nicotine (2.5 µM) increased MDR1 mRNA levels significantly by 3.95 fold respectively (figure-3.4).

Western Blot Analysis of P-gp in Caco-2 Cells

Western blot analysis indicated significant p-gp protein induction following treatment with morphine and nicotine. Morphine and nicotine successfully induced p-gp protein levels in Caco-2 cells (figure-3.5).
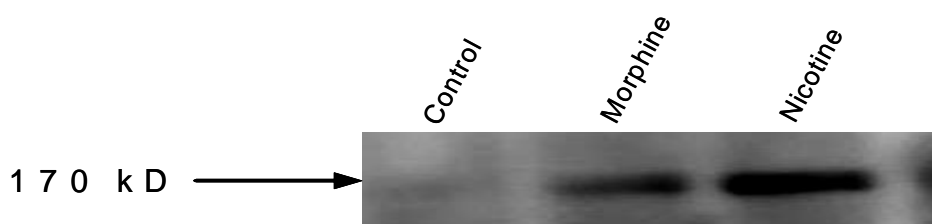
Figure 3.5   Immunoblot for the expression of CYP3A4 in Caco-2 cells. Total protein lysates probed for the efflux transporter P-gp. Growth medium (control: lane 1) or 3 µM morphine (lane 2), 2.5 µM nicotine (lane 3). Each lane contains 20 µg of protein lysate.

Uptake Study to Determine P-gp Functional Activity

To determine the functional activity of the efflux transporters, uptake studies were performed to measure the intracellular accumulation. LS180 and Caco-2 cells were exposed to morphine and nicotine for seven days and 15 days respectively for uptake studies. Following treatment, cells were incubated with radioactive labeled substrates [^{3}H] lopinavir, [^{3}H] digoxin for 30 minutes.
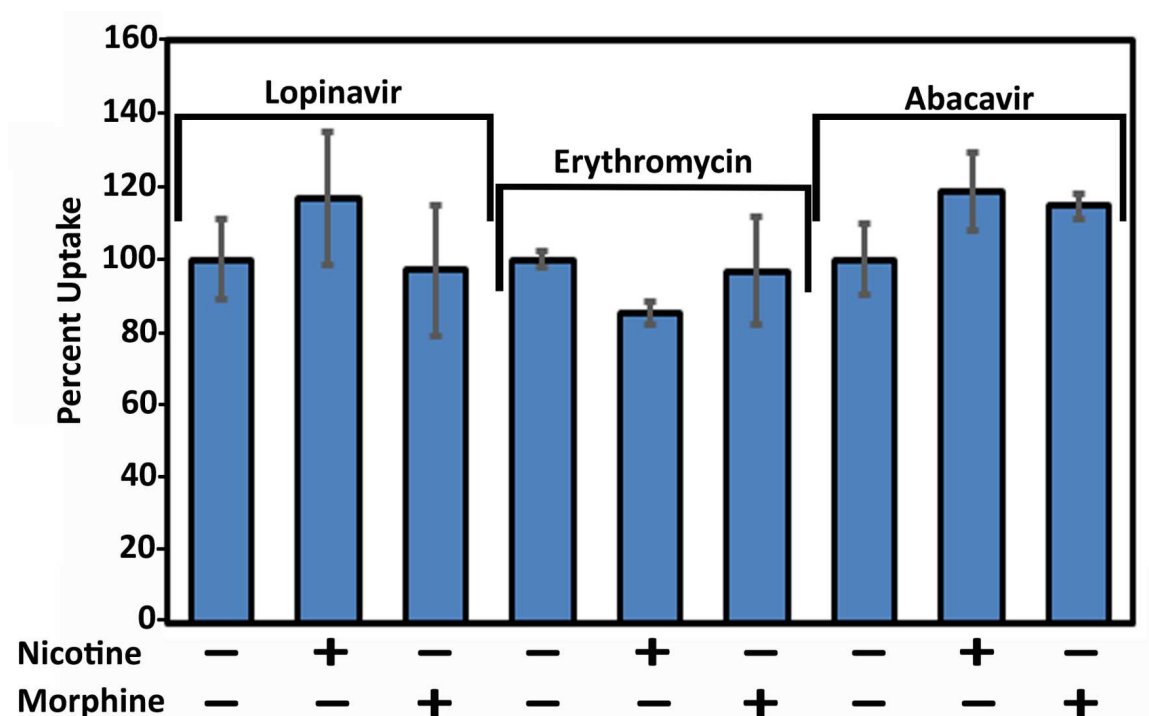
Figure 3.6 Uptake of radioactive substrates in LS180 cells Intracellular accumulation of [^{3}H] lopinavir, [^{14}C] erythromycin and [^{3}H] abacavir was measured by incubating the LS180 cells for 30 min in the absence (control) or presence of morphine(3 µM) and nicotine (2.5 µM). Values are means of quadruplicates with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P<$ 0.05) difference from the mean control value using Student's one-tailed t-test.

After 30 minutes, intracellular accumulation of radioactive substrates was decreased significantly when compared to control. Short term incubation of these radioactive substrates (lopinavir, erythromycin and abacavir) with morphine and nicotine had no effect on their accumulation of radioactive substrates in LS180 cells (figure-3.6). This data indicated that the reduction in intracellular accumulation was due to the altered induction of efflux transporters rather than inhibition.

Digoxin and Lopinavir Uptake in LS180 Cells
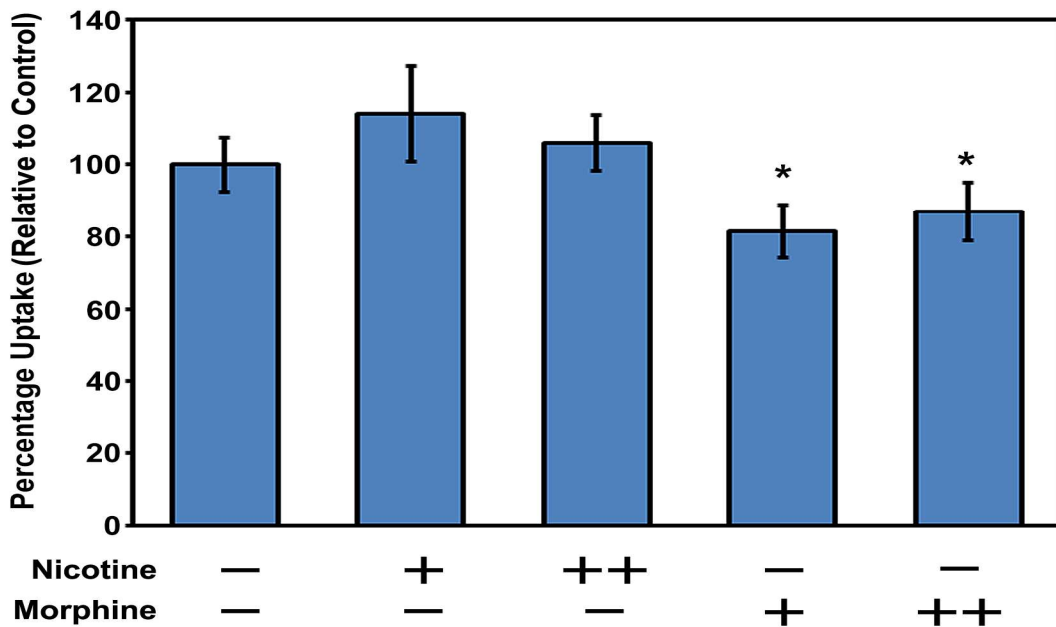
Figure 3.7 Digoxin uptake in LS180 cells Intracellular accumulation of [^{3}H] digoxin was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with morphine (3 μM) and nicotine (2.5 μM) for seven days. (+ indicates 2.5 μM and ++ 10 μM for nicotine; + indicates 3 μM and ++ 10 μM for morphine) Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant (*P*< 0.05) difference from the mean control value using Student's one-tailed *t*-test.
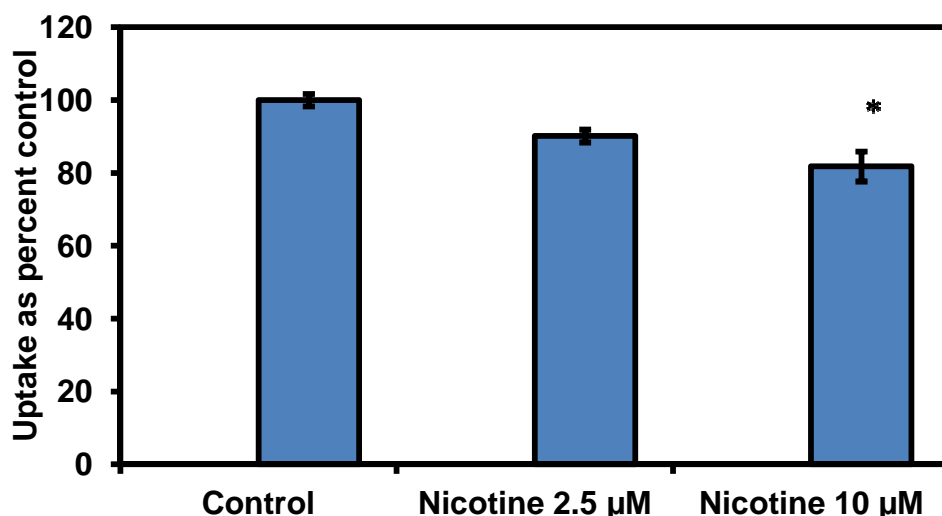
Figure 3.8 Lopinavir uptake following nicotine treatment in LS180 cells
Intracellular accumulation of [^{3}H] lopinavir was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with nicotine (2.5 μM) and nicotine (10 μM) for seven days. Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant (*P* < 0.05) difference from the mean control value using Student's one-tailed *t*-test.
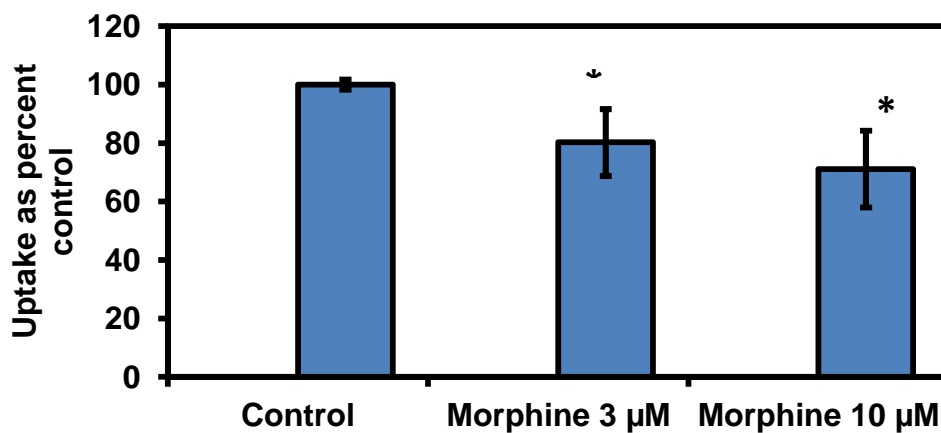
Figure 3.9 Lopinavir uptake following morphine treatment in LS180 cells
Intracellular accumulation of [^{3}H] lopinavir was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with morphine (3 μM) and morphine (10 μM) for seven days.

As shown in figure (3.7, 3.8, 3.9), morphine reduced the intracellular accumulation of [^{3}H]lopinavir and [^{3}H]Digoxin significantly suggesting the induction of p-gp functional activity. However, nicotine had no significant effect on intracellular accumulation of p-gp substrates when compared to control.

Uptake Studies in Caco-2 Cells

In another set of studies, Caco-2 cells exposed to morphine and nicotine also showed a significant reduction in intracellular accumulation of [^{3}H] lopinavir and [^{3}H] saquinavir (figure-3.10 and 3.11). A known potent inducer of efflux transporters, rifampicin 25 µM was used as a positive control to study its effects on the uptake of radioactive substrates.
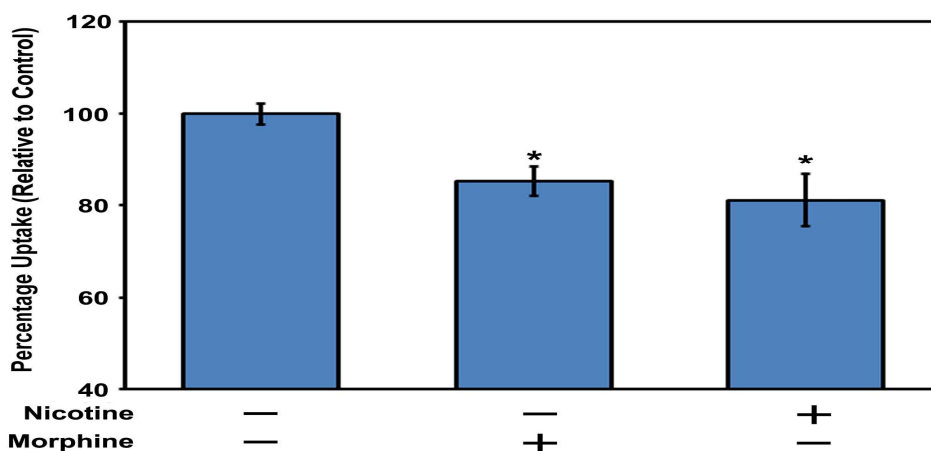
Figure 3.10   Lopinavir uptake following morphine and nicotine treatment in Caco-2 cells Intracellular accumulation of [^{3}H] lopinavir was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with nicotine (2.5 µM) and morphine (3 µM) for 15 days. Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P < 0.05$) difference from the mean control value using Student's one-tailed t-test.

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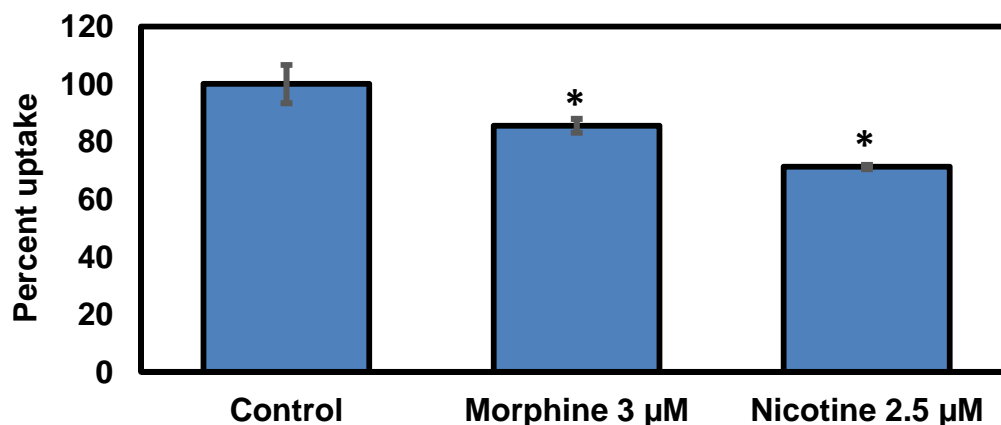
Figure 3.11 Saquinavir uptake following morphine and nicotine treatment in Caco-2 cells Intracellular accumulation of [³H] saquinavir was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with morphine (3 μM) and nicotine (2.5 μM) and for 15 days.

These results confirmed that the observed decline in the uptake of radioactive substrates following chronic morphine and nicotine treatment was due to the altered expression of p-glycoprotein. Since Caco-2 cells were exposed to longer time than LS180 cells, expression and functional activity mediated by MDR1 was much higher than in Caco-2 cells when compared to LS180 cells.

Calcein-AM Assay in LS180 Cells

To determine the functional significance of this increase in P-gp expression, the cellular uptake of P-gp substrate calcein-AM was evaluated at the selected concentration ranges. A significant decrease in calcein accumulation by LS180 cells was observed in the cells exposed to morphine and nicotine confirming the reduced functional activity of P-gp. Figure 3.12 demonstrated the decrease in P-gp activity following LS180 cells

treatment with morphine and nicotine. This data clearly established that exposure to

morphine and nicotine contributes to increased efflux activity.
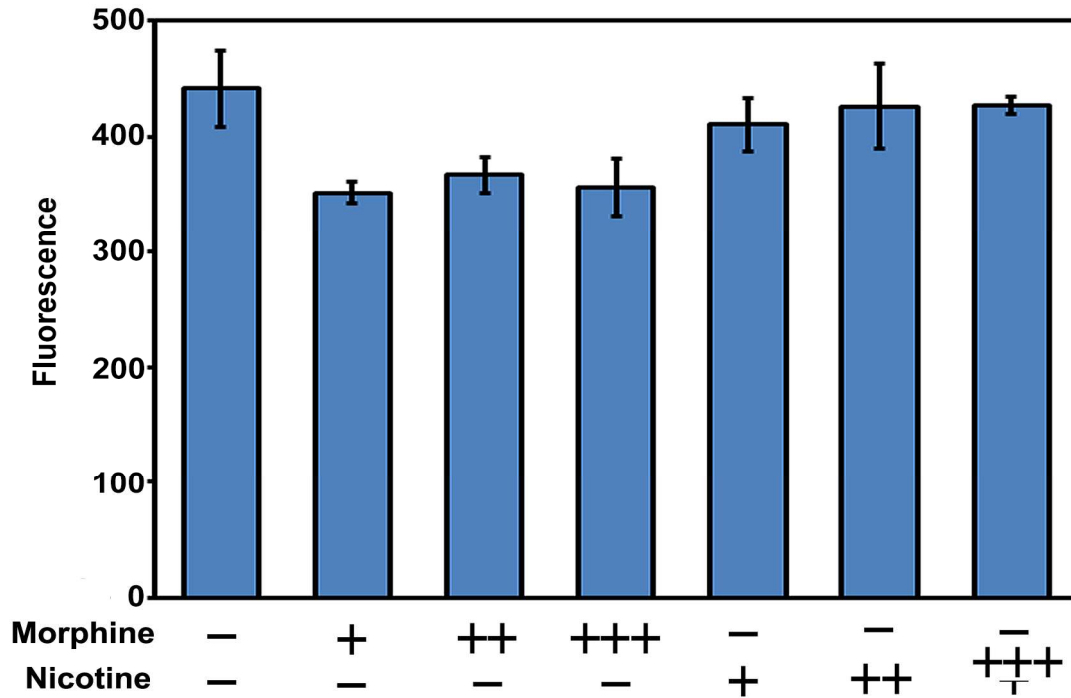
Figure 3.12: Calcein-AM assay study in LS180 cells Intracellular accumulation of calcein was measured by incubating LS180 cells with calcein-AM for 60 min without treatment (control) or treatment with morphine (1 μM, 3 μM and 10 μM) and nicotine (1 μM , 2.5 μM and 10 μM) for seven days. Values are means of 6 samples with standard deviation indicated by error bars.

CYP3A4 Expression and Functional Activity

We also evaluated the effect of morphine and nicotine on CYP3A4 mRNA expression in LS180 cells. Morphine and nicotine had no significant effect on CYP3A4 mRNA levels in LS180 cells when compared to control (figure-3.13). This may be due to lower basal CYP3A4 levels in LS180 cells. Also, 72 hour treatment duration with morphine and nicotine was not significant enough to induce CYP3A4 mRNA levels in LS180 cells.
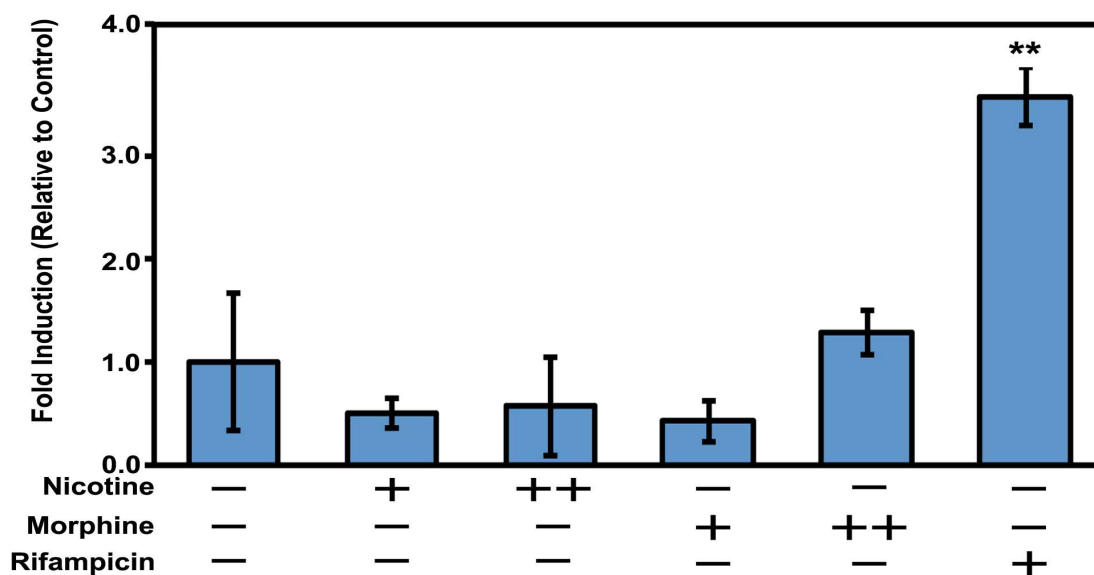
Figure 3.13   Quantification of CYP3A4 mRNA in LS180 cells (+ indicates 2.5 μM and ++ 10 μM for nicotine; + indicates 3 μM and ++ 10 μM for morphine; + indicates 25 μM for rifampicin)   (* indicates significant difference compared to control; (** indicates significant difference compared to control; ** $p<0.01$, $n = 3 \pm$ S.D)

CYP3A4 Induction Studies

Morphine and nicotine significantly enhanced CYP3A4 protein expression when compared to control (figure-3.14). Morphine and nicotine displayed a higher increase in CYP3A4 protein levels than CYP3A4 mRNA transcript levels.
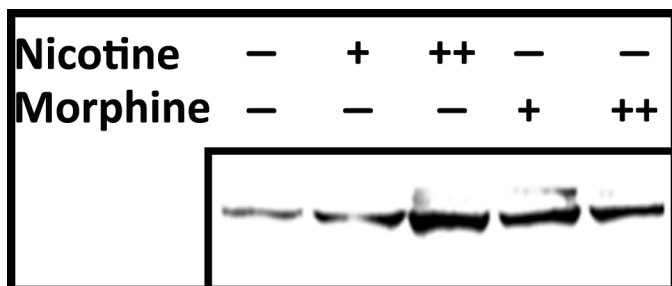
Figure 3.14 Quantification of CYP3A4 protein in LS180 cells. Total protein lysates probed for CYP3A4 Growth medium (control: lane 1), 2.5 μM nicotine, N1 (lane 2) 10 μM nicotine, N2 (lane 3) 3 μM morphine, M1 (lane 4), 10 μM morphine, M2 (lane 3). Each lane contains 20 μg of protein lysate.

Vivid CYP3A4 Assay in HepG2 Cells

After studying the gene expression, CYP3A4 enzyme activity was measured by performing VIVID CYP3A4 assay kit containing blue fluorescent substrate following treatment of HepG2 cells with morphine and nicotine. Following these treatments, CYP3A4 activity was measured as the rate of fluorescent metabolite production over the course of reaction with VIVID assay. Induction was calculated as the activity observed after treatment with inducer relative to treatment with 0.1 % DMSO (control).
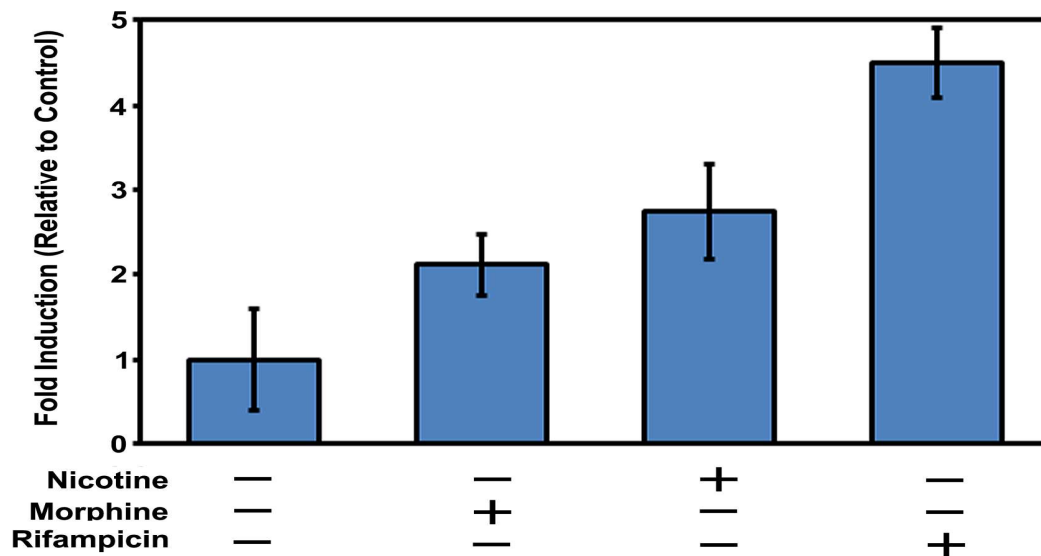
Figure 3.15   Vivid CYP3A4 assay in HepG2 cells Enhanced activity of CYP3A4 HepG2 in the presence of morphine and nicotine. HepG2 cells were treated with morphine (3 μM), nicotine (2.5 μM) and rifampicin (50 μM). Asterisk * indicates significant difference relative to control; $p<0.05$, $n = 8 \pm$ S.D.

Figure 3.15 clearly demonstrates the higher CYP3A4 activity in HepG2 cells exposed to morphine and nicotine. Nicotine produced almost three fold higher CYP3A4 activity when compared to control. This study confirmed the ability of morphine and nicotine to induce CYP3A4 protein levels.

Cell Viability Studies

Cell viability studies were performed to determine the cytotoxic potential of morphine and nicotine in LS180 and Caco-2 cells. Cells were treated with morphine (3 μM, 10 μM), nicotine (2.5 μM, 10 μM), DMSO (0.1%), rifampicin (25 μM). LS180 cells

exposed to morphine and nicotine for 7 days did not show any significant cytotoxicity (figure-3.16 A).

A)
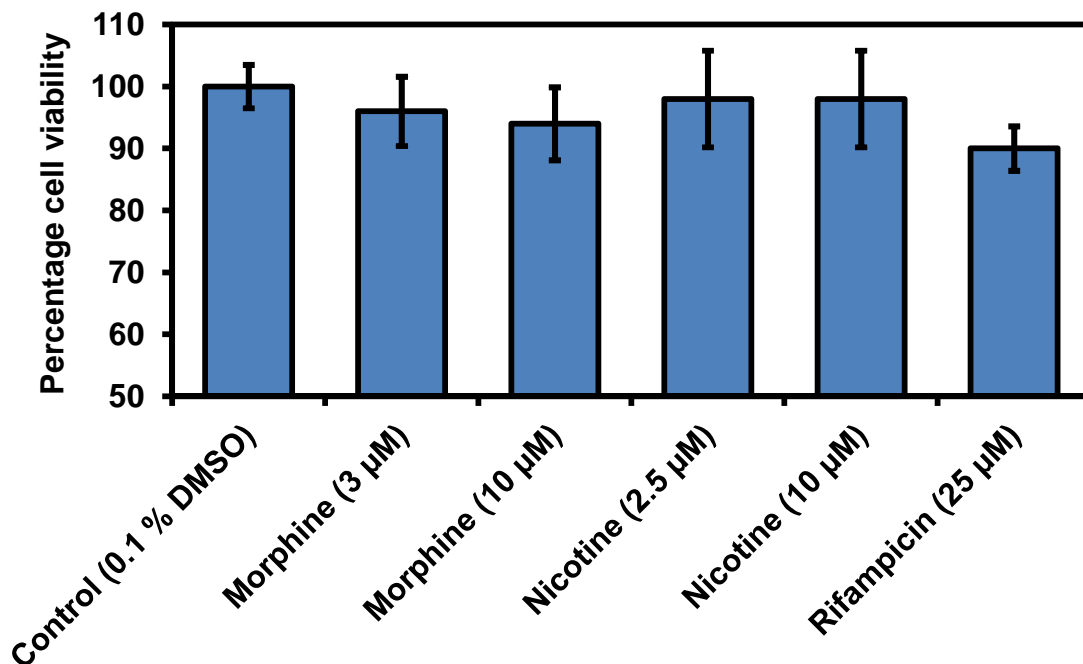
Figure 3.16   Cell viability studies A) Viability of LS180 cells following treatment with morphine (3, 10 μM), nicotine (2.5, 10 μM) and rifampicin (25 μM).

In a different study, treatment of Caco-2 cells with morphine (3 μM), and nicotine (2.5 μM), did not produce any significant toxicity (figure-3.16 B). However, rifampicin (25 μM) reduced the cell viability by 84% following treatment.
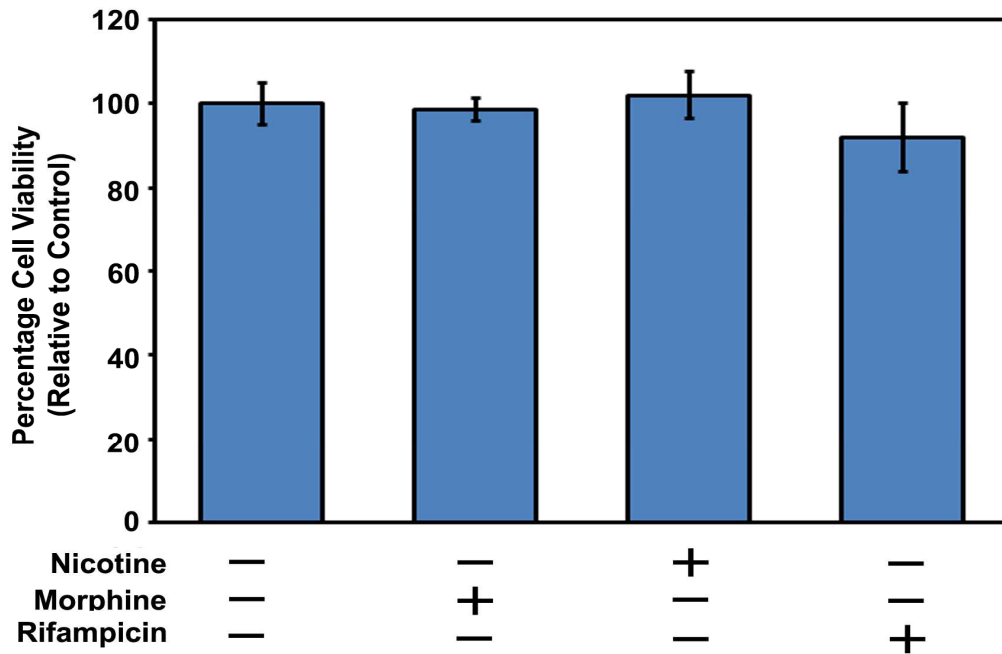
Figure 3.16 Cell viability studies B) Viability of Caco-2 cells following treatment with morphine (3 µM), nicotine (2.5 µM) and rifampicin (25 µM). (* indicates significant difference relative to control; p<0.05, n = 8 ± S.D)

Induction of MRP2 Expression in LS180 Cells

To determine the expression of MRP2 mRNA following treatment, RT-PCR was performed to quantify mRNA levels. Morphine (3 µM and 10 µM) increased MRP2 mRNA by 1.5 and 3.35 fold respectively (figure 3.17).
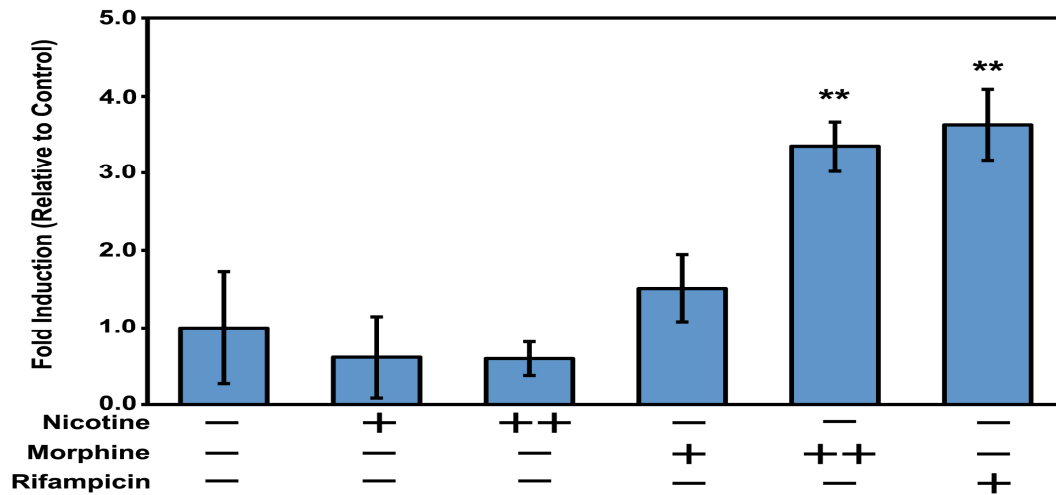
Figure 3.17 Quantification of MRP2 mRNA in LS180 cells (+ indicates 2.5 μM and ++ indicates 10 μM for nicotine; + indicate 3 μM and ++ indicates 10 μM for morphine; + indicates 25 μM for rifampicin) (* indicates significant difference compared to control; $p<0.05$, (** indicates significant difference compared to control $p<0.01$, $n = 3 \pm S.D$)

A)

B)
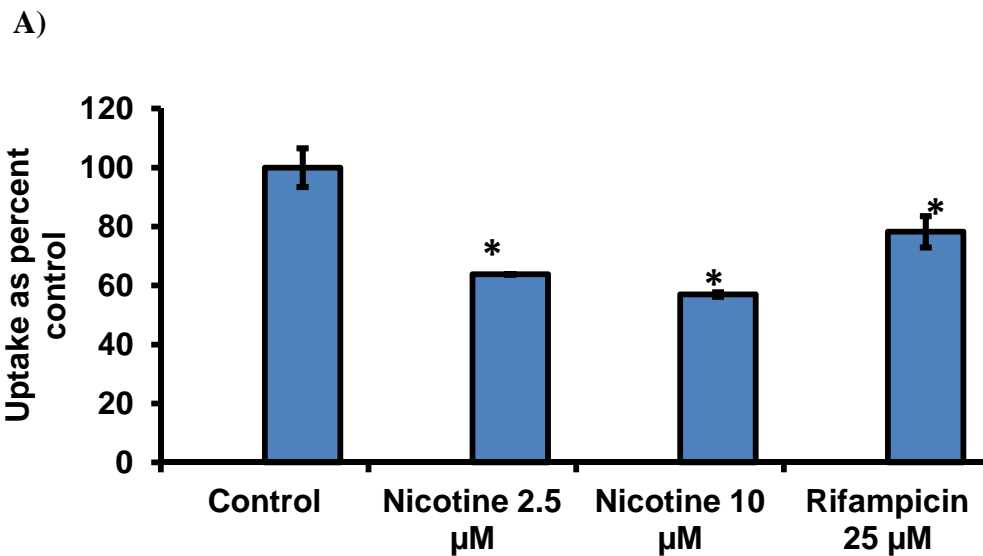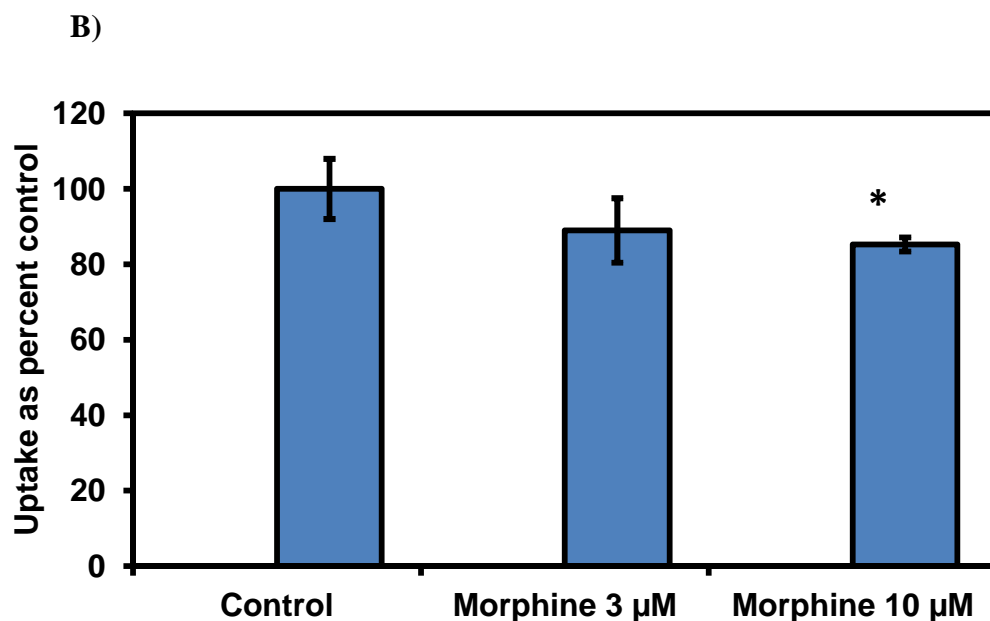
Figure 3.18: Quantification of [^{14}C] Erythromycin accumulation following nicotine and morphine treatment in LS180 cells. A) Intracellular accumulation of [^{14}C] Erythromycin was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with nicotine (2.5 µM and 10 µM) for seven days. B) treatment with morphine (3 µM and 10 µM) for seven days. Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P< 0.05$) difference from the mean control value using Student's one-tailed t-test.

Radioactive erythromycin was used as positive control to quantify MRP2 mediated efflux in LS180 cells. Exposure to morphine and nicotine for 72 hours reduced the intracellular accumulation of radioactive erythromycin when compared to control (figure-3.18 A, B).

ABCG2 mRNA Induction Studies in LS180 Cells

ABCG2 mRNA levels enhanced by 4 and 3.43 fold by nicotine (2.5 µM and 10 µM) respectively whereas morphine (3 µM and 10 µM) increased ABCG2 mRNA levels

by 4.33 and 4.88 fold respectively (figure-3.19). Interestingly, nicotine showed much

higher induction of ABCG2 mRNA than MDR1 and MRP2 expression in LS180 cells.
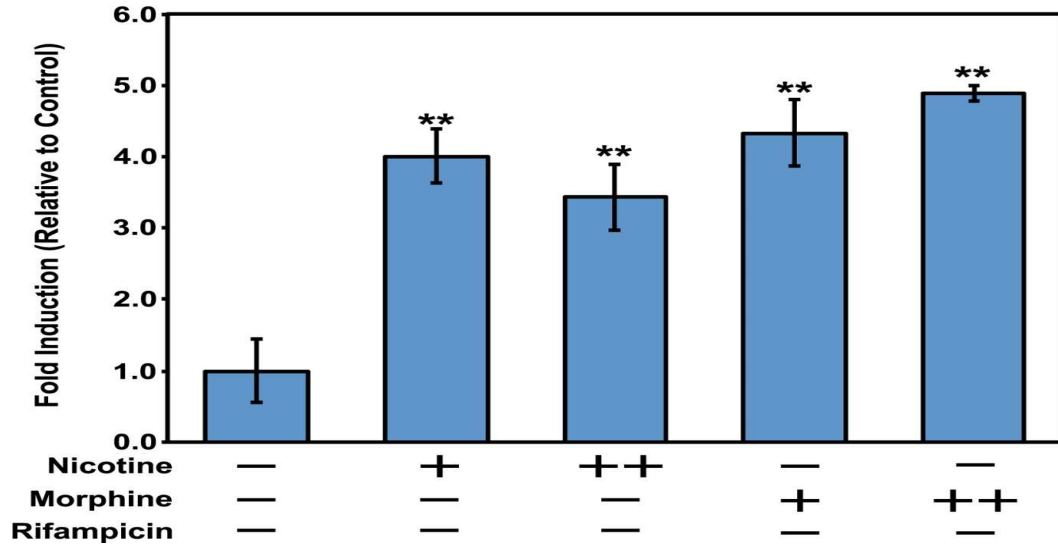
Figure 3.19 Quantification of ABCG2 mRNA in LS180 cells (+ indicates 2.5 μM and ++ indicates 10 μM for nicotine; + indicate 3 μM and ++ indicates 10 μM for morphine; + indicates 25 μM for rifampicin) (* indicates significant difference compared to control; $p<0.05$, (** indicates significant difference compared to control; $p<0.01$, $n = 3 \pm$ S.D)

BCRP Protein Induction in LS180 Cells

We investigated whether ABCG2 mRNA activation was translated into protein

induction in this study. Western blot analysis showed that compared to control, there was

an increase in BCRP protein expression following exposure to morphine (3 μM), nicotine

(2.5 μM) and rifampicin (25 μM) (figure-3.20).
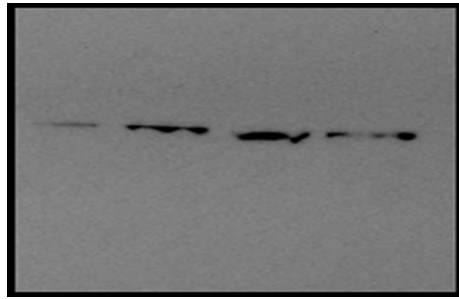
Figure 3.20 Immunoblot for the expression of BCRP in LS180 cells. Total protein lysates probed for the efflux transporter BCRP. Growth medium (control: lane 1), 3 µM morphine (lane 2), 2.5 µM nicotine (lane 3) and 25 µM Rifampicin. Each lane contains 20 µg of protein lysate.

Abacavir Uptake in LS180 cells Following Treatment

We investigated whether augmented BCRP efflux function was observed in LS180 cells exposed to nicotine and morphine. Uptake of [³H] abacavir was reduced significantly in LS180 cells exposed to nicotine and morphine when compared to control (figure-3.21 A, B).

A)
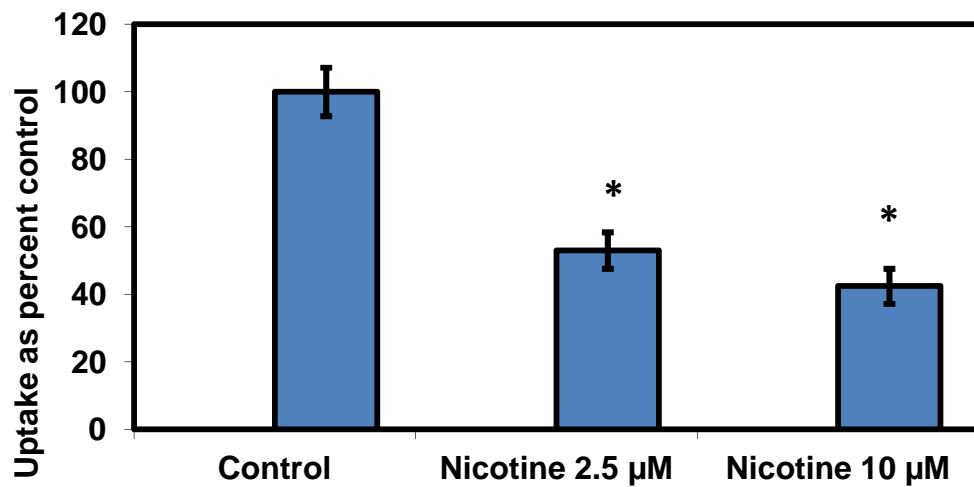
B)
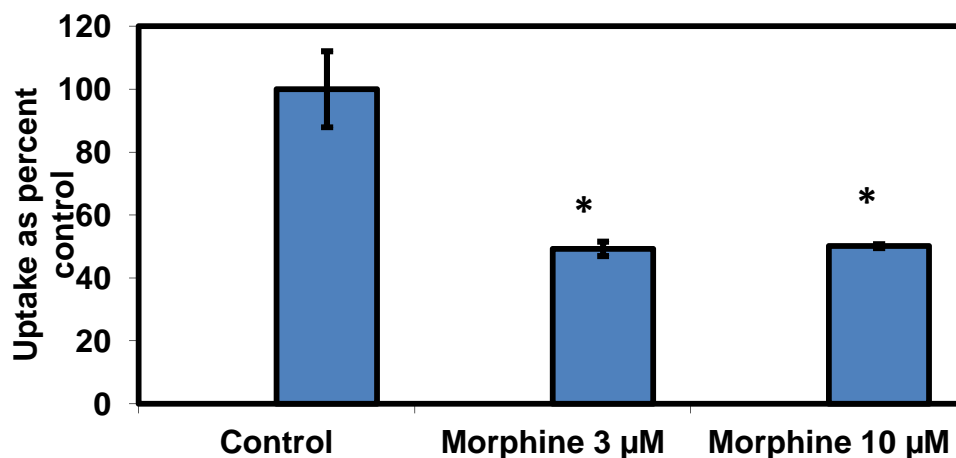
Figure 3.21: Quantification of [^{3}H] abacavir accumulation following nicotine and morphine treatment in LS180 cells. A) Intracellular accumulation of [^{3}H] abacavir was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with nicotine (2.5 µM and 10 µM) for seven days. B) treatment with morphine (3 µM and 10 µM) for seven days. Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant (*P*< 0.05) difference from the mean control value using Student's one-tailed *t*-test.

Transport Studies

Apical to basolateral transport studies were performed after treating LS180 cells with morphine and nicotine (10 µM). [^{3}H] lopinavir and [^{3}H] abacavir transport were used as model substrates. Results from transport studies also support the data from uptake studies. Transport of abacavir reduced significantly when compared to lopinavir following chronic exposure to morphine and nicotine (figure 3.22 and figure 3.23)
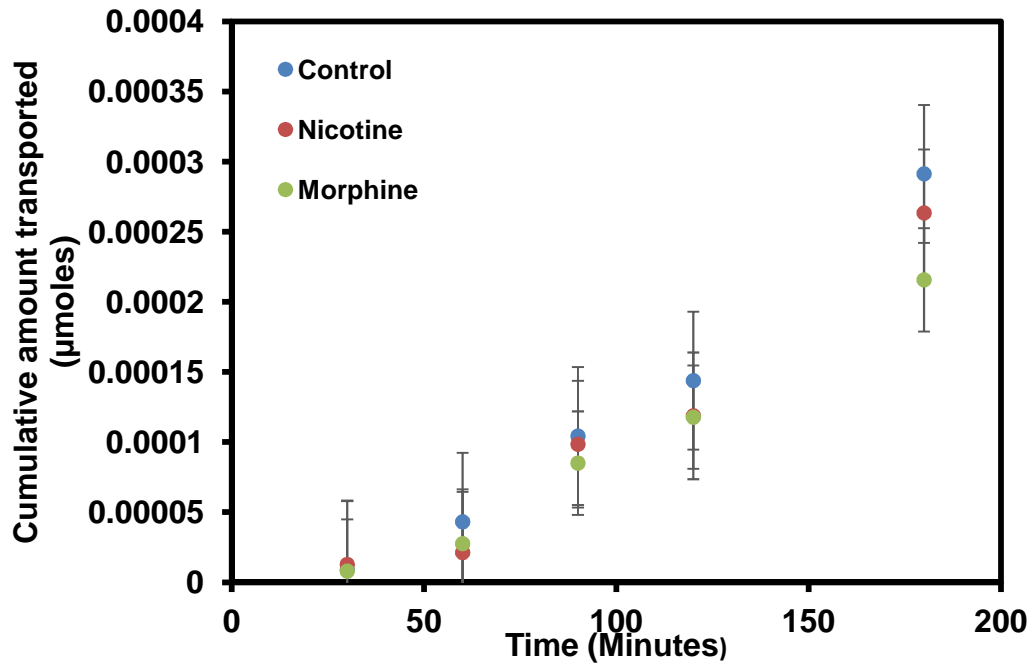
Figure 3.22: A-B transport of [^{3}H] lopinavir following nicotine and morphine treatment in LS180 cells.
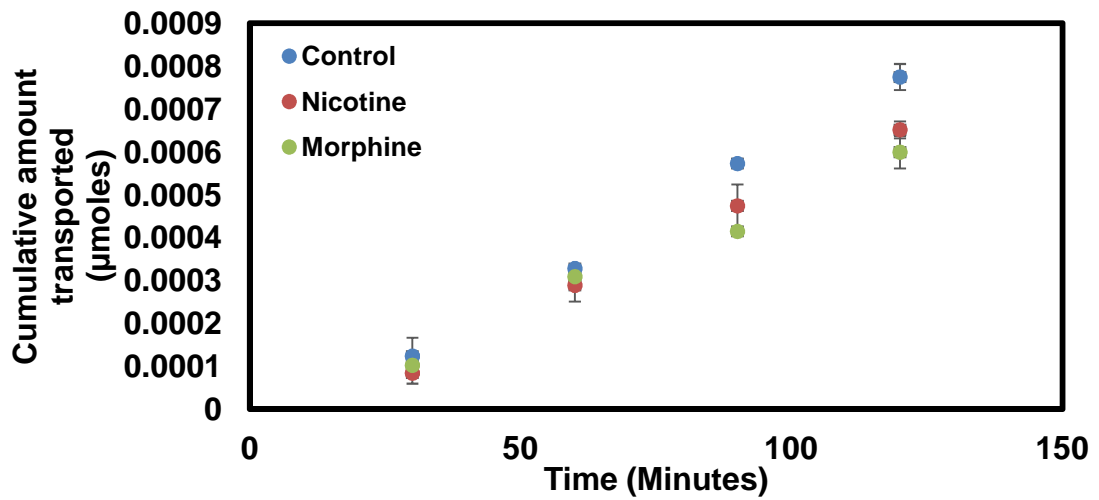
Figure 3.23: A-B transport of [^{3}H] abacavir following nicotine and morphine treatment in LS180 cells.

Potential Model to Study Induction Based Drug-Drug Interactions

LS180 cells are an intestinal colon carcinoma cells that express characteristics of small intestine. Therefore, this cell line was selected to study induction of efflux transporters and metabolizing enzymes. Although, LS180 cells constitutively express p-gp, PXR and CYP3A4, but in our studies morphine and nicotine had no significant induction on CYP3A4 mRNA levels. Recent studies reported that LS180 cells exposed to vinblastine (100 nM) displayed enhanced MDR1 and CYP3A4 expression and activity. Therefore, we treated LS180 cells with vinblastine and performed real time PCR to quantify MDR1 and CYP3A4 mRNA expression. Our studies demonstrated that nicotine and lopinavir enhanced MDR1 and CYP3A4 mRNA levels when compared to control (figure-3.22). This vinblastine selected LS180 cells can be a potential model to study MDR1 and CYP3A4 induction mechanisms.
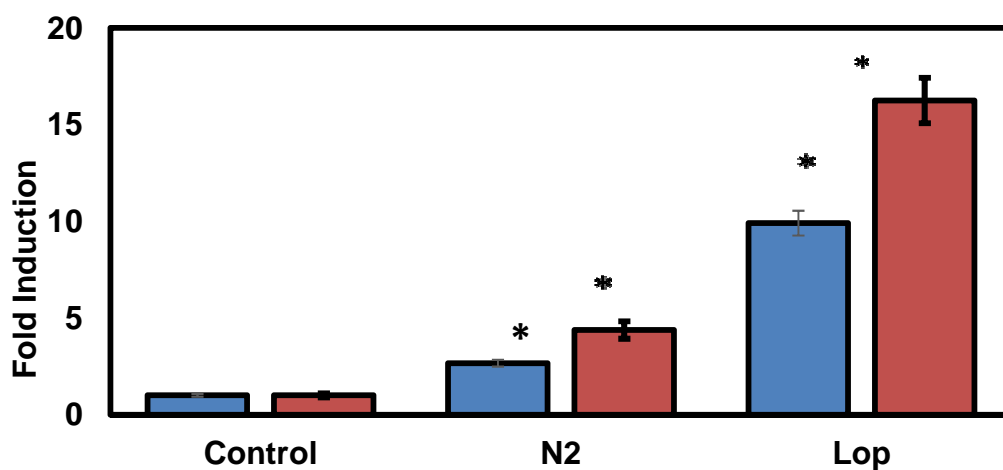
Figure 3.24 Quantification of MDR1 and CYP3A4 mRNA in LS180 cells (N2 indicates 10 μM for nicotine, 10 μM for lopinavir)   (* indicates significant difference compared to control; *p*<0.05, *n* = 3 ± S.D)

Conclusions

In summary, our results confirmed that chronic morphine and nicotine exposure of LS180 cells can differentially induce the expression and activity of P-gp, MRP2, BCRP and CYP3A4. Even though, the induction of expression and functional activity by morphine and nicotine was not high enough to produce this interaction in *in vitro* scenarios, the possibility of these drug-drug interaction could occur as it should be noted that these drugs are abused for years and the intracellular concentrations of HIV protease inhibitors can be declined drastically leading to therapeutic failure. These observations illustrate the *in vitro* mechanistic effect of drug-drug interactions between morphine and nicotine and HIV protease inhibitors. Clinical studies often fail to assess the mechanisms behind the therapeutic failure of HIV patients. These studies may explain the variable complex and plasma concentrations and treatment outcomes of HIV infected patients as well as HIV infected patients addicted to drugs of abuse. Morphine and nicotine effects on MRP2 and BCRP expression and functional activity are also of clinical significance as there is limited information available on their interactions. Previous studies have provided evidence that HIV protease inhibitors are substrates for p–glycoprotein and CYP3A4 and thereby co-administered drugs that are substrates, inhibitors and inducers modulate the expression of p-gp and CY3A4 resulting in the alteration of their intracellular accumulation. This reduced accumulation may potentially promote the emergence of mutant viruses at the sanctuary sites.

Table-3   Induction of efflux transporters by morphine and nicotine

Sample	MDR1	CYP3A4	MRP2	BCRP
Control	1 ± 1.1	1 ± 1.15	1 ± 1.26	1 ± 0.77
Nicotine 2.5 μM	0.68 ± 0.94	0.49 ± 0.24	0.61 ± 0.93	4.00 ± 0.67
Nicotine 10 μM	0.56 ± 0.71	0.56 ± 0.83	0.59 ± 0.39	3.43 ± 0.81
Morphine 3 μM	1.77 ± 0.43	0.41 ± 0.34	1.51 ± 0.76	4.33 ± 0.82
Morphine 10 μM	3.2 ± 0.59	1.28 ± 0.37	3.34 ± 0.57	4.87 ± 0.19

CHAPTER-4

INFLUX TRANSPORTERS IN LUNGS: EXPRESSION OF FOLIC ACID CARRIERS IN HUMAN BRONCHIAL EPITHELIAL CELL LINE, CALU-3

Rationale

Drugs can be delivered either via the pulmonary route by intratracheal instillation to treat systemic diseases or via inhalation of aerosolized drugs to treat local respiratory diseases[114]. Mechanisms of drug delivery across bronchial and alveolar epithelium have been investigated over the years, but little is known about the mechanism underlying the pathways regulating the transport of peptide and folate related compounds across pulmonary epithelium. Also, role of these transporters in the biopharmaceutics of the inhaled drugs is progressively being recognized. For example, several anti-infectious such as penicillin and cephalosporin antibiotics active against bacterial infections are substrates for PEPT1/PEPT2 transporters. Also, folate substrates such as methotrexate are substrates for folic acid transporters. Presence of these transporters across pulmonary epithelium can potentially affect the absorption and distribution of the drugs which are substrates for these influx transporters. Moreover, peptide and folate prodrugs can influence the kinetics of the pulmonary drugs. Therefore, the aim of this specific aim is to determine the expression and functional activity of folic acid receptor/transporter and peptide transporters in Calu-3, human bronchial epithelial cell line, Calu-3, *in vitro*.

Introduction

Folic acid transport is an important physiological process that maintains the folic acid homeostasis in the body. Folates are members of vitamin B9 family. Folate and folic acid are two forms of the same water soluble vitamin which occurs naturally in food such as leafy vegetables and fruits. Folic acid plays an essential role in cell growth and differentiation, red blood cell production, protein metabolism and several other biochemical processes[115]. Folates are essential cofactors for one carbon donor substrates involved in nucleotide and methionine synthesis[116]. Several therapeutic drugs such as methotrexate, 5-fluorouracil, raltitrexed, pemetrexed have been targeted to interfere with folate pathways and folate dependent enzymes for the treatment of cancerous as well as non-cancerous tissues[117]. Clinical studies have revealed that plasma levels of folate and genetic polymorphisms in folate dependent enzymes are often associated with increased risk of cancer, heart disease, peripheral vascular disease and dementia[118]. Previous studies also demonstrated the protective role of dietary folate on lung carcinogenesis[119]. Mammalian cells cannot synthesize folic acid de novo, and therefore rely on exogenous nutritional sources for their metabolic requirements. Since folates are highly hydrophilic bivalent anions, they can only minimally navigate through the biological membranes by simple diffusion process. Therefore, their internalization through mammalian cell membranes must occur by means of a carrier mediated process. Structurally and functionally diverse carriers transport folic acid across epithelia and into systemic tissues. Three major classes of folate transport mechanisms are reported for cellular entry of

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folates i.e., folate receptor (α, β and γ), folate transporter (reduced folate carrier 1) and proton-coupled folate transporter (PCFT). RFC1, a member of major facilitator superfamily encodes for a sequence of 60 kDa protein whereas folate receptors belong to a family of 32-36 kDa membrane anchored glycoproteins [120]. RFC1 is a low affinity, high capacity bidirectional transporter involved in folate uptake by many tissues like intestine, pancreas and liver. Folate receptor alpha (FR-α) is a high affinity, low capacity system that has been utilized to target polymer based systems and drugs to tumors that overexpress high levels of this receptor [121]. RFC1 has higher affinity for reduced folate forms like methotrexate whereas folate receptor has higher affinity for folic acid than reduced folate forms. Recently, Qiu et al identified a human proton coupled, high affinity folate transporter (PCFT) in HeLa, HepG2 and Caco-2 cells[122]. PCFT is a 50 kDa electrogenic, proton coupled high affinity transporter that   mediates the translocation of folates across the membranes. Prodrugs targeting specific high affinity carriers have been utilized for improving systemic concentrations of drugs with low oral bioavailability. Targeting specific carriers in the airway epithelium may improve drug uptake and delivery across pulmonary epithelial cells. Folic acid conjugation has been used successfully to deliver several macromolecules due to its small size, high affinity, selectivity, availability and lack of immunogenicity. Folate conjugation has also been employed for tumor targeted gene delivery in lungs. Folate targeted liposomes, nanoparticles and dendrimers have several advantages for tissue specific targeting since folic acid has higher affinity coupled with overexpression of folate carriers in certain

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forms of tumors [123]. Pulmonary delivery of various therapeutic substances following local and systemic administrations is of high clinical significance and has widely been investigated for a variety of respiratory diseases, such as asthma, pulmonary hypotension, chronic obstructive pulmonary disease and cystic fibrosis[124].
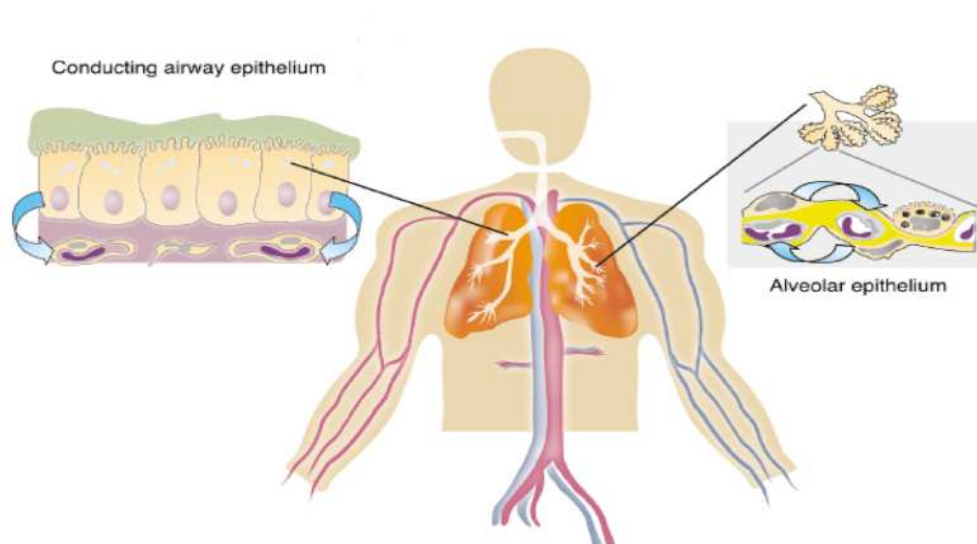
Figure- 4.1 Airway and alveolar epithelium

Inhalation route has been successfully indicated as a potential alternative to parenteral administration of proteins and peptidomimetic drugs due to advantages like large surface area of lung (~70-140 m^2), relatively permeable mucosa, and escape from first pass metabolism, lower enzymatic activity and higher vascularization. Bronchial epithelium is a major barrier to transport of macromolecules as it restricts paracellular diffusion (figure 4.1). However, little information is known about the mechanism of folic acid transport across the bronchial epithelium. Therefore, the objective of this study was to investigate the presence of folate transporters in human bronchial epithelial cell line,

Calu-3 and to functionally characterize the role of folic acid transporters across human bronchial epithelium. Several cell lines (A549, Calu-3, BEAS-2B and 16 HBE14o-), primary cultures (hAEpC) and isolated lung perfusion models have been established to investigate pulmonary drug transport mechanisms. Calu-3 cell line, derived from bronchial epithelium, has been employed as a model for the air way epithelium in a number of drug transport and metabolism studies. These cells form polarized monolayers with tight junctions producing high TEER values. They also produce several cell adhesion proteins (ZO-1 and E-cadherin) and mucosal secretions [125]. *In vitro-in vivo* studies indicated a good correlation (0.94) between permeability characteristics of Calu-3 cells with that of rat lung [126]. Therefore, Calu-3 cell line was selected as a model to investigate the transport of folic acid across respiratory epithelium.

Materials and Methods

Cell Culture

Calu-3 cell line, an airway epithelium derived cell line from human lung carcinoma was procured from ATCC. Calu-3 cells between passages 15-40 were employed for all the studies. Cells were cultured in DMEM-F12 (Dubelcco's Minimum Essential Medium) supplemented with 10% heat inactivated fetal bovine serum, non-essential amino acids (NEAA), HEPES, sodium bicarbonate, penicillin (100units/ml) and streptomycin (100 µg/ml) were purchased from Sigma Chemical Co. Cells were maintained at $37^{o}C$, in a humidified atmosphere of 5% CO_2 and 90% relative humidity.

The medium was replaced every alternate day. Cells were subcultured with 0.25% trypsin containing 0.537 mM EDTA and plated onto 12 well plates.

RT-PCR

Isolation of total RNA from cells was carried out using Trizol-LS® reagent according to manufacturer's instructions. In brief, Calu-3 cells grown in a culture flask (75 cm^2 growth area) were lysed by adding 800 µl of Trizol reagent. The lysate was then transferred to Eppendorf tubes. RNA was extracted by the phenol–CHCl$_3$–isopropranolol method and dissolved in 50 µl of RNase–DNase-free water. RNA was mixed with 1.25 µl of oligo dT$_{15}$ primer to prepare the first strand cDNA. After denaturation, it was reverse transcribed to cDNA using 1 µl (10 units) of Moloney Murine Leukemia Virus Reverse Transcriptase per reaction mixture. After the first strand cDNA synthesis, 1 µl of cDNA was used for PCR. The primers were designed by. amplification of cDNA was performed using primers mentioned in Table-4. Briefly, the PCR mixture has a final volume of 50 µl and contains the relevant template cDNA, 250 nM each of forward and reverse primers for the gene of interest, 1X Mg free PCR buffer (Promega), 3.75 mM MgCl$_2$, 0.025U Taq Polymerase, and 200 µM dNTPs, The polymerase chain reaction conditions of denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec with a final extension at 72°C for 10 min were used for the studies. PCR products were separated by 2% agarose gel in tris-acetate-EDTA

buffer along with 100 bp ladder. Bands were visualized by ChemiImager 8900 digital imaging.

Table-4   PCR primers for folic acid transporters and receptor

Gene	NCBI no.	Sequence (5'-3')		Product length (bp)
GAPDH	NM_002046.3	Forward :	GTCCACCACTGACACGTTGG	729
		Reverse :	GGGAAGGTGAAGGTCGGAGT	
FRα	NM_016724.2	Forward :	GCATTTCATCCAGGACACCT	407
		Reverse :	TCATGGCTGCAGCATAGAAC	
RFC	NM_194255.1	Forward :	GCTCCTACCAGTTCCTCGTG	621
		Reverse :	AGACACTGCAAACCCAGCTT	
PCFT	NM_080669.3	Forward :	CTCCACGTCGGCTACTTCGT	625
		Reverse :	CCATCCCCAGGATGTTGAAG	

.

Western Blot

Whole cell protein was extracted with reagent containing 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 1% Triton X - 100 and protease inhibitor cocktail at pH 7.4. Confluent cells were washed thrice with PBS and harvested using a cell scraper in 5 mL of PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes and the pellet was resuspended in freshly prepared lysis buffer for 15 minutes on ice. The extracted protein was then obtained by centrifugation and stored at -80°C, until used. Protein content was determined using Bradford method. Polyacrylamide gel electrophoresis (PAGE) was run with 25 and 50 µg of each protein at 120 V, 250 mAmp.

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Transfer was carried out on polyvinylidene fluoride (PVDF) membrane at 25 V for 1 h 30 min, on ice. Immediately after transfer, the blot was blocked for 3 hours in freshly prepared blocking buffer (2.5 % non-fat dry milk and 0.25 % bovine serum albumin prepared in TBST pH-8). After a light wash for 10 sec, the blots were exposed to primary antibodies overnight (dilution - 1:500 for PCFT, 1:400 for FR-alpha, 1:300 for RFC) Antibodies for PCFT and RFC were gifts from Professor Sylvia B. Smith whereas goat polyclonal antibody for FR-alpha (SC-16386) was obtained from Santa Cruz Biotechnology. The blots were then exposed for 2 hours to secondary antibodies obtained from Santa Cruz Biotechnology (1: 5000 for PCFT, 1: 5000 for FR-alpha, 1: 5000 for RFC). The blots were finally washed three times with TBST and developed using SuperSignal West Pico chemiluminescence substrate. The blots were exposed for 30 sec after which the image was taken in Gel Doc Imager.

Uptake Studies

Uptake studies were conducted in 12 well plates with confluent cell monolayers. At 11-14 days post seeding, the cells reached confluency. Then, the medium was removed and cells were rinsed three times, 5 min each with 2 ml of DPBS (pH 5.0, 129 mM NaCl, 2.5mM KCl, 7.4mM Na_2HP0_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM MgSO4 and 5.3 mM glucose) and equilibrated for 30 minutes with the buffer. All the uptake experiments were carried out by incubating a fixed amount of [^{3}H]folic acid (0.5µCi/ml, concentration-20 nM) at 37^0C with or without other test compounds. At the

end, the test solutions were removed and uptake process was stopped by adding ice cold stop solution (210 mM KCl and 2 mM HEPES; pH 7.4). After three washings with stop solution, cells were lysed overnight in 1 ml of 0.1% Triton-X solution in 0.3% NaOH. Following overnight lysis, 500 µl cell lysate from each well was transferred to scintillation vials containing 5 ml of scintillation cocktail. Samples were analyzed by liquid scintillation counter and the uptake values were normalized to the protein content in each well. Amount of protein present in the cell lysate was measured by Bradford reagent using bovine serum albumin as standard.

Time dependency: Time dependent uptake of [^{3}H]folic acid was performed at different time periods from 1 to 60 minutes. Cells were incubated with [^{3}H]folic acid at different time periods and the reaction was terminated by adding stop solution. In addition, time dependent uptake of [^{3}H]folic acid was carried out in presence of 10 µM methotrexate.

pH dependency: The effect of variation of incubation buffer pH was examined on the folic acid uptake. Incubation buffer was adjusted to 5.0, 6.0, 7.4 and 8.0 for pH dependence studies.

Concentration dependency: Stock solutions (5mg/ml) of unlabeled folic acid were prepared in DMSO (less than 2%v/v in water). Different concentrations (0.01 µM-25 µM) of folic acid were then prepared by diluting adequate quantities of stock solutions

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with DPBS buffer. Then fixed amount of [^{3}H]folic acid (0.5 µCi/ml) was spiked to the different dilutions and uptake at different concentrations was carried out according to previously described procedures.

Sodium and chloride dependency: To determine the effect of Na$^+$ and Cl$^-$ ions, uptake of folic acid was performed with sodium free and chloride free DPBS buffer. Equimolar quantities of Choline chloride and dibasic potassium phosphate (K$_2$HPO$_4$) were used as replacement for sodium chloride (NaCl) and dibasic sodium phosphate (Na$_2$HPO$_4$) respectively to obtain sodium free buffer. Chloride free buffer was prepared by substituting with equimolar quantities of monobasic sodium phosphate (NaH$_2$PO$_4$), monobasic potassium phosphate (KH$_2$PO$_4$) and calcium acetate in place of sodium chloride (NaCl), potassium chloride (KCl) and calcium chloride (CaCl$_2$).

Temperature dependency: To determine whether uptake was temperature dependent, cells were incubated with [^{3}H]folic acid for 15 minutes at 37°C, room temperature and 4°C.

Energy dependency: To investigate whether the transport of folic acid is energy dependent, 1 mM ouabain (Na$^+$ /K$^+$ -ATPase inhibitor) and 1 mM sodium azide (metabolic inhibitor) were added. All the inhibitors were preincubated for one hour prior to a study and uptake was conducted for 15 minutes.

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Substrate specificity: To examine specificity, uptake of [^{3}H]folic acid was examined in the presence of 10 µM structurally related compounds (unlabeled folic acid and methotrexate). Uptake was also studied in the presence of 1 mM unlabeled vitamins (ascorbic acid, thiamine and nicotinic acid). These experiments were conducted to elucidate the structural properties of the substrates required for interaction with the carrier system.

Effect of membrane transport inhibitors and receptor inhibitors: Uptake of [^{3}H]folic acid was carried out to investigate the presence of a receptor mediated process or an anion exchange process on the apical side. Prior to the initiation of an uptake experiment, the cells were incubated for one hour with anion transport inhibitors; probenecid (1 mM) and amiloride (1 mM) as well as receptor mediated endocytosis inhibitors; colchicine (10 µM) and cytochalasin D (10 µM). After one hour treatment, uptake of [^{3}H]folic acid was carried out for 15 minutes.

Effect of sulfasalazine and thiamine pyrophosphate on folic acid uptake: PCFT and RFC mediated uptake of folic acid uptake was determined in Calu-3 cells following preincubation with sulfasalazine (10 µM, 50 µM and 100 µM) and thiamine pyrophosphate (100 µM and 500 µM) for 1 hour. Folic acid uptake was determined for 15 minutes.

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Statistical Analysis

All results were expressed as mean ± standard deviation. Statistical analysis between two groups of data was carried out using Student's t-test. A difference between mean values was considered significant if the P-value was less than 0.05.

Results and Discussion

Carrier mediated transport of folic acid is ubiquitously present in mammalian cells since folic acid is required for cellular proliferation, cell survival and tissue regeneration. Pulmonary route for local and systemic diseases has been successfully employed to deliver several macromolecules. Pulmonary residence time, the drawback associated with pulmonary drug delivery may be controlled by selective drug targeting with carriers. Studies involving the mechanism and functional aspects of folate uptake in the respiratory tract may be useful for selective drug targeting through prodrugs.

The present study investigates the presence of a carrier mediated system involved in the regulation of folic acid transport across Calu-3, human bronchial epithelium cell line. Calu-3 cell line has been validated as a metabolic and transport model to study the mechanisms of respiratory drug delivery at respiratory epithelium. Calu-3 cell line has been employed as a model based on its tight junctions, permeability of low molecular weight lipophilic compounds, expression of efflux pumps and influx transporters and metabolic enzymes. Calu-3 cell line is a well-established model and previous studies in our laboratory have demonstrated transport of insulin and HIV protease inhibitors across

Calu-3 cells[127]. Therefore, we selected Calu-3 cell line as a model cell line to investigate the presence of a carrier mediated system for folic acid and to delineate the mechanism involved in the uptake of folic acid by bronchial epithelium.

RT-PCR for Folic acid Carriers

Resulting cDNA was used as a template for PCR reaction. Primers specific to FR-alpha, RFC-1 and PCFT were designed for PCR. Amplified PCR products obtained were separated by 2% agarose gel electrophoresis and bands were visualized. As shown in Figure 4.2, bands were detected at approximately and 729, 407, and 625 bp for GAPDH, FR-alpha and PCFT respectively.
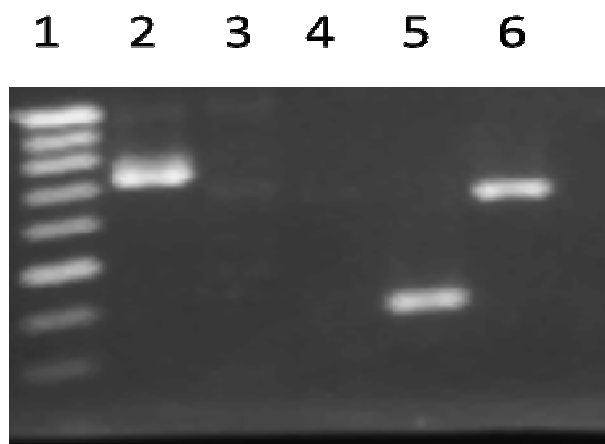
Figure 4.2   PCR for folic acid transporters and receptor. Molecular weight marker (lane 1), GAPDH (lane 2), RFC (lane 4), FR-α (lane 5) and PCFT (lane 6).

Western Blot Analysis

To further characterize the folate transporters, Western blot analysis were carried out to determine the protein expression of PCFT, RFC and FR-alpha. Studies revealed specific bands at 65 KDa and 37 KDa for PCFT and FR-alpha respectively (Figure 4.3). RFC was not expressed in the protein homogenate extracted from Calu-3 cells. β-actin was used as a positive control. These results correlated well with the PCR results.
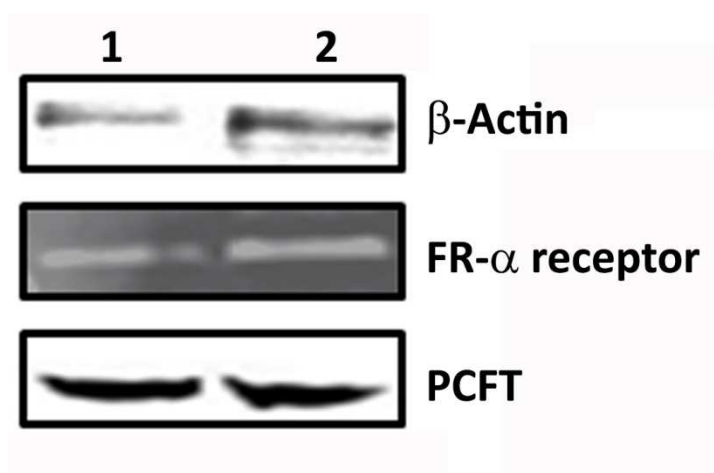
Figure 4.3 Western blot for PCFT and FR-α

Time Dependent Study of [^{3}H] Folic Acid

Uptake of [^{3}H] folic acid by Calu-3 cells was examined as a function of time. Uptake was found to be linear for up to 60 minutes as depicted in Figure 4.4. Therefore, all the subsequent uptake studies were conducted over 15 minutes. At 15 min, the uptake of [^{3}H]folic acid was 0.3 pmole/mg protein.
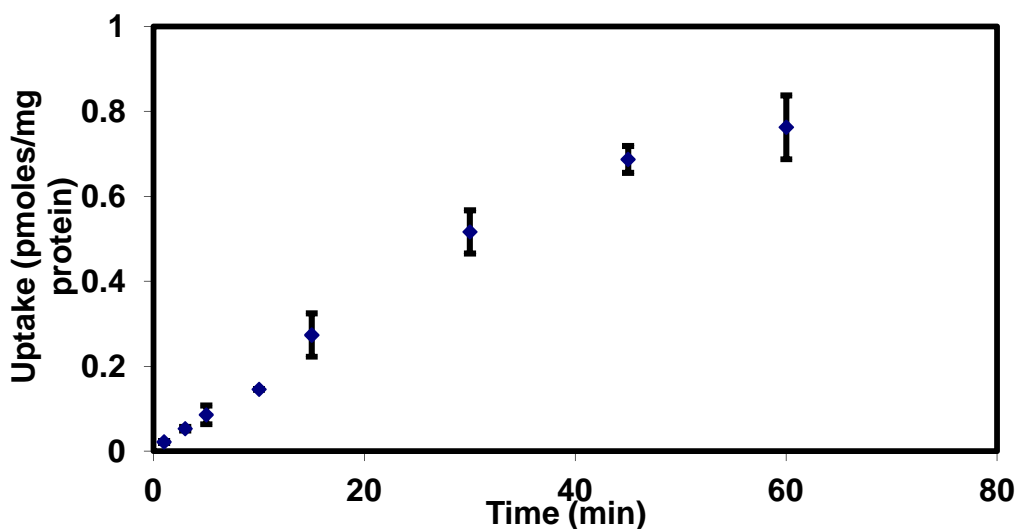
Figure 4.4 Time dependent study of [³H]folic acid

Uptake of [³H]Folic acid at Different pH

Uptake of [³H]folic acid significantly decreased in Calu-3 cells when the pH of incubation buffer was increased from 5.00 to 8.00. Uptake was higher at pH-5 (0.030 ±0.002 pmol/min/mg) relative to other pH values (Figure 4.5). At pH-7.4, uptake rate of [³H]folic acid was found to be 0.0086 ±0.0006 pmol/min/mg. Therefore, all further studies were conducted at pH-5.00. This effect may indicate the presence of an inwardly directed proton gradient being involved in the uptake of folic acid. Folate transporter which is effective at low pH was also found in human rat intestinal brush border membrane vesicles [128] and human retinal pigmental cells (ARPE-19) [129].
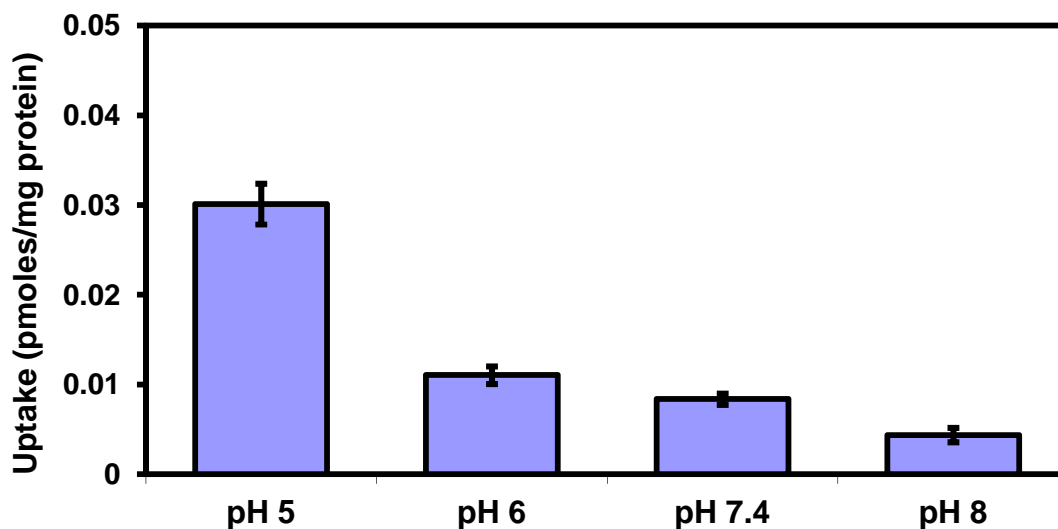
Figure 4.5 pH dependent study of [3H]folic acid

Concentration Dependent Study of [3H]Folic Acid

To determine the saturation kinetics, uptake of [3H]folic acid was examined in the presence of various concentrations (0.01 µM-10 µM) of unlabeled folic acid. Unlabeled folic acid significantly inhibited the uptake of [3H]folic acid. Uptake data was fitted to a modified Michaelis-Menton equation and kinetic parameters for folic acid uptake were determined by non-linear regression analysis of the data. Apparent K_M and Vmax were calculated to be $0.33 \pm 0.03\mu M$ and 22.04 ± 0.0009 pmol/min/mg protein respectively (figure-4.6). This data is further supported by substrate specific inhibition of folic acid in the presence of folate analogues like methotrexate. Previous studies demonstrated a Km value of 1.4 µM for a carrier mediated transport system for folic acid across human colonic epithelial cell line NCM460 [130].

The chart contains a fitted equation table:

y = (m1*m0)/(m2+m0)		
	Value	Error
m1	0.022045	0.00094151
m2	0.33345	0.038228
Chisq	3.9724e-07	NA
R	0.99895	NA
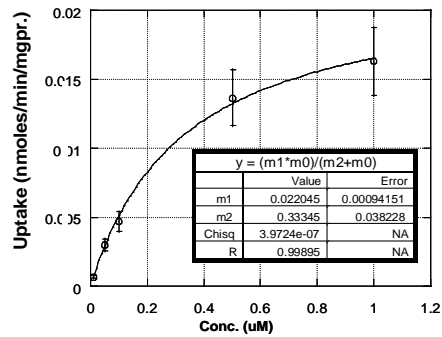
Figure 4.6   Kinetic parameters for folic acid uptake

Effect of transmembrane ion gradient on the uptake of folic acid was investigated by repeating the experiments in Na and Cl free medium. The absence of Na^+ and Cl^- in the incubation buffer had no significant effect on the uptake of folic acid (data not shown).

Energy Dependent Uptake of Folic Acid

To investigate whether transport of folic acid is energy dependent, 1 mM ouabain (Na^+ /K^+ -ATPase inhibitor) and 1 mM sodium azide (metabolic inhibitor) were added. All inhibitors were preincubated for one hour prior to a study and uptake was conducted over 15 minutes.
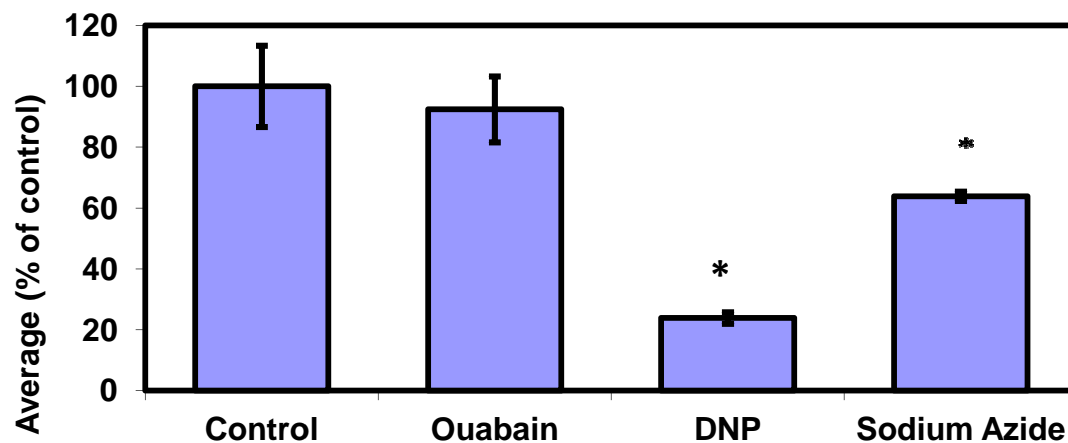
Figure 4.7 Energy dependent study of [^{3}H]folic acid. Uptake of [^{3}H]folic acid in the presence of ouabain, 2,4 dinitrophenol and sodium azide (1 mM). Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P < 0.05$)

Substrate Specificity Study

To examine specificity, uptake of [^{3}H]folic acid was examined in the presence of 10 µM structurally related compounds (unlabeled folic acid and methotrexate). Uptake was also studied in the presence of 1 mM unlabeled vitamins (ascorbic acid, thiamine and nicotinic acid) (figure-4.8). These experiments were conducted to elucidate structural properties of substrates required for interaction with the carrier system.
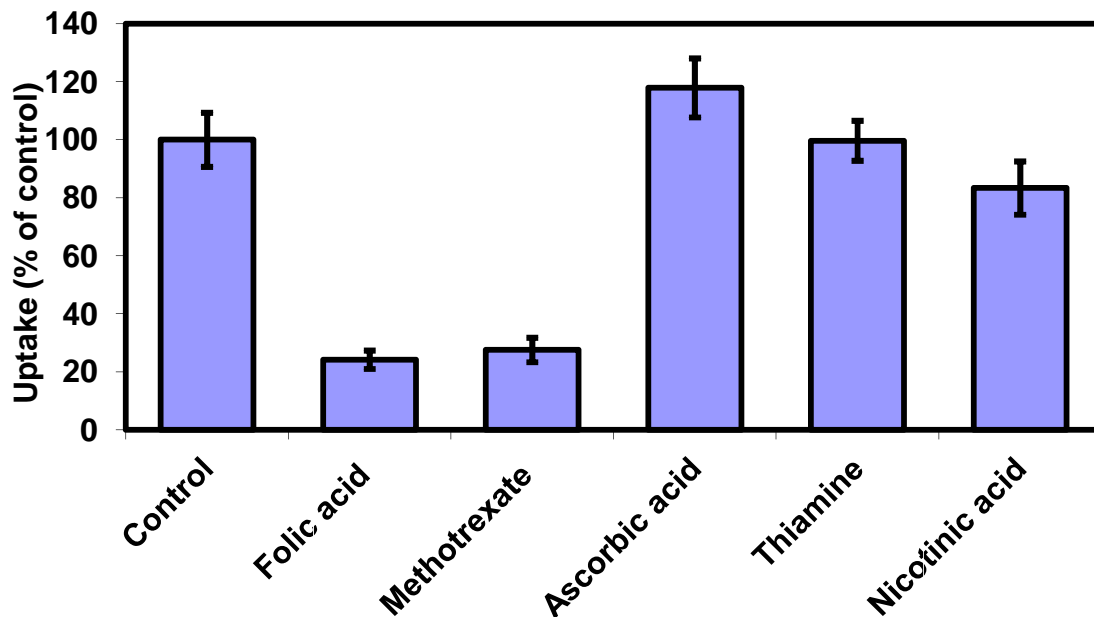
Figure 4.8 Substrate specificity of [³H]folic acid. Intracellular accumulation of [³H]folic acid in the presence of folic acid, methotrexate, ascorbic acid, thiamine and nicotinic acid (10 µM). Values are means of triplets with standard deviation indicated by error bars.
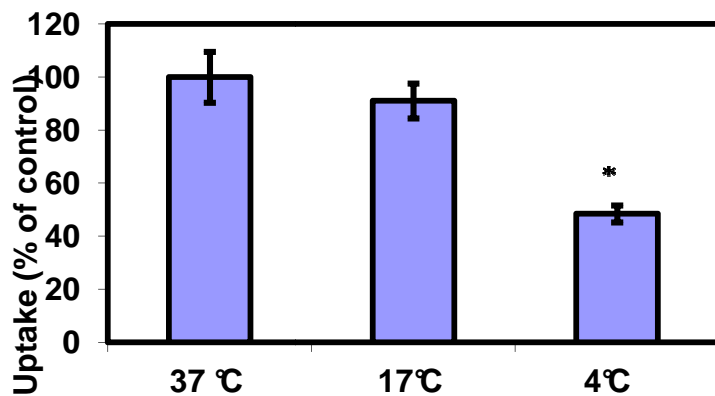
Figure 4.9 Temperature dependent study of [³H]folic acid. Intracellular accumulation of [³H]folic acid at different temperatures. Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P < 0.05$) difference from the mean control value using Student's one-tailed t-test.

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Uptake of Folic Acid in Presence of Colchicine

In a separate experiment, possible role of receptor mediated endocytosis was investigated by treating Calu-3 cells with colchicine. As shown in figure 4.10, colchicine significantly reduced the uptake of folic acid.
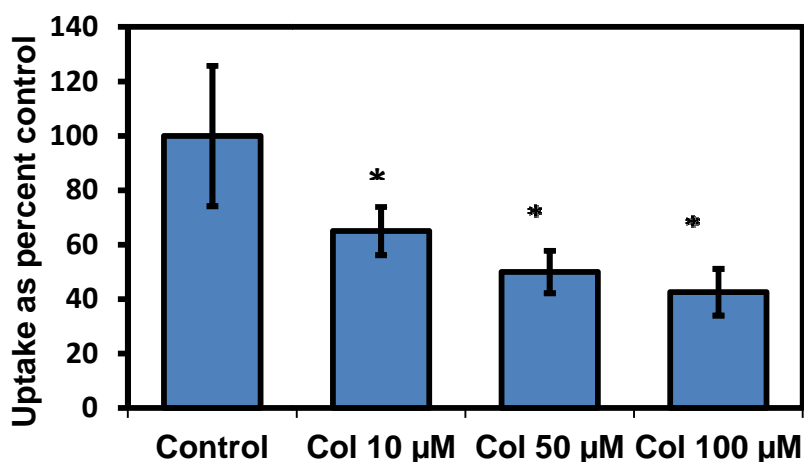
Figure 4.10 Uptake of [³H]folic acid in presence of colchicine, receptor mediated endocytosis inhibitor. Asterisk (*) indicates a significant (*P*< 0.05) difference from the mean control value using Student's one-tailed *t*-test.

Uptake in the Presence of Anion Exchange Inhibitors

To examine any possible involvement of an anion exchange mechanism for folate uptake, known anion exchange inhibitors such as SITS and DIDS were added. Preincubation of Calu-3 cell monolayers with SITS (0.5 and 1 mM), DIDS (0.5 and 1

mM caused significant inhibition (46.6% and 76.04% respectively) in folic acid uptake as illustrated in Figure 4.11.
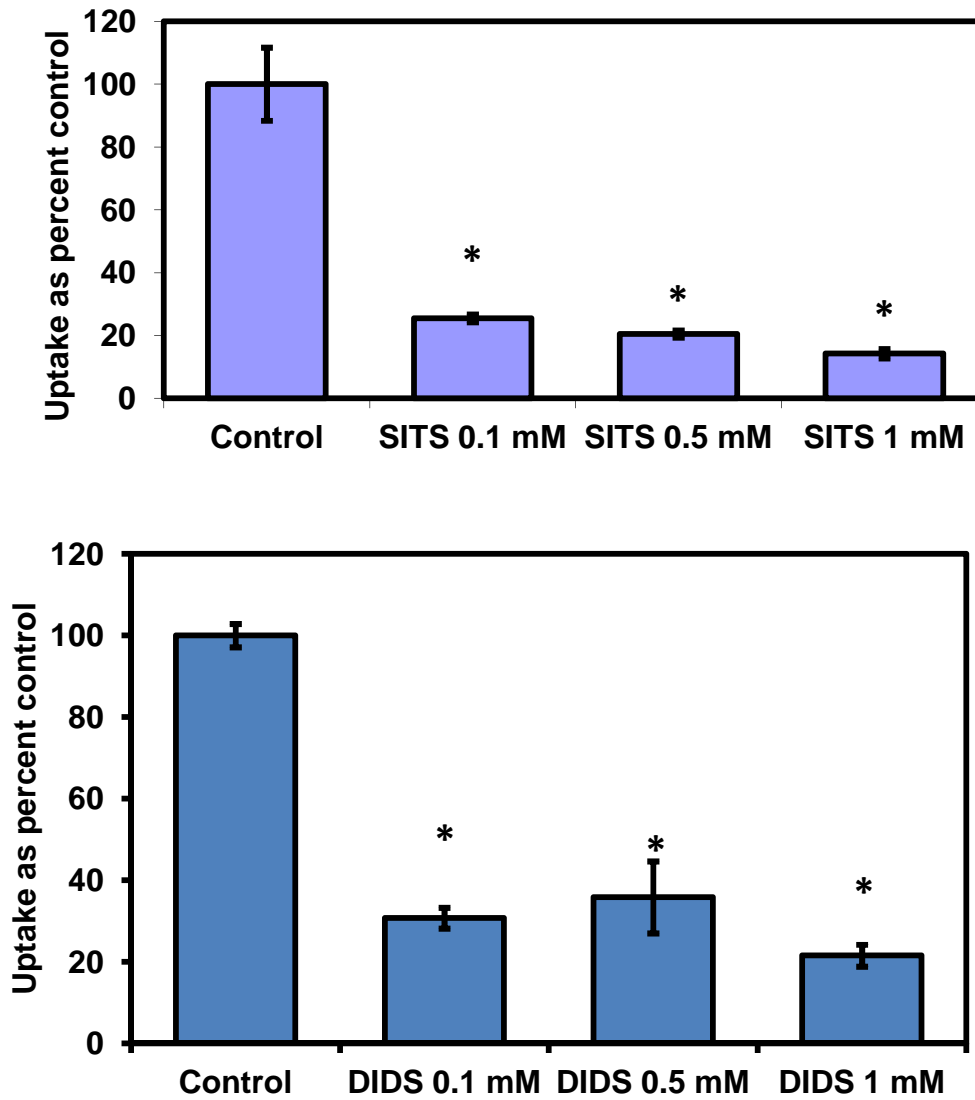
Figure 4.11 Uptake of [^{3}H]folic acid in presence of SITS and DIDS, anion transport inhibitors. Asterisk (*) indicates a significant ($P< 0.05$) difference from the mean control value using Student's one-tailed *t*-test.

Uptake of Folic Acid in Presence of Sulfasalazine and Thiamine Pyrophosphate

Sulfasalazine and thiamine pyrophosphate were proven to be specific inhibitors of PCFT and RFC respectively. To determine the inhibitory effect of sulfasalazine and thiamine pyrophosphate on PCFT mediated uptake, Calu-3 cells were incubated with increasing concentrations of sulfasalazine for one hour. Uptake of [^{3}H]folic acid was performed for 15 minutes at 37°C. Uptake was found to decrease significantly by 89.7%, 63.4% and 22.9% for 10 µM, 50 µM and 100 µM respectively as shown in figure 4.12. These results further indicate the presence of PCFT mediated uptake in Calu-3 cells.

A)

B)
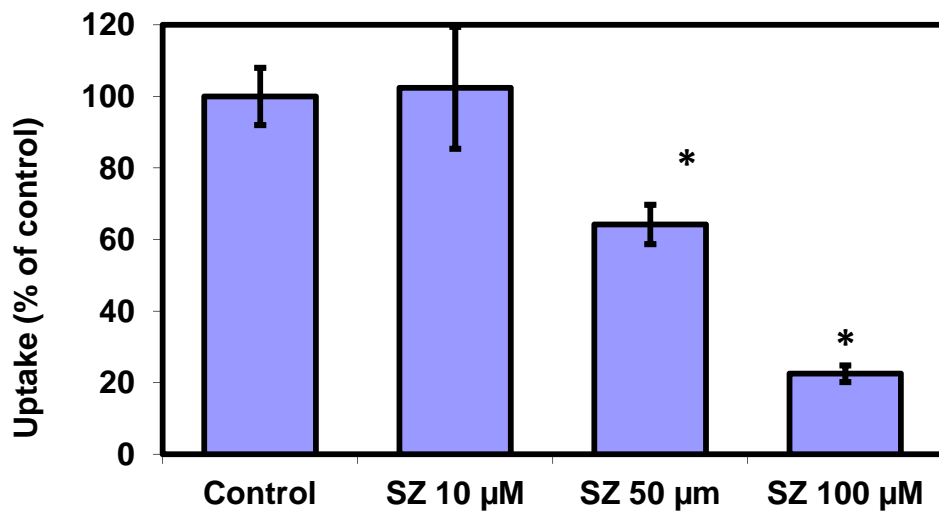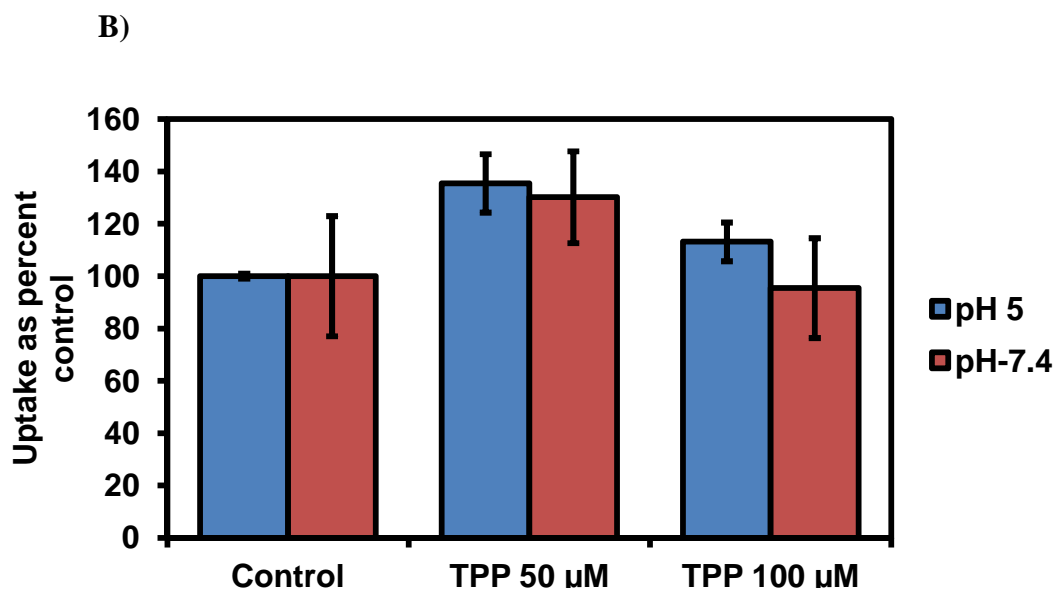
Figure 4.12 Uptake of [³H]folic acid in presence of A) Sulfasalazine and B) Thiamine pyrophosphate

Functional Activity of Peptide Transporter

Several peptidomimetic antibiotics are frequently used for local drug therapy in pulmonary infections. Glycylsarcosine, radioactive substrate of peptide transporter was used as a model substrate to study the functional activity in Calu-3 cells. Uptake of [¹⁴C] Gly-sar was performed at acidic pH 5.00. Uptake of [¹⁴C] Gly-sar was inhibited by 1 mM cold Gly-sar, cefradine and cefadroxil indicating the presence of peptide transporter at the apical membrane of human bronchial epithelial cells, Calu-3 (figure-4.13). Studies by Gronberg et al also indicated the presence of peptide transporter in lungs.
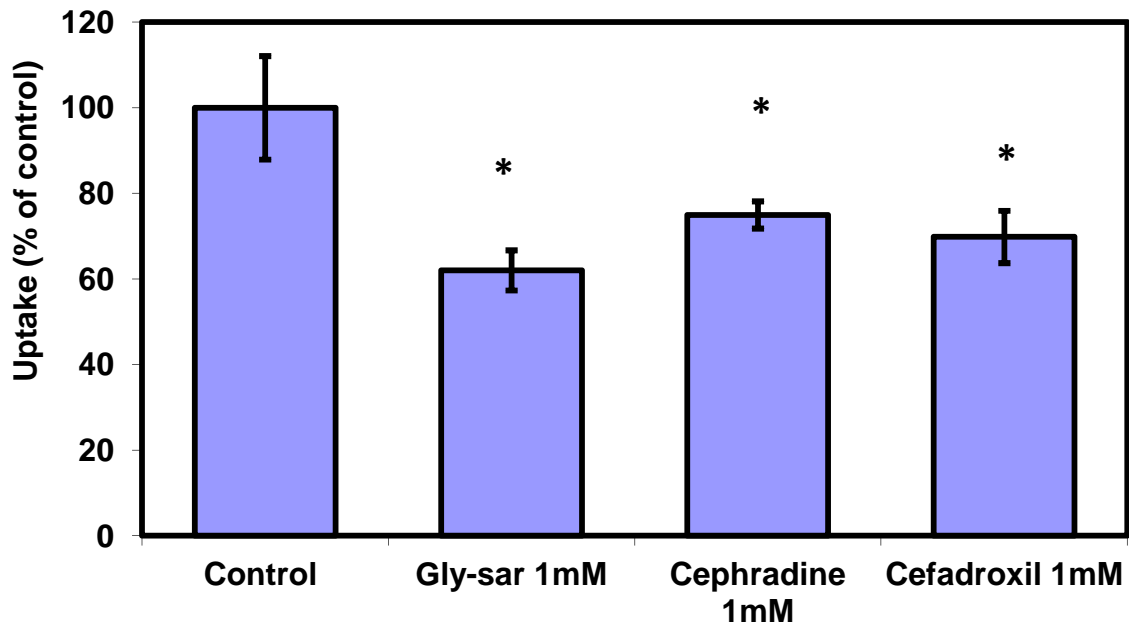
Figure 4.13   Uptake of [³H]Gly-sar in presence of peptide substrates

Effect of Nicotine on Peptide and Folic acid Transporter Activity

We wanted to investigate the effect of nicotine on activity of influx transporters peptide and folic acid transporter. Calu-3 cells were treated with nicotine for 11 days and uptake of [³H]Gly-sar and [³H]folic acid was determined. As shown in figure- 4.14 and 4.15, uptake of radioactive Gly-sar and folic acid was reduced significantly after exposing Calu-3 cells to nicotine. This data indicated that chronic nicotine treatment inhibited the activity of influx transporter. This data conclude that there can be involvement of inhibition of activity of influx transporters in cells treated with nicotine.
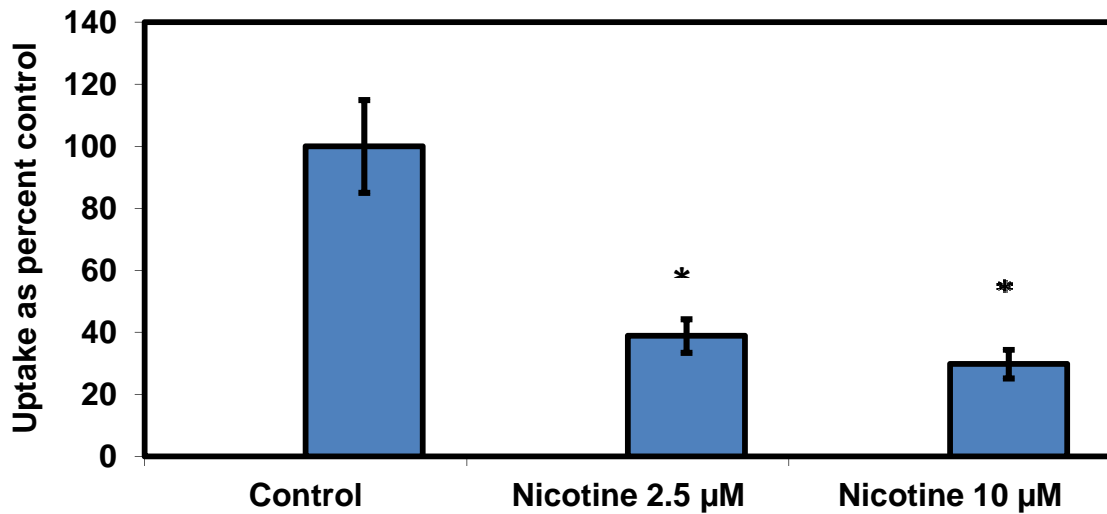
Figure 4.14 Uptake of [³H]Gly-sar after nicotine treatment in Calu-3 cells. Intracellular accumulation of [³H] Gly-sar was measured by incubating LS180 cells for 30 min without treatment (control) or treatment with nicotine (2.5 µM and 10 µM). Values are means of triplets with standard deviation indicated by error bars. Asterisk (*) indicates a significant (*P*< 0.05) difference from the mean
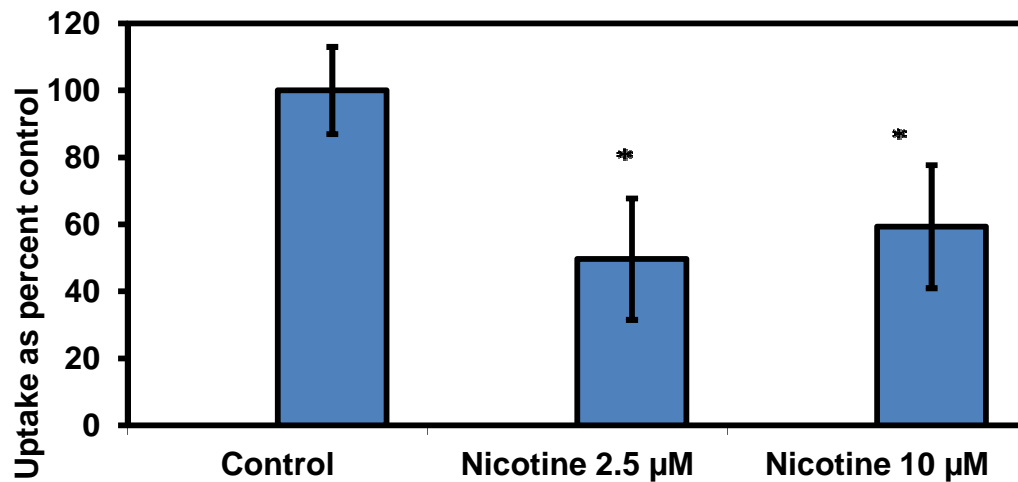
Figure 4.15   Uptake of [³H]folic acid after treatment with nicotine in Calu-3 cells

Conclusions

In conclusion our results confirmed the molecular identity of PCFT and FR-alpha in human bronchial epithelial cell line, Calu-3. Also, we functionally characterized the transport of folic acid mediated by PCFT across Calu-3, human bronchial epithelial cell line. Our findings may offer new strategies to treat chronic pulmonary diseases by designing folate linked compounds. Data from this study also revealed the effect of nicotine on the activity of influx transporters, peptide and folic acid. Reduced Gly-sar and folic acid uptake indicate that chronic nicotine exposure through cigarette smoking can modulate the uptake of inhaled drugs in lungs. More experiments should be designed to study the effect of chronic cigarette smoking on the expression and activity of influx transporters in lungs.

CHAPTER-5

TO STUDY THE ROLE OF EFFLUX TRANSPORTERS IN HUMAN BRONCHIAL EPITHELIAL CELL LINE, CALU-3

Rationale

Efflux transporter proteins have the potential to critically alter the systemic exposure and bioavailability of the drug substrates and therefore influence the modulation the absorption and disposition of the drugs. Efflux transporters have the ability to recognize and transport a diverse range of endogenous substrates, xenobiotics and pharmaceutically relevant drugs. Many efflux proteins such as p-glycoprotein, MRP2, BCRP and lung resistance proteins mediate the multidrug resistance of the chemotherapeutic agents. Several inhaled drugs are substrates of efflux transporters and hence their localization and activity influence the delivery of these drugs to the site of their action (figure-5.1). Current studies aim to investigate the localization and activity of these efflux transporters in lungs. Since, local concentration of the drugs is the most important factor for the eradication of bacteria and viruses, role of efflux transporters across bronchial and alveolar epithelium needs to be thoroughly investigated. Calu-3, a human bronchial epithelial cell line has been employed as an *in vitro* model for efflux studies. Therefore, overall aim of this chapter was to investigate the molecular presence and activity of the efflux transporters. Specifically p-glycoprotein and MRP mediated activity of the model p-gp and MRP substrates were examined. This research will give insights in the preclinical development of pulmonary drugs.

Introduction

Pulmonary route has been successfully utilized as an alternative route for the systemic delivery of chemotherapeutic agents both for local delivery and systemic delivery. Several efforts have been made to deliver these agents for systemic administration as well as local conditions. In particular, inhaled insulin has been explored extensively for the treatment of diabetes mellitus. Lungs have a comparatively larger surface area (70 m^2) than other mucosal tissues such as nasal, buccal, rectal and vaginal routes. In addition, the lower thickness of the alveolar epithelium (0.1-0.5 µm), rich vascularization, rapid absorption followed by rapid onset of action, lower enzymatic degradation and escape of first pass liver metabolism makes it as an attractive route for delivery of proteins. About 90% of the absorptive surface area of the lungs is due to the alveoli. Alveolar epithelial with tight intercellular junctions form a major barrier for the absorption of high molecular weight substances. Small molecular weight compounds less than 40 kDa are absorbed by paracellular transport whereas larger molecular weight agents are absorbed by transcytosis. Previous studies have shown that proteins that have a molecular weight up to approximately 30 kDa have bioavailability between 20-50%. The lower bioavailability is due to the degradation of proteins by the proteolytic enzymes present in the lungs. Penetration enhancers such as chelators, surfactants, bile salts and fatty acids are often used to enhance the pulmonary absorption. These penetration enhancers might alter the integrity of the mucosal membrane, inhibit the proteolytic activity and affect the membrane lipids and proteins. Inhaled particles get filtered and

subsequently deposited on the airways due to progressive branching of the tracheobronchial tree. These particles are then cleared by two mechanisms: mucociliary clearance and alveolar macrophages. Mucociliary escalator results from the upward movement of the mucus secretions (produced by the goblet cells and mucus secreting glands) by the cilia that beat at about 1000 to 1500 per minute. Mucus gets cleared at a rate of 0.5 to 20 mm/minute towards the trachea and then swallowed into the gastrointestinal track. Inhaled toxic particles get phagocytosized by alveolar macrophages present in the alveoli. Also, these macrophages secrete several inflammatory mediators such as interleukins, leukotrienes, granulocyte colony-stimulating factors and proteases that degrade the proteins. However, to elicit proper systemic effect, the major challenge is to develop formulations that can deliver the aerosol particles to the deep lung. Particle size and velocity are the two main factors that govern the deposition of particles to the deep lung. For efficient deposition, the particles should have mass median aerodynamic diameter between 1 and 3 µm. For targeted deposition to the alveolar region, the mass median aerodynamic diameter should not be more than 3 µm. Aerosol particles with diameter greater than 6 µm gets deposited in the oropharynx. Three major types of devices have been used to deliver aerosol particles to the lungs: metered dose inhalers, nebulizers and dry powder inhalers. To control the release of drug from the administered dose, proteins are encapsulated in particulate delivery systems such as liposomes, microparticles, nanoparticles and dendrimers. PEG, PLGA, PLA and chitosan are most commonly used for encapsulating the proteins. These polymers improve the physical as

well as chemical stability and protect the protein molecules from loss of confirmation. Exubera was the first approved inhaled formulation of insulin developed by Pfizer for the treatment of hyperglycemia in type 1 and 2 diabetic patients. However, this product was discontinued due to its potential to develop side effects and its inability to deliver precise insulin doses. AIR Insulin System (Eli Lilly), AER_X Insulin Diabetes Management System (Novo Nordisk), Technosphere Insulin System (Mannkind) are some of the delivery devices that are currently in phase-III clinical trials for the delivery of inhaled insulin.

Efflux transporters play an important role in preventing accumulation of potentially toxic xenobiotics in the lung [131]. Gumbleton et al described the spatial expression of the several efflux transporters in lungs[132] (figure-5.1).
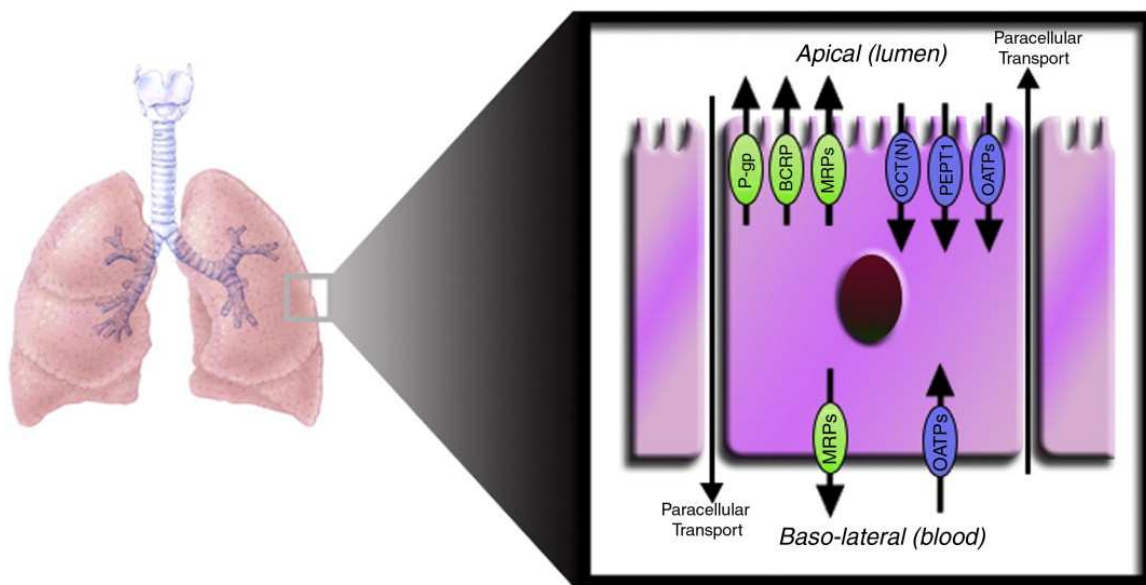
Figure 5.1 Localization of efflux transporters in lungs

Efflux pumps along with mucociliary clearance, alveolar macrophages and bactericidal surfactants act as a protective barrier in the lungs. Recent studies have shown that majority of the BCRP substrates were basic lipophilic amines which readily accumulate in lung tissue. P-glycoprotein has also been shown to play a role in the pulmonary accumulation of fluoroquinolones[133].

Also, efflux transporters such as P-gp and MRPs have been attributed to drug resistance in lung cancers[134]. BCRP is also involved in efflux of various chemotherapeutic agents employed in lung cancer which include mitoxantrone, doxorubicin, topotecan[135] and gefitinib[136]. Elevated mRNA levels have been correlated with resistance to these anticancer agents in lung cancer. BCRP expression in lungs might play a role in the disposition of drugs in cancer chemotherapy. Therefore, our objective is to identify and characterize the expression of BCRP in human bronchial epithelial cells.

Various cell lines were utilized to investigate the expression and modulation of BCRP. Calu-3 cell line, derived from bronchial epithelium, has been employed as a model for the air way epithelium in a number of drug transport and metabolism studies. Several advantages of Calu-3 cell line in comparison to other models of the airway epithelium, such as tracheal epithelial sheets or primary tracheal cell cultures have been reported. Calu-3 cells form polarized monolayers with high tight junctions producing high TEER values. These cells express in vivo features of the airway epithelium (cilia, mucus production), several transport and metabolic systems relevant to drug absorption. These cells also produce several cell adhesion proteins (ZO-1 and E-cadherin) and

generate mucosal secretions. *In vitro-In vivo* studies indicated good correlation (0.94) between permeability properties of Calu-3 cells with the rate of drug absorption from the rat lung (figure-5.2). Previous investigations established the functional activity of various efflux proteins belonging to ABC transporter super family such as MDR1 [137] and MRP-1 in Calu-3 cells. Therefore, Calu-3 cell line has been selected as a model cell line to investigate the BCRP expression and to estimate its functional activity.
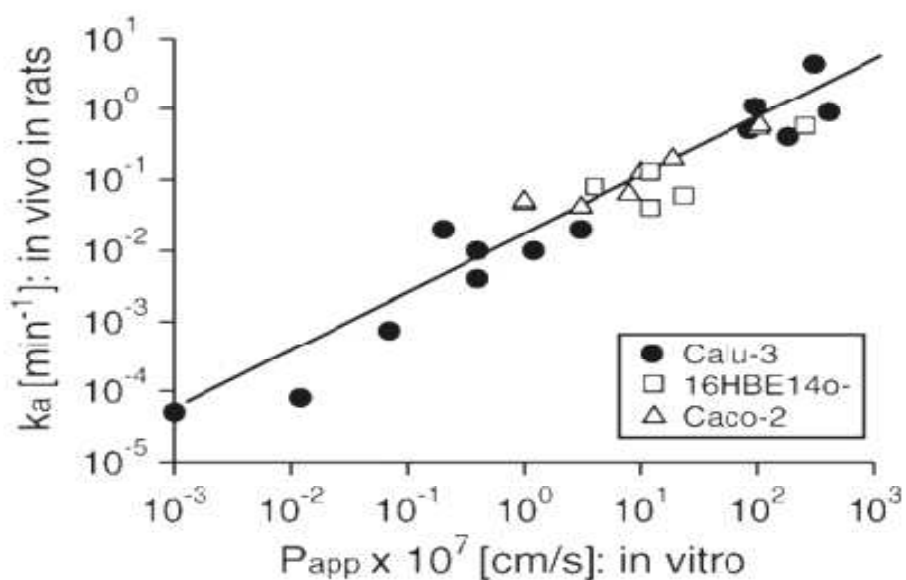
Figure 5.2   *In vitro and In vivo* correlation of permeability of Calu-3 cells

Materials and Methods

Materials

[^{3}H]Ritonavir (3 Ci/mmol) was purchased from Moravek biochemicals (Brea, CA, USA). Cells between passages 20-40 were used for all the studies.

Cell Culture

Cells were cultured in DMEM-F12 medium supplemented with 10% heat inactivated fetal bovine serum, MEM non-essential amino acids, HEPES, sodium bicarbonate, penicillin (100 µg/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. Medium was replaced every alternate day until 5 days and subsequently every day until 11 days. Cells were subcultured by trypsinization with 0.25% trypsin containing 0.537 mM EDTA.

Uptake Studies

At 11-13 days post seeding, cells were rinsed three times with DPBS (pH 7.4, 129 mM NaCl, 2.5mM KCl, 7.4mM Na_2HP0_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$ and 5.3 mM glucose) and equilibrated for 15 minutes with the buffer. [³H]Ritonavir was used as a model substrate to characterize functional activity. Uptake studies were performed by incubating a fixed amount of 0.5 µCi/ml of [³H]ritonavir alone and in the presence of P-gp and MRP inhibitors at 37°C. Following incubation, the reaction was stopped by addition of ice cold stop solution (210 mM KCl, 2 mM HEPES; pH 7.4). After three washings with stop solution, cells were lysed by keeping them overnight in 1 ml of 0.1% Triton-X solution in 0.3% NaOH. Following overnight incubation, 500 µl of the cell lysate from each well was transferred to scintillation vials containing 5 ml of scintillation cocktail. Samples were analyzed by liquid scintillation counter and uptake was normalized to the protein content in each well. Amount of protein in the cell lysate was measured by the Bio-Rad protein estimation kit with bovine serum

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albumin as standard. Functional activity was assessed by studying the uptake of [^{3}H]-ritonavir in presence of various inhibitors.

Statistical Analysis

All results were expressed as mean ± standard deviation. All the experiments were done in triplicate. Statistical analysis between two groups of data was carried out with a student's t-test. A difference between mean values was considered significant at the P-value less than 0.05.

Discussion

Cellular drug resistance conferred by multidrug resistance (MDR) proteins is a major challenge in cancer chemotherapy. Tumor cells can acquire resistance to a single drug or to a class of cytotoxic drugs or to a broad spectrum of structurally and functionally diverse chemotherapeutic agents. The cellular mechanisms of MDR involve reduced drug uptake, activation of DNA repair and detoxification process, defective apoptotic signals [138] and most commonly, active transport of drugs out of the cells mediated by efflux pumps [139]. P-glycoprotein (P-gp/ABCB1) has been widely investigated as a MDR transporter for many years.

MDR1 Inhibition Studies

To study the existence of p-glycoprotein mediated efflux in Calu-3 cells, uptake of ritonavir, a well-known p-gp substrate was examined. Intracellular accumulation of [³H]-Ritonavir in the presence and absence of p-gp efflux inhibitors ketoconazole (25 µM, 50 µM and 100 µM) and quinidine (75 µM) was depicted in figure 5.3.
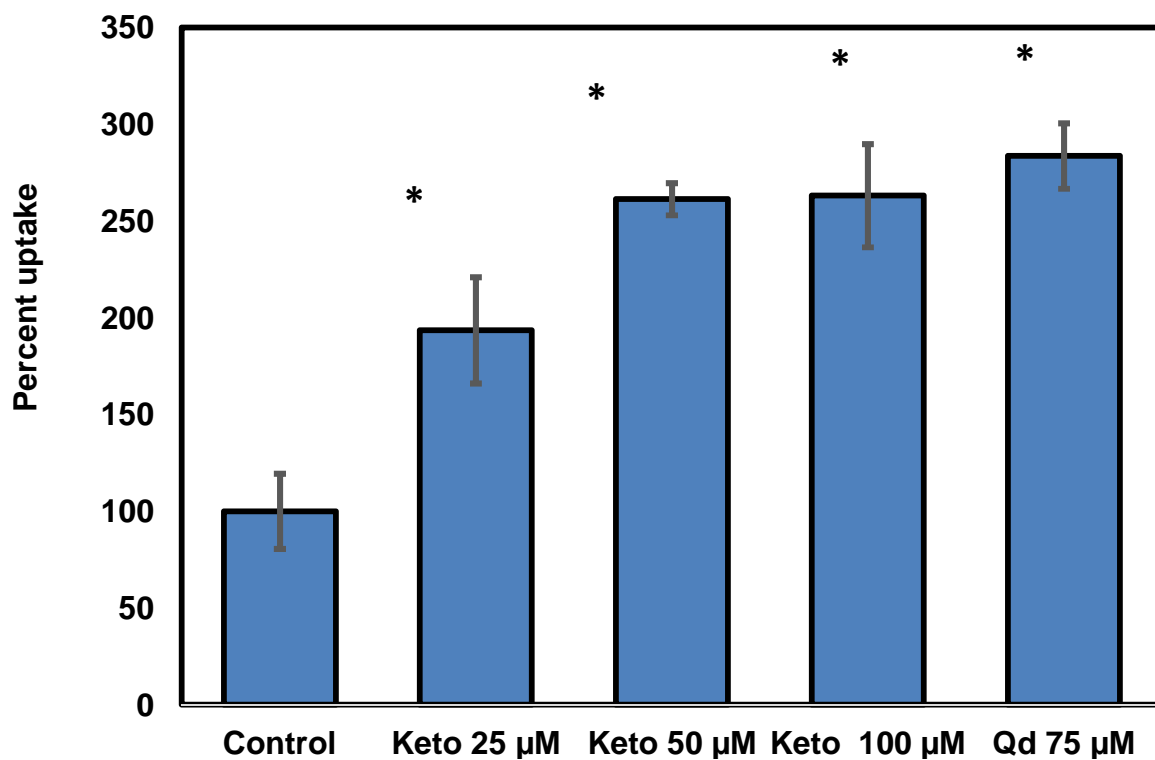
Figure 5.3   Uptake of radioactive ritonavir in presence of ketoconazole and quinidine

A 2.6 fold increase of intracellular ritonavir uptake was noticed in presence of 50 µM ketoconazole when compared to control. However, there was an increase of 2.83 fold uptake of ritonavir in presence of 75 µM quinidine, a known p-gp inhibitor (figure-5.3).

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Ketoconazole inhibited the efflux mediated by p-gp in a concentration dependent manner. This data indicate the role of p-gp mediated efflux of HIV protease inhibitor ritonavir in Calu-3 cells.

MRP Expression in Calu-3 Cells

Cellular RNA was isolated and RT-PCR was performed to determine MRP2 expression in Calu-3 cells. Figure 5.4 demonstrates MRP2 mRNA expression and a clear band was noticed at 412 bp.
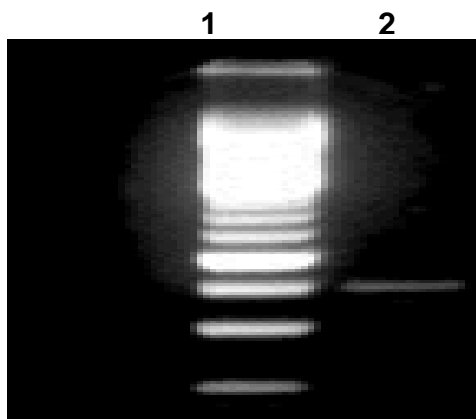
Figure 5.4 RT-PCR results for MRP2 expression in Calu-3 cells. Molecular weight ladder (lane 1) and MRP2 (lane 2)

Uptake Studies

MK571, a leukotriene D4 antagonist is a specific inhibitor of MRP mediated transport active against MRP2, MRP1 and MRP3. Uptake of ritonavir was performed in the presence of various concentrations of MK-571. As demonstrated in figure-5.5, MK-571 inhibited the MRP2 mediated efflux of ritonavir in concentration dependent manner.

Uptake of ritonavir was increased significantly by 5.26 fold with 75 µM MK571 which

confirmed the presence of MRP2 mediated transport of ritonavir in Calu-3 cells.
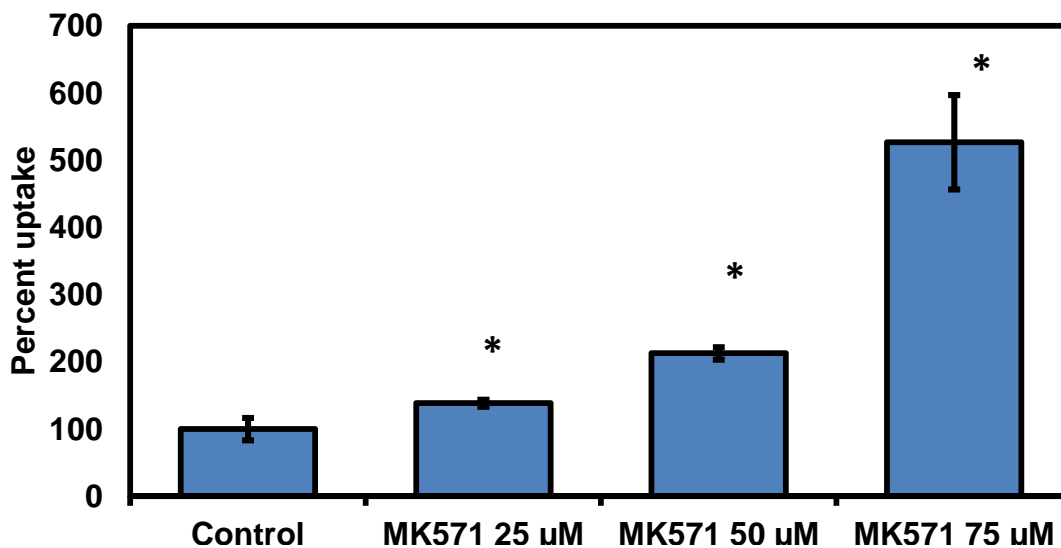
Figure 5.5 Uptake of [³H] ritonavir in presence of MK-571. Intracellular accumulation of [³H] ritonavir was measured by incubating Calu-3 cells for 30 min in the absence (control) or presence of MK571, MRP inhibitor. Values are means of quadruplicates with standard deviation indicated by error bars. Asterisk (*) indicates a significant (*P* < 0.05) difference from the mean control value using Student's one-tailed *t*-test.

Basolateral Uptake of [³H]Ritonavir in Calu-3 Cells

To determine the MRP functional activity on the basolateral side, basolateral

uptake studies were performed in presence of MRP inhibitors, MK-571 and

sulfinpyrazone. In these experiments, [³H]-Ritonavir was added to the basolateral

chamber of a transwell and accumulation of radioactive ritonavir in the apical chamber

was analyzed.
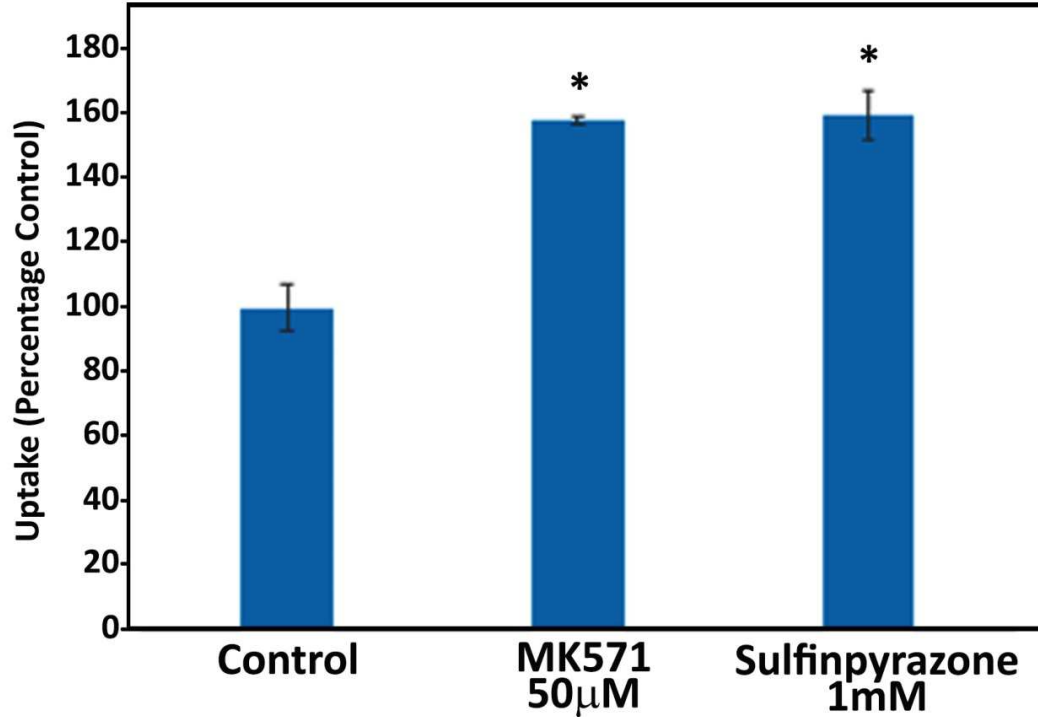
Figure 5.6 Basolateral uptake of [³H] ritonavir in presence of MK571 (50 μM) and sulfinpyrazone (1 mM). Intracellular accumulation of [³H] ritonavir was measured by incubating Calu-3 cells in the absence (control) and presence of MK571, MRP inhibitor and sulfinpyrazone on the basolateral side. Values are means of quadruplicates with standard deviation indicated by error bars. Asterisk (*) indicates a significant ($P < 0.05$) difference from the mean control value using Student's one-tailed *t*-test.

As shown in figure-5.6, MK-571 inhibited the MRP1 mediated efflux by 1.5 fold resulting in enhanced intracellular accumulation of ritonavir. Addition of 1 mM sulfinpyrazone (MRP inhibitor) to the basolateral chamber caused inhibition of ritonavir uptake by 1.45 fold when compared to control.

Transport Studies of [^{3}H]Ritonavir

The amount of [^{3}H]ritonavir transported after 180 minutes across the Calu-3 monolayers was higher in the basolateral-apical direction than apical-basolateral direction. Also, the amount of [^{3}H]ritonavir transported enhanced in the presence of MK571, a known MRP inhibitor. These results are also supported by the uptake results that show the MRP2 mediated transport of ritonavir.
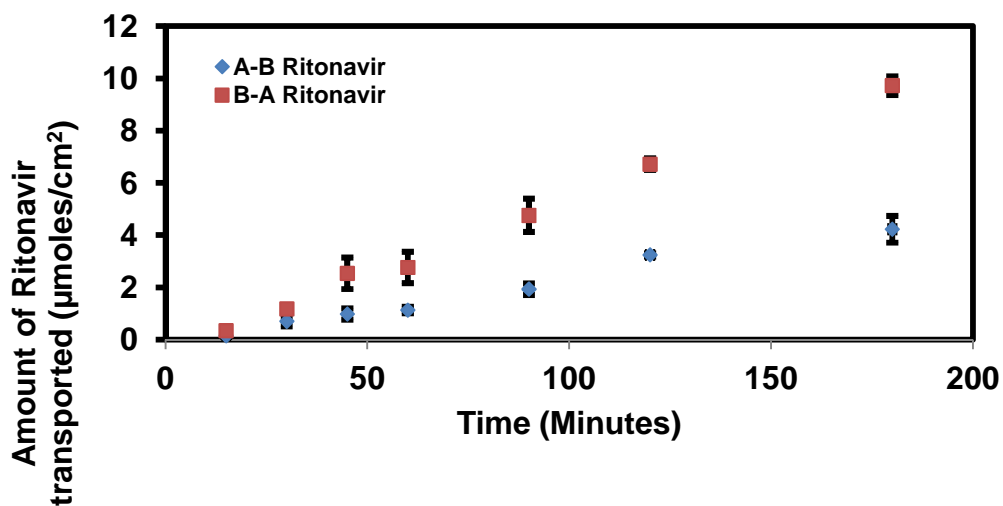
Figure 5.7 A-B and B-A transport of [^{3}H]-Ritonavir in Calu-3 cells
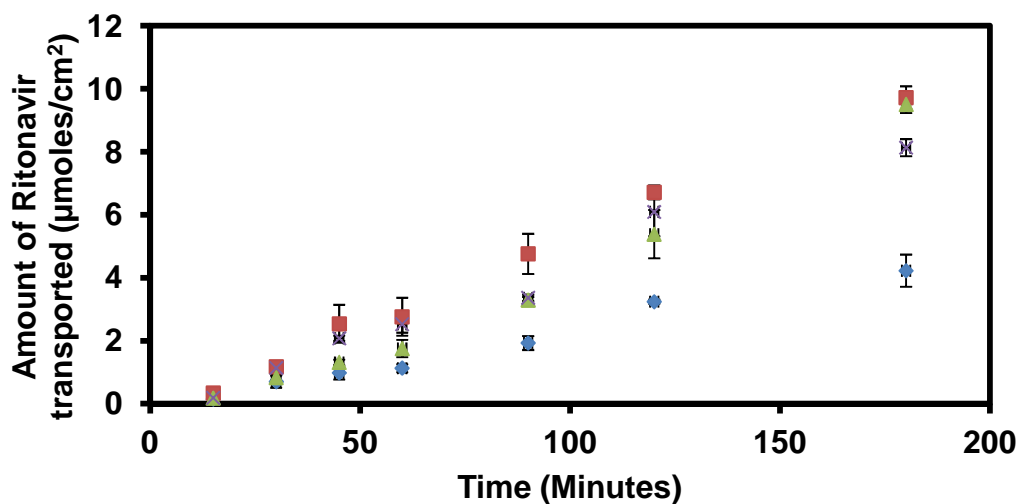
Figure 5.8   A-B and B-A transport of [³H]-ritonavir in presence of MK-571 (50 µM)

Conclusions

This study demonstrates the presence and functional activity of MRP2 and MRP1 in human bronchial epithelial cell line, Calu-3. Also, this study clearly indicates that ritonavir is actively effluxed by p-glycoprotein and MRP2 and there is possibility of drug-drug interaction *in vivo* due to the localization of these efflux transporters. However, role of MRP2 on the absorption of inhaled drugs needs to be investigated.

CHAPTER-6

TO CHARACTERIZE THE MOLECULAR AND FUNCTIONAL ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN IN HUMAN BRONCHIAL EPITHELIAL CELLS, CALU-3

Rationale

Breast cancer resistance protein (BCRP), a 72 kDa protein belongs to the subfamily G of the human ATP binding cassette transporter superfamily. Overexpression of BCRP was found to play a major role in the development of resistance against various chemotherapeutic agents. BCRP plays an important role in absorption, distribution and elimination of several therapeutic agents. BCRP expression and functional activity across human bronchial epithelium and its impact on pulmonary drug accumulation has not been established. Efflux transporters are believed to play an important role in preventing accumulation of potentially toxic xenobiotics in the lung [131]. Efflux pumps along with mucociliary clearance, alveolar macrophages and bacteriocidal surfactants act as a protective barrier in the lungs. Recent studies have shown that majority of the BCRP substrates were basic lipophilic amines which readily accumulate in lung tissue. P-glycoprotein has also been shown to play a moderate role in the pulmonary accumulation of amine drugs. Also, efflux transporters such as P-gp and MRPs have been attributed to drug resistance in lung cancers. BCRP is also involved in efflux of various chemotherapeutic agents employed in lung cancer which include mitoxantrone, doxorubicin, topotecan [135] and gefitinib [136]. Elevated mRNA levels have been correlated

with resistance to these anticancer agents in lung cancer. BCRP expression in lungs might play a role in the disposition of drugs in cancer chemotherapy. Therefore, our objective is to identify and characterize the expression of BCRP in human bronchial epithelial cells.

Introduction

Breast cancer resistance protein (BCRP) was initially identified in a breast cancer derived cell line that showed drug resistance even in the presence of verapamil (a potent P-gp inhibitor) [140]. This protein was termed BCRP as it was first isolated from a human MCF-7 breast cancer cell line in an attempt to elucidate non-P-glycoprotein mechanisms of drug resistance. This efflux pump was also identified in a mitoxantrone-resistant human colon carcinoma cell line S1-M1–80 and hence gained the name MXR [48].

BCRP/MXR is the second member of subfamily G of ATP-binding cassette (ABC) transporter superfamily. It is also referred to as ABCP (P stands for placenta), to indicate high levels of expression in placental tissue. BCRP, MXR and ABCP are homologous proteins differing only in one or two amino acid sequence. BCRP structurally diverges from the other prominent ABC transporters. BCRP is termed as half transporter having only six transmembrane helices and only one nucleotide binding domain. With the help of low resolution crystallography, it has been shown that functional BCRP has a homodimeric structure [50, 141].

BCRP is a high efficiency efflux pump with broad substrate specificity [142]. BCRP expression is maximum in placenta and significantly high in the intestine and liver.

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BCRP expression in colon, brain, lungs, ovary and testis has also been reported [143]. Various categories of drugs such as tyrosine kinase inhibitors, antivirals, HMG-CoA reductase inhibitors, carcinogens and flavonoids have been reported to be either substrates and/or inhibitors of this transporter system [142]. Studies with ABCG2 knockout mice have revealed the physiological significance of this efflux transporter across barriers such as blood-brain, blood-testis and blood-fetal barriers [144]. It plays a protective role across these barriers by active efflux of xenobiotics, pollutants, chemicals and toxins.

Various cell lines were utilized to investigate the expression and modulation of BCRP [143, 145]. Calu-3 cell line, derived from bronchial epithelium, has been employed as a model for the air way epithelium in a number of drug transport and metabolism studies [125, 146]. Several advantages of Calu-3 cell line in comparison to other models of the airway epithelium, such as tracheal epithelial sheets or primary tracheal cell cultures have been reported. Calu-3 cells form polarized monolayers with high tight junctions producing high TEER values [126]. These cells express in vivo features of the airway epithelium (cilia, mucus production), several transport and metabolic systems relevant to drug absorption. These cells also produce several cell adhesion proteins (ZO-1 and E-cadherin) and generate mucosal secretions [125]. In vitro-In vivo studies indicated good correlation (0.94) between permeability properties of Calu-3 cells with the rate of drug absorption from the rat lung [126]. Previous investigations established the functional activity of various efflux proteins belonging to ABC transporter super family such as MDR1 [137] and MRP-1 [23] in

Calu-3 cells. Therefore, Calu-3 cell line has been selected as a model cell line to investigate the BCRP expression and to estimate its functional activity.

Materials and Methods

Cell Culture

Calu-3 cells were cultured in DMEM-F12 medium supplemented with 10% heat inactivated fetal bovine serum, MEM non-essential amino acids, HEPES, sodium bicarbonate, penicillin (100 µg/ml) and streptomycin (100 µg/ml). Cells were maintained at 37^oC, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. Medium was replaced every alternate day until 5 days and subsequently every day until 11 days. Cells were subcultured by trypsinization with 0.25% trypsin containing 0.537 mM EDTA.

RT-PCR

Isolation of total RNA from cells was carried out with Trizol-LS® reagent (Invitrogen) according to manufacturer's instructions. RNA was mixed with 1.25 µl of oligo dT_{15} primer and denatured at 70^oC for 10 minutes and 4^oC for 5 minutes. Following denaturation, it was reverse transcribed to cDNA with 1 µl (10 units) of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) per reaction mixture. After the first strand cDNA synthesis, 1 µl of cDNA was used for the PCR. GAPDH and ABCG2 primers as shown in table-1 were designed using OligoPerfect™ Designer (Invitrogen Corp. Carlsbad, CA). Briefly, the PCR mixture was adjusted to a final

volume of 50 µl and contained template cDNA, 250 nM each of forward and reverse primers for the gene of interest, 1X Mg free PCR buffer (Promega), 3.75 mM $MgCl_2$, 0.025U Taq Polymerase (Promega), and 200 µM dNTPs, The polymerase chain reaction conditions consisted of denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 sec and 72°C for 1 min with a final extension at 72°C for 10 min. Ten microliters of PCR products obtained from PCR were separated by 2% agarose gel in tris-acetate-EDTA buffer along with 100 bp ladder.

Table-5 PCR primers for GAPDH and ABCG2

Gene	Primers (5′to 3′)	Product size (bp)	GenBank No.
GAPDH	GGGAAGGTGAAGGTCGGAGT GTCCACCACTGACACGTTGG	729	NM_002046
ABCG2	GCTGCAAGGAAAGATCCAAG TTCCTGAGGCCAATAAGGTG	508	NM_004827

Western Blot Analysis

Calu-3 cells grown in culture flasks were washed with phosphate-buffered saline (PBS) three times for ten minutes each. Cells were scrapped and homogenized in lysis buffer (PBS without calcium and magnesium, 1% Triton-x and protease inhibitor cocktail). The lysate was kept on ice for 30 minutes and then homogenized. Cell lysate

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was then centrifuged at 20,000 rpm for 10 minutes at 4°C. The supernatant was collected and stored at -80°C until further use. Protein content was measured with Bradford reagent. Three concentrations (25 µg, 50 µg and 75 µg) of protein were employed for gel electrophoresis. Protein samples were incubated at 100°C for 3 minutes and 15 µl sample was loaded onto a 4-12% NuPage Bis-Tris gel. Electrophoresis was carried out at 120V and subsequently transblotted at 15V for 90 minutes onto a polyvinylidene fluoride membrane. Blot was then blocked overnight with 3% nonfat dry milk and 1.5% bovine serum albumin. The membrane was incubated with BXP-21 monoclonal antibody (1:200 dilution) for 2 hours at room temperature and then probed with secondary antibody tagged with horse radish peroxidase. Bands were visualized with ChemiImager 8900 digital imaging system (Alpha Innotech, San Leandro, CA).

Immunocytochemistry

Calu-3 cells grown on slides were used for these studies. Cells were washed with cold PBS for 10 minutes thrice and then fixed with 4% paraformaldehyde for 10 minutes. Fixed cells were washed and then permeabilized with 2% saponin and incubated for 2 min at room temperature. Nonfat dry milk (3%) and bovine serum albumin (1.5%) were used for blocking for 2 hours. Membrane was incubated with mouse monoclonal antibody (BXP-21) for 2 hours. Following incubation with primary antibody, FITC tagged secondary antibody was added to cells and incubated for 1 hour at room temperature. For the negative control, slides without primary antibody were employed.

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Mounting medium was then added to chamber slides without any air bubbles and then stored at 4°C until further use. Slides were visualized using a confocal fluorescence microscope.

Uptake Studies

At 11-13 days post seeding, cells were rinsed three times with DPBS (pH 7.4, 129 mM NaCl, 2.5mM KCl, 7.4mM Na_2HP0_4, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 0.7 mM $MgSO_4$ and 5.3 mM glucose) and equilibrated for 15 minutes with the buffer. [^{3}H]-Mitoxantrone (166 nM) was used as a BCRP substrate to characterize functional activity. Time dependent uptake of [^{3}H]-mitoxantrone was performed to determine the incubation time for all the studies. Uptake studies were performed by incubating a fixed amount of 0.5 µCi/ml of [^{3}H]-mitoxantrone alone and in the presence of BCRP inhibitors at 37^0C. GF120918 (0.5, 1 and 5 µM), quercetin (50 µM) and saquinavir (50 µM) were added as BCRP inhibitors to determine the functional activity of BCRP. Following incubation, the reaction was stopped by addition of ice cold stop solution (210 mM KCl, 2 mM HEPES; pH 7.4). After three washings with stop solution, cells were lysed by keeping them overnight in 1 ml of 0.1% Triton-X solution in 0.3% NaOH. Following overnight incubation, 500 µl of the cell lysate from each well was transferred to scintillation vials containing 5 ml of scintillation cocktail. Samples were analyzed by liquid scintillation counter and uptake was normalized to the protein content in each well. Amount of protein in the cell lysate was measured by the Bio-Rad protein estimation kit with bovine serum

albumin as standard. Functional activity of BCRP was assessed by studying the uptake of [^{3}H]-mitoxantrone in the presence of 0.5, 1 and 5 µM GF120918.

Hoechst 33342 Accumulation and Cytotoxicity Studies

Hoechst 33342, a fluorescent substrate was added to assess the efflux mediated by BCRP. Culture medium was first removed from the 96 well plates and washed with DPBS buffer at pH-7.4. Concentration dependent accumulation of Hoechst 33342 dye was measured to determine the experimental concentration for the remaining studies. Cells were incubated with varying concentrations of Hoechst 33342 dye ranging from 1 µM to 100 µM for 15 minutes. After 15 min incubation, reaction was arrested with stop solution (210 mM KCl and 2 mM HEPES; pH 7.4) and lysed with 1 ml of lysis buffer (0.1% Triton-X solution in 0.3% NaOH). Amount of intracellular dye was measured with a fluorescence spectrophotometer. Excitation and emission filters were set at 370 and 450nm and the intracellular mean fluorescence was normalized to protein content. For the remaining studies, cells were incubated with 5 µM Hoechst 33342 (100 µl) in DPBS buffer with or without BCRP inhibitors GF120918 (5 µM and 10 µM) and fumitremorgin C (1 µM and 5 µM). Similarly, ATP dependent accumulation study of Hoechst 33342 was performed in the presence of ouabain (1mM) and 2, 4, dinitrophenol (1 mM). Cytotoxicity studies of Hoechst 33342 dye alone and in the presence of BCRP inhibitors and ATP modulators was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. 10 % DMSO was selected as a positive control for the

cytotoxicity studies. Cytotoxicity tests were performed according to manufacturer's protocol.

Statistical Analysis

All results were expressed as mean ± standard deviation. All the experiments were done in triplicate. Statistical analysis between two groups of data was carried out with a student's t-test. A difference between mean values was considered significant at the P-value less than 0.05.

Results and Discussion

Pulmonary delivery has been utilized to administer several small molecule drugs, as well as therapeutic peptides and proteins. Small molecules delivered through inhalation route have higher bioavailability due to the lack of first pass metabolism and resistance to peptidases in the lungs. Lungs are constantly exposed to harmful xenobiotics and air borne toxins [147]. Therefore, the organ displays protective efflux mechanisms to protect it from the external environment. Air-lung epithelial barrier is the most significant barrier to absorption for the inhaled drugs. Studies have shown that p-glycoprotein expressed in airway and bronchial epithelial cells is involved in the removal of environmental xenobiotics and cytotoxic drugs into the lumen and blood. Earlier studies from our lab have shown that p-glycoprotein limits the transport of anti-HIV protease inhibitors across Calu-3 cells (Patel et al., 2002). MRP1 has also been shown to be expressed on the basolateral side of the airway epithelium of the normal lung tissue.

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However, BCRP expression in bronchial epithelial cells and its impact on the kinetics of inhaled drugs has not been investigated.

The present study provides the first evidence for the expression of BCRP across air-lung epithelial barrier. Calu-3 cell line has been validated as a metabolic and transport model to study the mechanisms of drug delivery at respiratory epithelium. Therefore, we selected Calu-3 cell line as a model cell line to investigate the BCRP expression studies. Previous studies have shown that Calu-3 cells exhibited higher TEER values and stable morphology between 11-13 days. Calu-3 cells grown for 11-13 days were used for the functional activity studies.

Detection of ABCG2 mRNA Levels in Calu-3 Cells

RT-PCR was carried out to determine the ABCG2 mRNA levels in Calu-3 cells. RNA was extracted from Calu-3 cells grown in DMEM-F12 medium with 10% FBS using trizol reagent. RT was performed using MMLV-RT enzyme and RNA was reverse transcribed to cDNA. Resulting cDNA was used as a template for PCR reaction. Primers specific to BCRP were designed using an Oligoperfect designer. GAPDH was used as an internal standard. Amplified PCR products obtained were separated by 2% agarose gel electrophoresis and bands were visualized. With BCRP specific primers, bands were detected at 508 bp following gel electrophoresis (Figure 6.1). Band for GAPDH was detected at 723 bp.
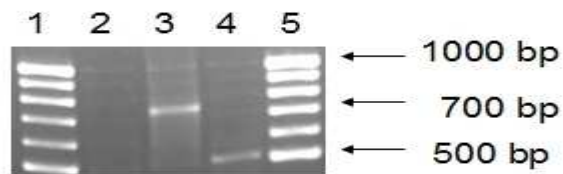
Figure 6.1 PCR image for ABCG2

BCRP Protein Detection in Calu-3 Cells

Western blot was performed to study the expression of BCRP protein in Calu-3 cells. BXP-21 a mouse monoclonal antibody raised against amino acids 271-396 of ABCG2 was used for the western blot. BXP-21 does not cross react with other efflux transporters such as p-glycoprotein, MRP-2 and MRP-1. Western blot was performed to determine the expression of BCRP protein in Calu-3 cells. Protein samples were electrophoresed by SDS-PAGE and transferred to a PVDF membrane. BXP-21 monoclonal antibody was used to detect BCRP protein in Calu-3 cells. Lanes 2, 3 and 4 were loaded with 25, 50 and 75 µg protein extracted from Calu-3 cells. Figure 6.2 illustrates the bands detected by chemiluminescence. Bands were observed at approximately 72 kDa corresponding to BCRP.
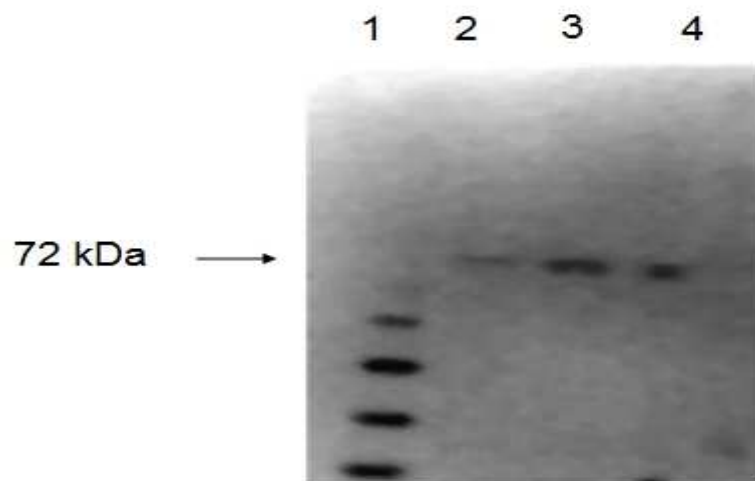
Figure 6.2   Western blot analysis of breast cancer resistance protein BXP-21 monoclonal antibody was used to detect BCRP protein in Calu-3 cells. Lanes 2, 3 and 4 were loaded with 25, 50 and 75 µg protein extracted from Calu-3 cells.

Immunocytochemical Detection of BCRP
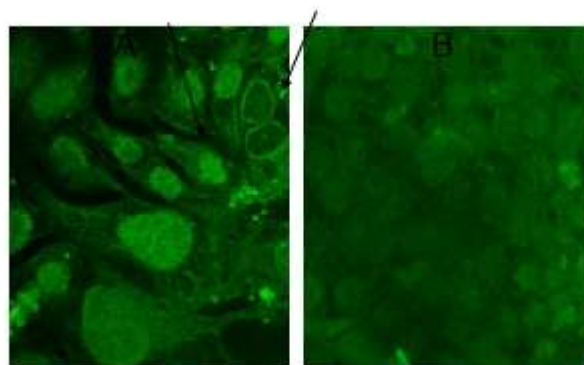
Figure 6.3 Confocal microscopy of breast cancer resistance protein

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BCRP expression was evaluated by immunocytochemical staining with a BXP-21 mouse monoclonal antibody. Calu-3 cells revealed strong plasma membrane localization (Fig. 6.3). In addition, some cytoplasmic staining was also observed with the use of BXP-21.

Uptake Studies with Radioactive Mitoxantrone

Time dependent uptake was performed to determine the uptake time for inhibition studies. Uptake of [^{3}H]mitoxantrone was found to be linear for 30 minutes as shown in Figure 6.4. So, a 15 minute time period was selected for all subsequent inhibition studies. Uptake of [^{3}H]mitoxantrone gradually increased significantly with increasing concentrations of GF120918.
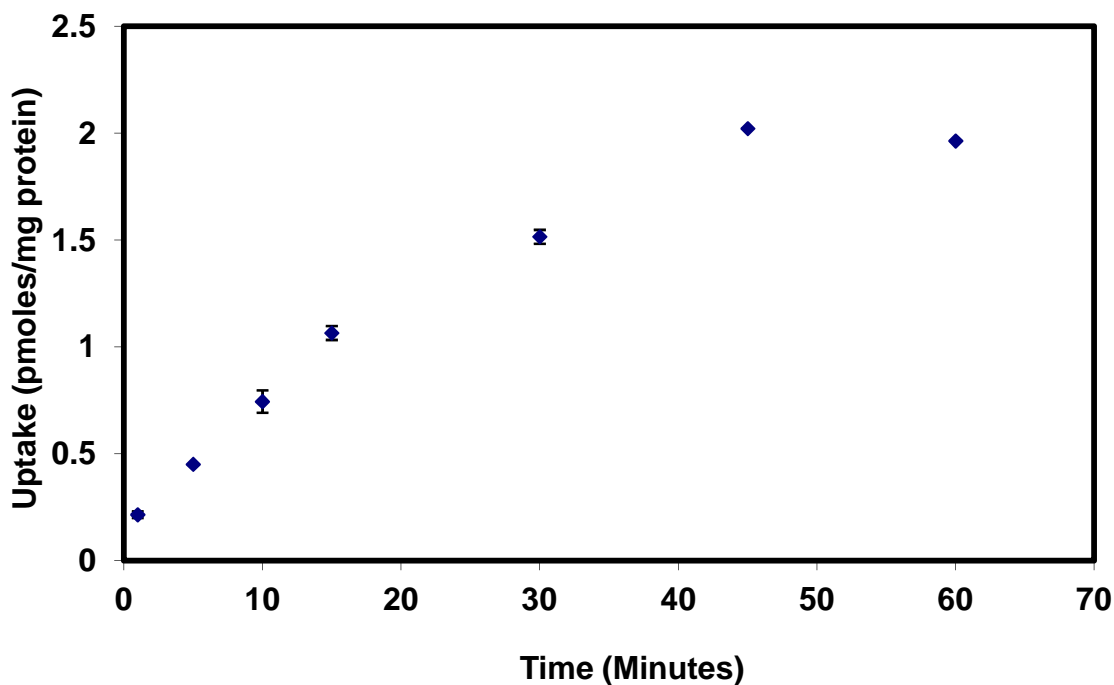
Figure 6.4 Time dependent study of [^{3}H]mitoxantrone

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As shown in Figure 6.5, uptake of [³H]mitoxantrone was found to be 112 ± 3.7%, 133 ± 4.2%, 145 ± 11.7% in the presence of 0.5, 1 and 5 µM GF120918 respectively as relative to control. Quercetin (50 µM) and Saquinavir (50 µM) enhanced the uptake of mitoxantrone to 141 ± 3.7%, 193 ± 2.6% respectively as compared to control (Figure 6.6).
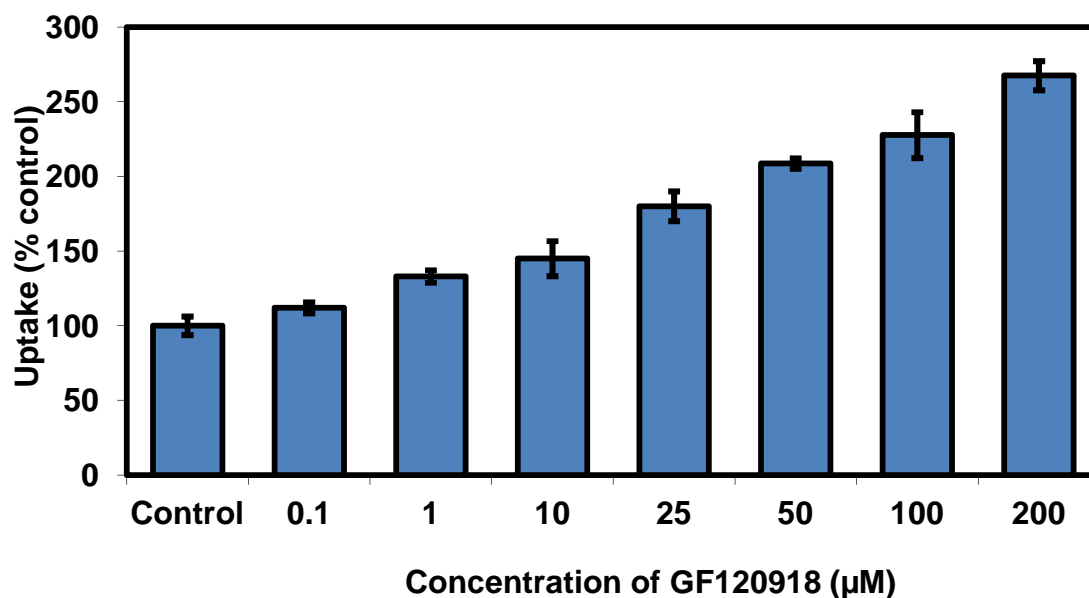
Figure 6.5   Concentration dependent study of [³H]mitoxantrone
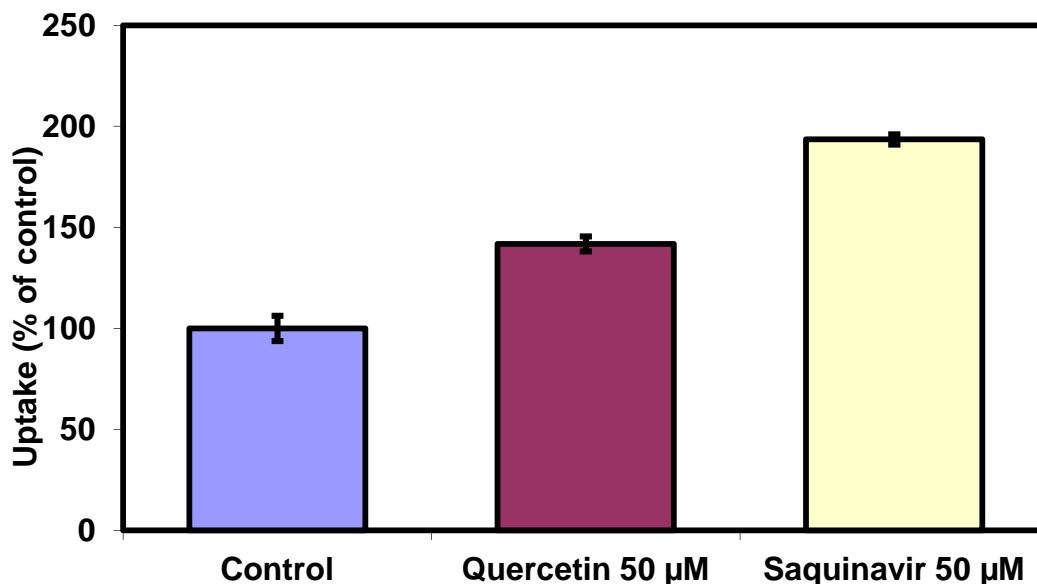
Figure 6.6   Uptake of [³H]mitoxantrone in presence of BCRP inhibitors

Hoechst 33342 Accumulation Studies

BCRP functional activity was evaluated by measuring the accumulation of Hoechst 33342 in Calu-3 cells. Concentration dependent accumulation of Hoechst 33342 was also performed to select the 5 µM experimental concentration. ABCG2 specific inhibitors GF120918 and fumitremorgin C were selected for inhibition studies. As shown in Fig. 6.8, Hoechst 33342 uptake was found to be $168.37 \pm 11.7\%$, $159.37 \pm 7\%$, $150.49 \pm 6.5\%$ and $164.34 \pm 7.23\%$ as compared to control in the presence of 5 µM GF120918, 10 µM GF120918, 1 µM fumitremorgin C and 5 µM fumitremorgin C. To study the energy dependency of Hoechst 33342 dye, uptake of the fluorescent dye was performed in the presence of 1 mM ouabain (Na^+ /K^+ -ATPase inhibitor) and 1 mM 2, 4-dinitrophenol (intracellular ATP reducer).
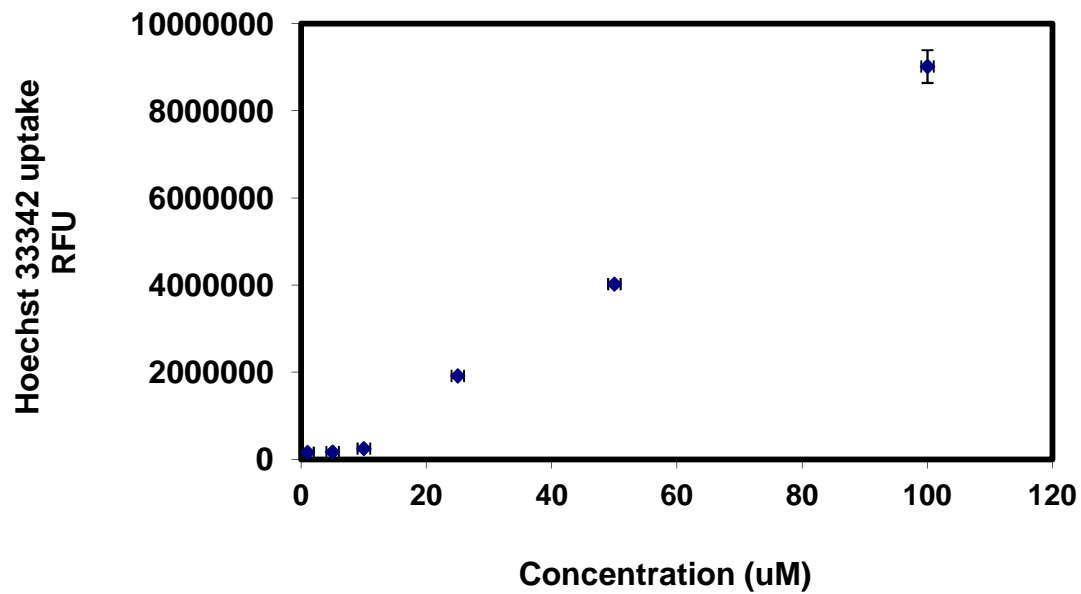
Figure 6.7 Concentration dependent study of Hoechst 33342 dye
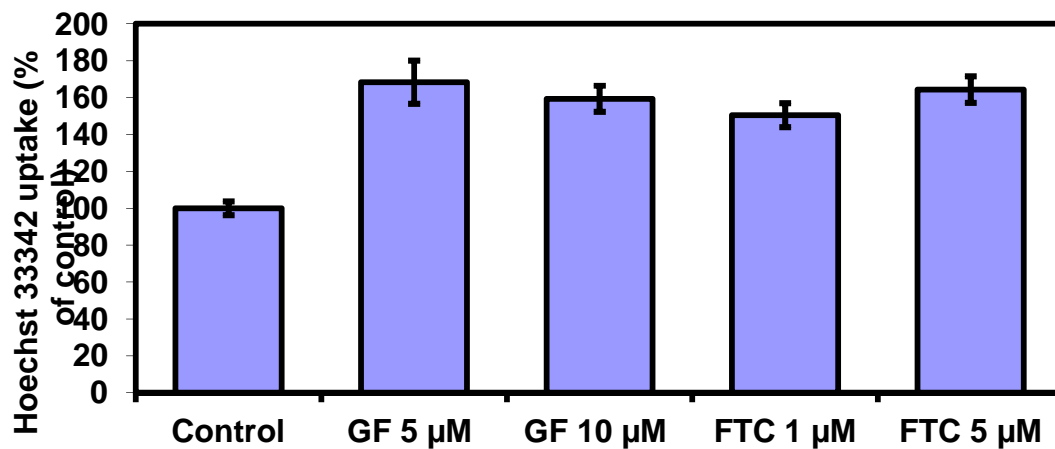
Figure 6.8 Hoechst 33342 uptake in presence of different concentrations of GF10218 and Fumitremorgin C

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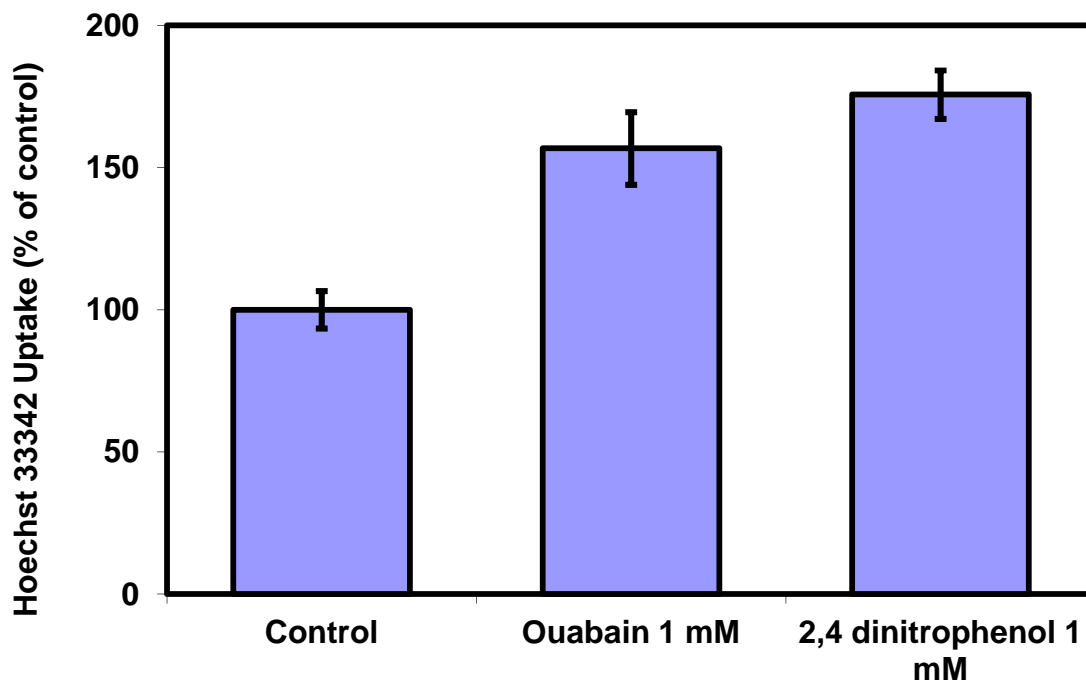
Figure 6.9 Energy dependent uptake of Hoechst 33342

In presence of ouabain and 2, 4-dinitrophenol, Hoechst dye uptake enhanced significantly (Figure 6.9). Uptake was found to be $156.43 \pm 12.76\%$ and $175.68 \pm 8.5\%$ respectively in presence of 1 mM ouabain and 1 mM 2, 4-dinitrophenol.

Cytotoxicity Studies

In the course of our studies, to determine whether the Hoechst 33342 dye, BCRP inhibitors and ATP modulators were cytotoxic to the cells, cytotoxicity studies were performed with MTT assay kit. Hoechst 33342 and the inhibitors were incubated with the

cells under the same conditions as the accumulation assay. Viability of Calu-3 cells was found to be unaffected in the presence of BCRP inhibitors and ATP modulators (6.10).
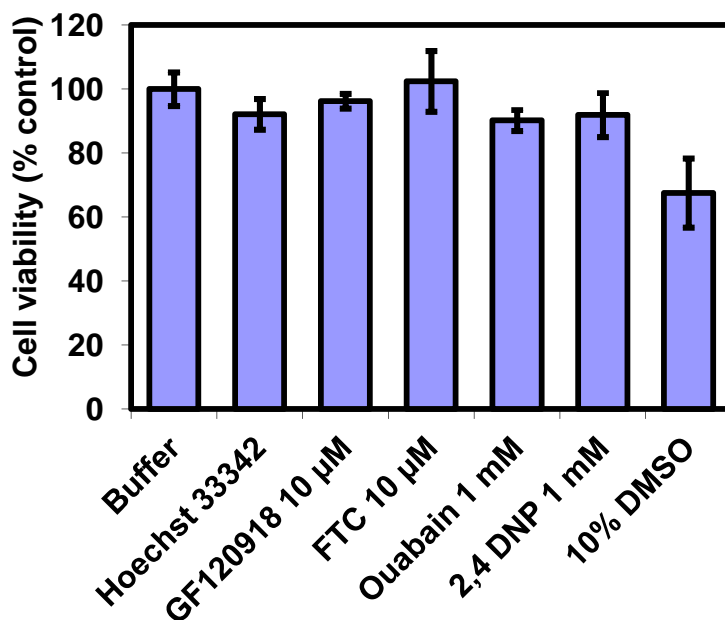
Figure 6.10   Cytotoxicity in presence of BCRP inhibitors

In our studies, radioactive mitoxantrone was used as a substrate to determine the functional activity of BCRP. Mitoxantrone had been shown to possess a high affinity substrate to BCRP and its accumulation correlated well with BCRP expression. Drug resistant cell lines that overexpress BCRP and cell lines transfected with BCRP cDNA were found to accumulate lower amounts of mitoxantrone [148]. Radioactive mitoxantrone

has been used as a radiolabeled substrate for BCRP and incubated for 1.5 hours, 2 hours and 3 hours to determine the functional activity. Radioactive mitoxantrone was used alone and in the presence of BCRP specific inhibitors in these studies. Time dependent uptake of mitoxantrone was performed to determine the optimal time of incubation. Based on the results, 15 minute incubation was selected for all the experiments. Results from the uptake studies reveal that uptake of mitoxantrone was increased in presence of BCRP inhibitors GF120918, quercetin and saquinavir. GF120918 is a potent BCRP inhibitor with an IC50 value of 50 nM. Fumitremorgin c is a more specific and potent inhibitor for BCRP efflux pump than GF120918. Miscellaneous potent inhibitors such as flavanoids [149] and protease inhibitors [150] were also used to confirm the BCRP inhibition. Uptake increased significantly in the presence of BCRP inhibitors indicating the presence of functionally active BCRP. HIV protease inhibitors were found to be potent BCRP inhibitors and maximum concentration of saquinavir used for studies was 50 µM (Weiss et al 2004). Zhang et al have shown that flavanoids strongly inhibited the BCRP mediated accumulation of mitoxantrone. Mechanism of interaction between BCRP and its substrates and inhibitors is very complex due to the presence of multiple binding sites on BCRP. Also, ATP hydrolysis is one of the major mechanism by which the inhibitors interact with BCRP. Flavanoids bind to the nucleotide binding domain of BCRP whereas mechanism of interaction between BCRP and HIV protease inhibitors is yet to be understood completely.

Litman et al correlated BCRP expression with reduced accumulation of several fluorescent drugs such as mitoxantrone, daunorubicin, bisantrene, topotecan, lysotracker and rhodamine 123 in BCRP overexpressing cells. P-gp and Mrp1 substrates were not effluxed from BCRP overexpressing cells which indicated the differentiation of BCRP mediated drug resistance. Kim et al studied the ability of BCRP to efflux the fluorescent dye, Hoechst 33342 in hematopoietic stem cells. In our studies, Hoechst 33342 dye accumulation assay was performed to determine the functional activity of BCRP. Hoechst 33342 was used at a concentration of 5 µM which when calculated was found to be approximately 3 µg/ml. Previous studies by Scharenberg et al and Kim et al used Hoechst 33342 dye at a concentration of 5 µg/ml and 4 µg/ml for BCRP mediated efflux studies. Concentration dependent accumulation studies of Hoechst 33342 varying from 1 µM to 100 µM were done to select the experimental concentration within the linear range. A concentration of 5 µM Hoechst 33342 dye was selected for the fluorescent dye accumulation studies. Hoechst 33342 dye accumulation elevated significantly in presence of BCRP inhibitors such as GF120918 and fumitremorgin C. Fluorescent dye studies with ouabain and 2, 4-dinitrophenol indicated the energy dependent efflux of Hoechst dye in Calu-3 cells. These results suggest the involvement of energy dependent efflux process. MTT assay revealed the lack of toxicity of Hoechst 33342 alone and in the presence of BCRP inhibitors and ATP modulators to Calu-3 cells.

Conclusions

In conclusion, our present study demonstrates for the first time the presence of BCRP expression and functional activity across human bronchial epithelial cell line, Calu-3. Further studies are needed to delineate the role of BCRP on the pharmacokinetics of inhaled drugs.

CHAPTER-7

TO INVESTIGATE THE EFFECT OF CHRONIC NICOTINE EXPOSURE ON THE LEVELS OF EFFLUX TRANSPORTERS AND METABOLIZING ENZYMES IN CALU-3 CELLS AND RAT LUNGS

Rationale

Lung cancer and AIDS are the major causes of morbidity all over the world. Nicotine is one of the most profoundly used addictive drugs in United States. According to world health organization, cigarette smoking is the foremost preventable cause of morbidity affecting almost one third of the global population. Nonsmokers exposed to tobacco smoke are at a similar risk as that of a light smoker. Also, conditions of the adults with preexisting pulmonary conditions such as allergies, chronic lung diseases and other opportunistic life threatening infections are intensified by cigarette smoking. Previous research reports have shown that nicotine is linked with tumor promotion and resistance to therapy in lung cancer. There is limited information available about nicotine mediated induction of chemoresistance and hence there is a need to investigate the effect of chronic nicotine exposure. In case of HIV infection, it is important to treat the opportunistic infections and to monitor the clinical efficacy of pulmonary drugs and HIV protease inhibitors. Since, lungs are one of the primary sanctuary sites for HIV virus, this chapter aims to explore the chronic effect of nicotine on the levels of efflux transporters and metabolizing enzymes (CYP3A4/CYP3A5) in vitro and rat lungs. Also, role of

nuclear receptor PXR was investigated. Recent research has shown that several protease inhibitors are successfully used in combination to treat lung cancer. According to research by National Cancer institute, nelfinavir, saquinavir and ritonavir inhibited growth of lung cancer cells in vitro. Researchers investigated six different protease inhibitors in 60 human cancer cell types and mouse models. Results have shown that nelfinavir, ritonavir and saquinavir inhibited growth of non-small cell lung cancer. Interactions can be mediated by MDR1, MRP2 and BCRP efflux transporters and CYP3A4/CYP3A5 metabolizing enzymes expressed in lungs. Drug interaction uptake studies in Calu-3 cells (human bronchial cancer cells) in our laboratory demonstrated functional activity of efflux transporters such as MDR1 and BCRP in Calu-3 cells. CYP3A4/CYP3A5 expression was identified and functional activity of cortisol was established in Calu-3, rat lung and human lung microsomes. In view of the above observations, chronic nicotine exposure can play a significant role in induction of efflux transporters and metabolizing enzymes resulting in drug resistance and subsequent therapeutic failure.

Introduction

Pulmonary drug delivery is a noninvasive route of drug delivery used for delivery of locally acting compounds as well as for the compounds targeting blood circulation. Pulmonary drug delivery is the ideal mode of delivery for rapid onset of action. Air borne suspension of fine particles are delivery by devices such as metered dose inhalers,

nebulizing solutions and dry powder inhalants. Lung is the only organ that receives the entire cardiac output. In order for the pulmonary drugs to reach their target, drugs has to cross the epithelial barrier in the lungs. Tracheal and bronchial epithelium constitutes the air way epithelium (Figure-7.1). Major cell types found in the lungs are ciliated columnar, goblet and basal cells. These ciliated columnar cells are responsible for the secretion of efflux proteins and also they have a higher cytochrome P450 metabolizing capacity.
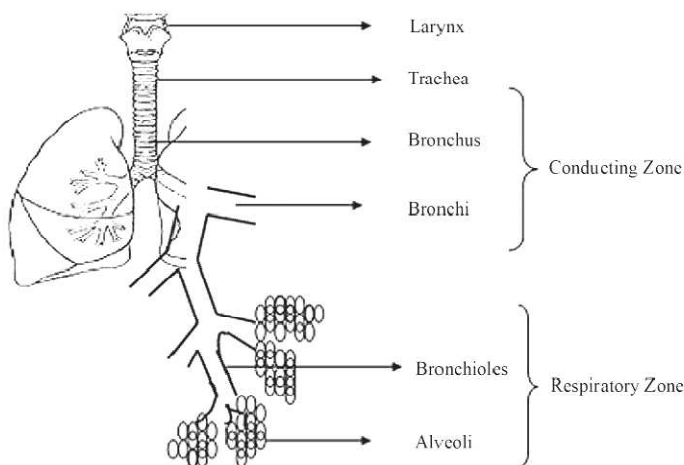
Figure 7.1   Anatomy of lungs

Therapeutic management becomes difficult in treatment of diseases. Several protease inhibitors are recently being investigated for their use in cancer therapy. Ritonavir, saquinvir and nelfinavir inhibited growth of non-small cell lung cancer.   HAART therapy has been used for treatment of HIV infections. Patients are often treated for opportunistic infections of the lung. Drug interactions are often indicated for the pulmonary therapies. Rifampicin accelerates the metabolism of HIV protease inhibitors

resulting in sub therapeutic serum levels whereas protease inhibitors slow the metabolism of rifampicin leading to increased serum levels and cytotoxicity. Rate of smoking in HIV population is 57% higher than in general population (33%). In addition, smoking is the main risk for 87% of cases associated with lung cancer. Nicotine readily diffuses through skin, lungs and mucous membranes. It has a half-life of about 60 minutes. Nicotine is metabolized to cotinine and nicotine oxide by lungs. Thus, the purpose of this study was to investigate whether nicotine can induce the expression of efflux transporters and metabolic activity of CYP3A4.

Materials and Methods

Calu-3 Cell Culture

Calu-3 cell line, an airway epithelium derived cell line from human lung carcinoma was procured from ATCC. Calu-3 cells between passages 15-40 were employed for all the studies. Cells were cultured in DMEM-F12 supplemented with 10% heat inactivated fetal bovine serum, non-essential amino acids (NEAA), HEPES, sodium bicarbonate, penicillin (100units/ml) and streptomycin (100 µg/ml) were purchased from Sigma Chemical Co. Cells were maintained at 37^oC, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. The medium was replaced every alternate day.

Preparation of Microsomes

Calu-3 cells grown for 11 days were washed thrice with phosphor buffered saline and scraped with a cell scrapper. The cell suspension was centrifuged at 1000 g and the pellet was suspended in cell homogenization buffer (50 mM Tris HCl buffer, pH-7.4, 0.25 M sucrose, 1 mM EDTA and protease inhibitor cocktail). The suspension was then homogenized. Nuclear and mitochondrial fractions were removed after centrifuging the samples at 3000 g for 10 min.
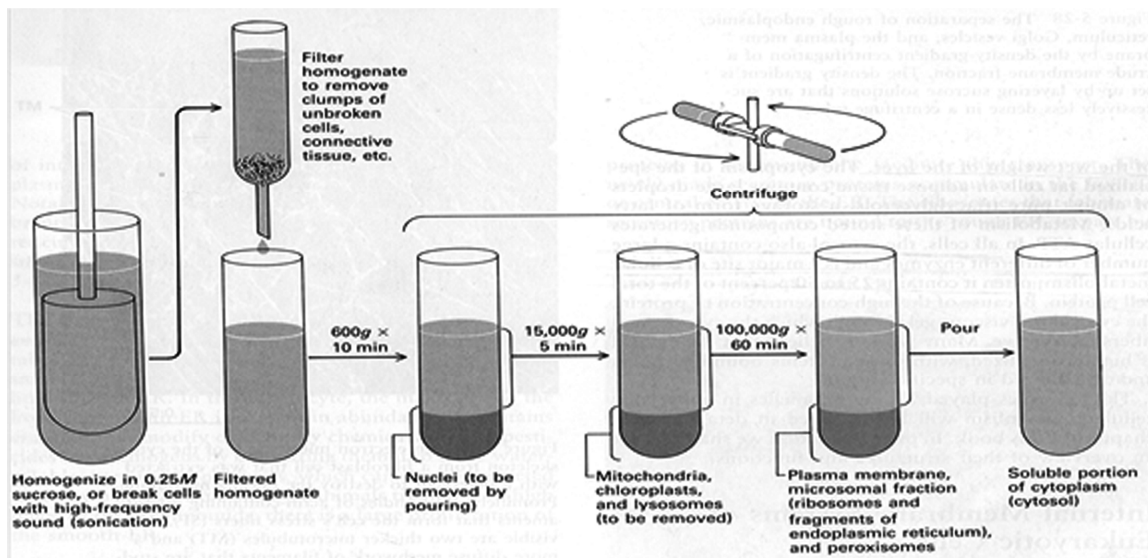
Figure 7.2 Microsomes extraction procedure

Later mitochondrial fraction was discarded after centrifuging the sample at 9000 g for 20 minutes. Supernatant obtained from the previous step was centrifuged at 105,000 g for one hour to obtain the microsomal pellet. Microsomal protein was stored at -80°c for further studies. Protein content of the microsomes was measured by adding Bradford

reagent. Microsomes were extracted after treating with nicotine for seven days according to the figure -7.2.

Western Blotting

Whole cell protein was extracted with reagent containing 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 1% Triton X - 100 and protease inhibitor cocktail at pH 7.4. Confluent cells were washed thrice with PBS and harvested using a cell scraper in 5 mL of PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes and the pellet was resuspended in freshly prepared lysis buffer for 15 minutes on ice. The extracted protein was then obtained by centrifugation and stored at -80°C, until used. Protein content was determined using Bradford method. Polyacrylamide gel electrophoresis (PAGE) was run with 25 and 50 µg of each protein at 120 V, 250 mAmp. Transfer was carried out on polyvinylidene fluoride (PVDF) membrane at 25 V for 1 h 30 min, on ice. Immediately after transfer, the blot was blocked for 3 hours in freshly prepared blocking buffer (2.5 % non-fat dry milk and 0.25 % bovine serum albumin prepared in TBST pH-8). After a light wash for 10 sec, the blots were exposed to primary antibodies overnight. The blots were then exposed for 2 hours to secondary antibodies obtained from Santa Cruz Biotechnology. The blots were finally washed three times with TBST and developed using SuperSignal West Pico chemiluminescence substrate. The blots were exposed for 30 sec after which the image was taken in Gel Doc Imager.

Real Time PCR

RNA was extracted from the control and nicotine treated lung tissues using Trizol reagent (Invitrogen). All samples were normalized to 1 µg of total RNA. 1 µg of total RNA was mixed with 1.25 µl oligo dT primer at 70ºc for 10 minutes and then reverse transcribed to cDNA using MMLV-reverse transcriptase enzyme. Real time PCR was according to a standard protocol published by Roche. Real time PCR primers were designed using oligo perfect designer. All the primers were designed such that the amplicons generated were between 100-200 bp long to increase the efficiency of simultaneous amplification of target and reference genes. Briefly, the PCR mixture has a volume of 20 µl. SYBR green kit from Roche has 2X concentration of PCR master mix and PCR grade water. To this master mix, 250 nM each of forward and reverse primers of gene of interest were added. The master mix was then transferred to a multiwall plate. 5 µg/µl cDNA sample was added to this master mix to prepare 20 µl of PCR sample. The samples were then carefully centrifuged at 3000 g for 2 minutes. Samples were analyzed and fluorescence was quantified.

Analysis

Samples for real time PCR were prepared in triplicate. Quantitative values were obtained above the threshold PCR cycle number (Ct) at which the increase in signal associated with an exponential growth for PCR products were detected. The relative mRNA levels in each sample were normalized according to the expression levels of β-actin. An

induction ratio(treated/untreated) was determined from the relative expression levels of the target gene using $2^{-\Delta Ct}$ ($\Delta Ct = Ct$ target gene-Ct β-actin). The average of the real time PCR measurements were used to calculate the mean induction ratio for each gene.

Cortisol Metabolism Studies

Cortisol was used as a model substrate to study the CYP3A4 mediated metabolism. 6-hydroxy cortisol was obtained from sigma. Briefly, microsomes isolated using standard methods were used for the metabolism studies. Fixed concentration of microsomal protein (0.5 mg/ml) was used for the studies. Microsomal protein solution (100 mM phosphate buffer (KH_2PO_4 100 mM and Na_2HPO_4, $2H_2O$ 100 mM) at pH 7.4) containing 6 mM $MgCl_2$ and 0.1 mM EDTA was incubated with NADP regenerating system (8 mM G6P, 0.1 UI/ml G6PD and 0.3 mM $NADP^+$ for 5 minutes at 37 ºc. were in a final volume of 1 ml. After activation, 200 µM cortisol was added to the above mixture and incubated for 30 minutes. The reaction was stopped by adding 500 µl methyl-tert-butyl ether. The sample was then vortexed and centrifuged at 10,000 g for 3 minutes. The upper layer was then separated and evaporated. The residue was dissolved in 200 µl mobile phase for further HPLC analysis.

HPLC Analysis of 6-hydroxycortisol

Analysis of 6-hydroxycortisol was performed according to published protocol. All samples will be analyzed by a reversed phase HPLC technique. A C_8 Luna column (250 x

4.6mm; Phenomenex, Torrance, CA) was employed for the quantification of metabolites. Mobile phase composed of 0.5 % w/v Ammonium phosphate monobasic and acetonitrile (75:25 v/v) was used to elute the samples. Flow rate will be maintained at 0.8 mL/min and detection wavelength set at 254 nm.

Rat Metabolism Studies

Human lung microsomes (10 mg/ml) obtained from smokers and nonsmokers were obtained from Xenotech LLC. Microsomes from control and nicotine treated rats were isolated from rat lungs. Microsomal protein concentration was obtained by Bradford reagent. Metabolism studies were performed according to the above mentioned protocol.

Oral Rat Studies

Male Sprague-Dawley rats weighing 200-300 g were utilized for these studies. Rats were fasted overnight before treating them with control or drug solution. Nicotine solution (5 mg/kg in 0.8 ml sterile saline) was administered by oral gavage twice a day for 5 days. After five days of exposure to nicotine, tissues were isolated and stored at -80ºc for further use.

Results and Discussion

Majority of the inhaled toxicants pass through the respiratory tract, exposing pulmonary epithelium to higher concentrations than liver cells. Higher concentrations can contribute to significant metabolism in lungs. In the same way, higher concentrations of

tobacco smoke can modulate the metabolism of CYP enzymes. Several CYP enzymes are expressed in the lungs of mammals, but studies on their modulation are very limited.

Role of pulmonary metabolism in the systemic clearance of the xeniobiotics has not been well studied. Total cardiac output reaches the lungs and therefore, systemic drugs can be metabolized in lungs. Pulmonary alveolar epithelium has a larger surface area and inhaled drugs are exposed to pulmonary enzymes resulting in significant metabolism. Expression of PXR and CAR was detected in human lungs

Expression of CYP3A4 and PXR mRNA expression

RT-PCR was performed to determine the mRNA expression in Calu-3 cells, rat lungs and normal lungs. Analysis showed that CYP3A4 was expressed in all the three tissues, Calu-3, rat lungs and human lungs at 500 bp (Figure-7.3). Strong expression of CYP3A4 was observed in human lung tissue when compared to Calu-3 cells and rat lungs.

Lane-1 - Molecular wt Marker.
Lane-2 - Calu-3
Lane-3 - Rat lung
Lane-4 - Human lung
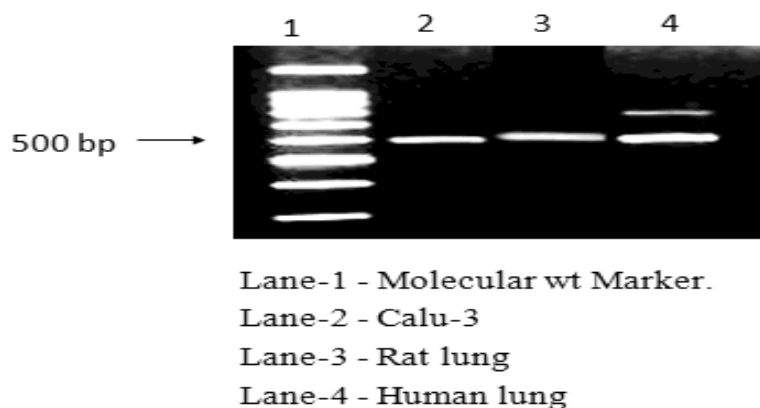
Figure 7.3 CYP3A4 mRNA expression in Calu-3 cells, rat lungs and human lungs

Nuclear Receptor Expression in Calu-3 Cells

PXR expression was confirmed in Calu-3 cells. PCR products obtained by using specific primers were detected at 320 bp (Figure-7.4). In another set of experiments, PXR, CAR and RXR expression was analyzed. Results from this experiment indicated the presence of PXR and RXR mRNA in Calu-3 cells. CAR expression was not detected in our experiment (figure-7.5).
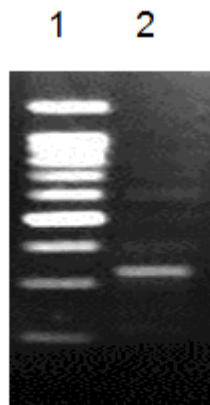
✓ Lane-1- Molecular wt marker
✓ Lane-2- Calu-3

Figure 7.4   PXR expression in Calu-3 cells

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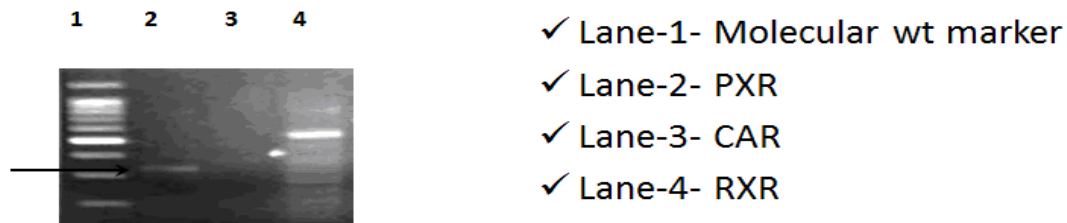
Figure 7.5   Nuclear receptors (PXR, CAR and RXR) mRNA expression in Calu-3 cells

Semi quantitative PCR was performed to quantify CYP3A4 mRNA levels in nicotine and rifampicin treated Calu-3 cells. Since we were not able to quantify by this method, real time PCR was later performed to study the induction levels (figure-7.6).
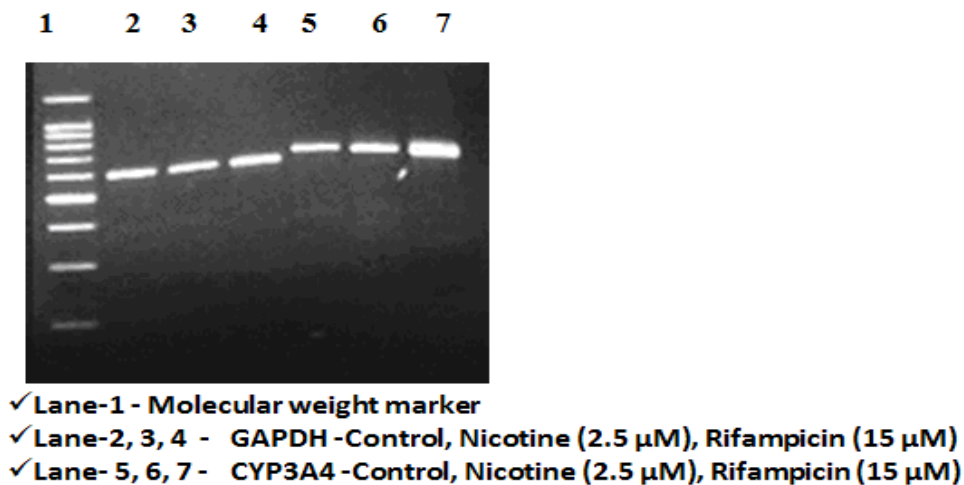
Figure 7.6   Semi quantitative RT-PCR analysis of CYP3A4 mRNA expression in Calu-3 cells after treatment with nicotine and rifampicin

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CYP3A4 Protein Expression

Western blot analysis indicated enhanced CYP3A4/CYP3A5 expression in rat lungs when compared to Calu-3 cells. Furthermore, strong CYP3A4/CYP3A5 protein expression was observed in nicotine treated lung microsomes. These results indicate induction of CYP3A4/CYP3A5 protein expression after treatment with nicotine.

Lane-1- Calu-3
Lane-2- Rat lung microsomes

Lane-1- Rat lung microsomes
Lane-2- Nicotine treated rat lung microsomes
Lane-3- Human lung microsomes
Lane-4- Human lung microsomes from smokers
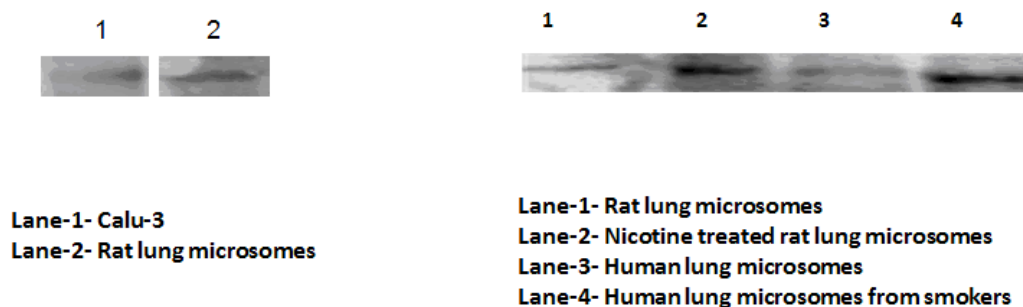
Figure 7.7   Immunoblot for CYP3A4 expression in Calu-3 cells

Real Time PCR Studies to Quantify MDR1 and ABCG2 mRNA

Calu-3 cells exposed to nicotine for 72 hours were used for real time PCR studies. MDR1 mRNA levels enhanced significantly when Calu-3 cells were exposed to nicotine. Both the nicotine concentrations (2.5 µM and 10 µM) had significant induced mRNA levels by 2 to 3 fold approximately when compared to control (figure-7.8). ABCG2 mRNA levels were enhanced by 2 fold when compared to control (figure-7.9).

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Rifampicin, a positive control for MDR1 and CYP3A4 induction also showed the induction in Calu-3 cells. Higher concentrations of nicotine enhanced CYP3A4 and CYP3A5 mRNA levels (figure -7.10).
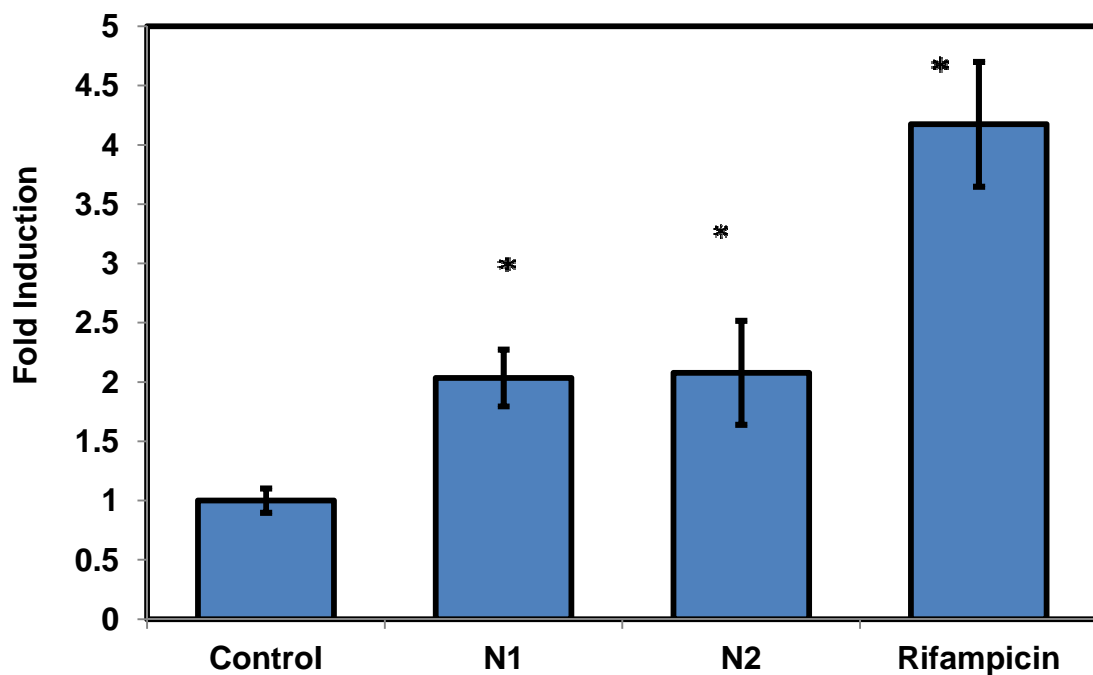
**Figure 7.8   Quantification of MDR1 mRNA protein expression in Calu-3 cells (N1 and N2 indicates 2.5 µM and 10 µM for nicotine respectively, 25 µM for rifampicin) (* indicates significant difference compared to control; *p<0.05, n = 3 ± S.D)*

A significant correlation between the expression of resistance genes, treatment failure and a decrease in overall survival has been clearly demonstrated in lung cancer

patients, supporting the implication of efflux transporter genes. Previous studies showed that vinca alkaloids and taxanes, known MDR1 substrates upregulated p-gp expression when lung cancer cell lines were exposed to vincristine. *In vivo* lung cancer xenograft models showed that cytotoxic drugs can induce drug resistance mediated by MDR1 and MRP genes. This induction explained treatment failure in non-small cell lung cancer patients.
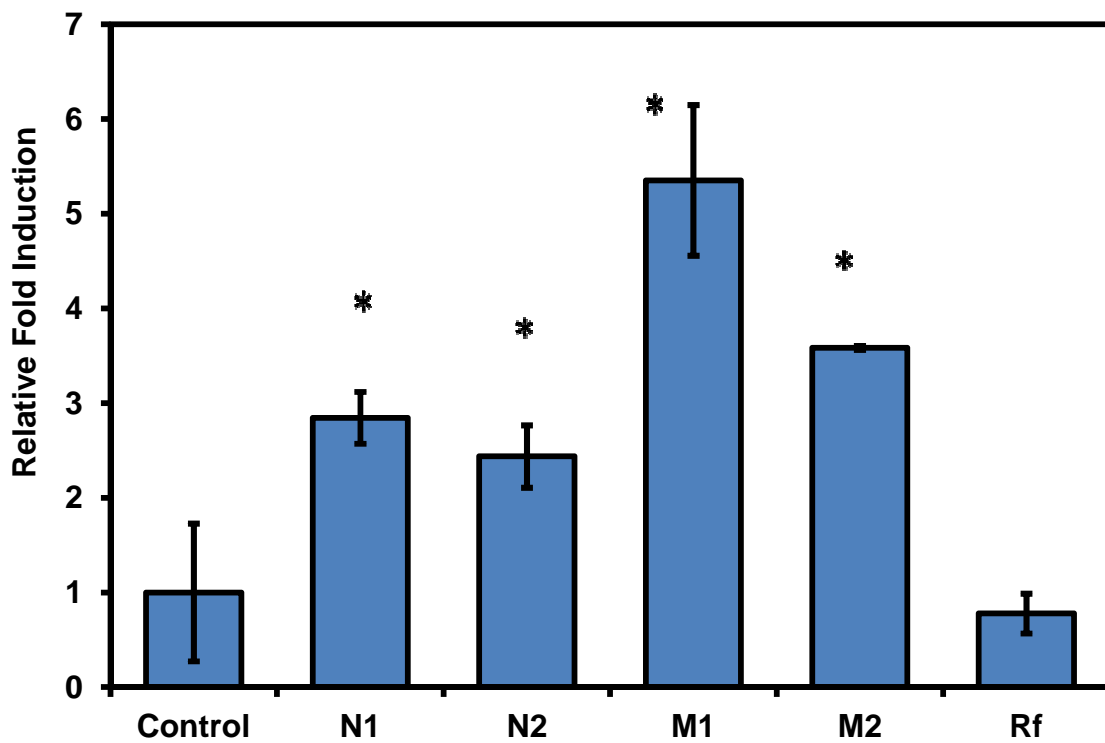
Figure 7.9 Quantification of ABCG2 mRNA expression in Calu-3 cells (N1 and N2 indicates 2.5 µM and 10 µM for nicotine; M1 and M2 indicates 3 µM and 10 µM for morphine; Rf indicates 25 µM for rifampicin)   (* indicates significant difference compared to control; $p<0.05$, $n = 3 \pm$ S.D)

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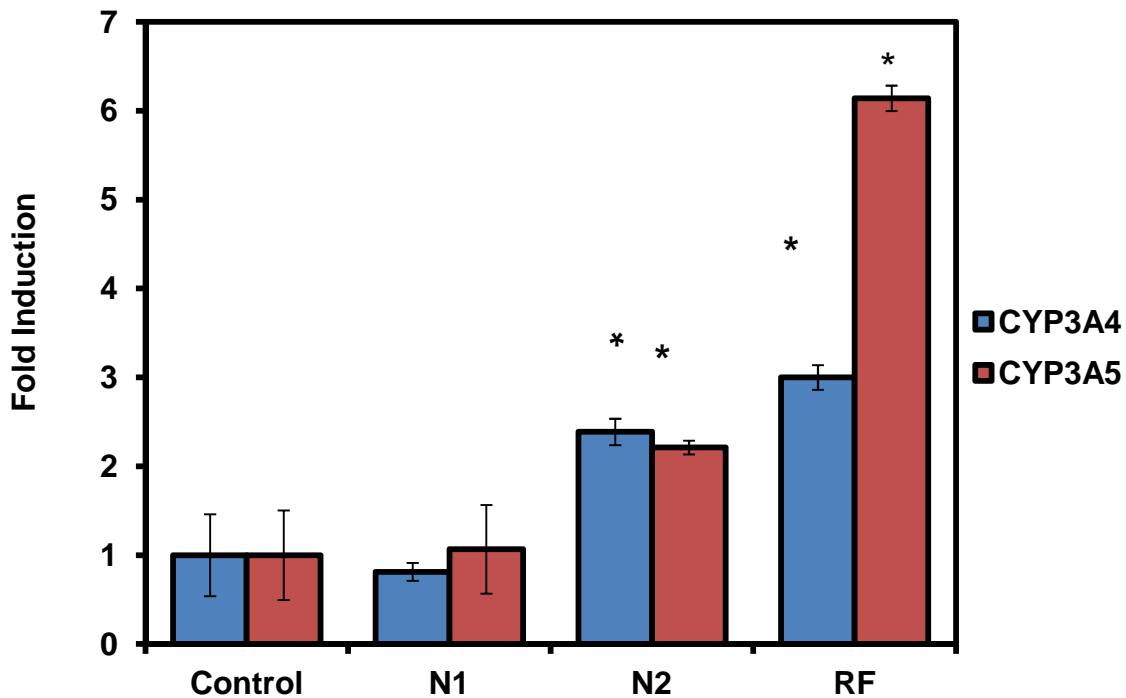
Figure 7.10   Quantification of CYP3A4 and CYP3A5 mRNA protein expression in Calu-3 cells. (N1 and N2 indicates 2.5 μM and 10 μM for nicotine, RF indicates 25 μM for rifampicin)   (* indicates significant difference compared to control; *p<0.05, n = 3 ± S.D)

PXR Induction in Calu-3 Cells

     PXR mRNA was quantified in nicotine treated Calu-3 cells. As shown in figure 7.11, PXR mRNA levels were enhanced by 4 and 15 fold respectively with nicotine 2.5 μM and 10 μM concentrations.
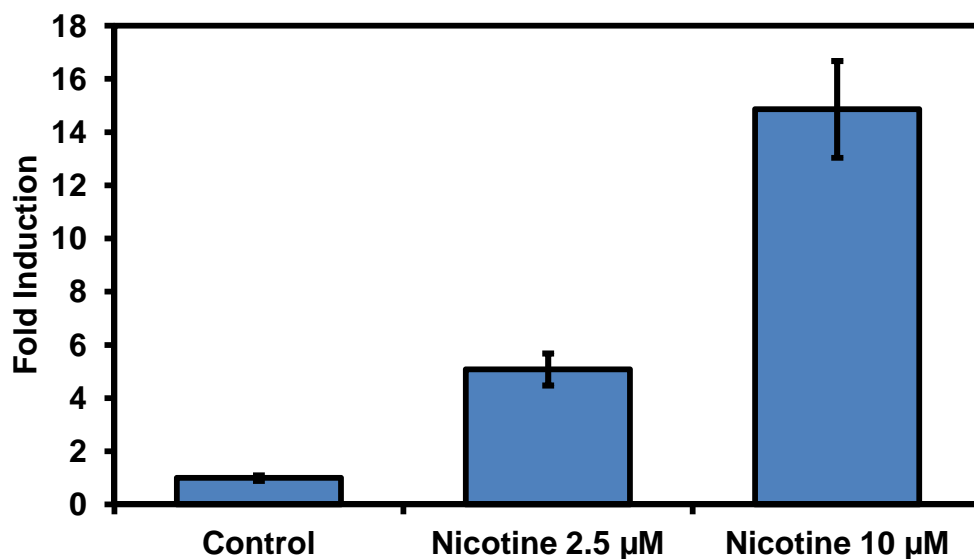
Figure-7.11 PXR quantification in Calu-3 cells

Cortisol Metabolism Studies

The 6β-hydroxylation of cortisol was used as a control to investigate the CYP3A4/A5 activity. Increased amount of metabolite 6β-hydroxycortisone was observed in nicotine treated rat lung microsomes. Concentration of 6β-hydroxycortisone was found to be 1.55 ± 0.16 nmoles/min.mg protein whereas concentration of 6β-hydroxycortisone was found to be 3.93 ± 0.61 nmoles/min.mg protein in nicotine treated rat lung microsomes. CYP3A4 mediated metabolism activity was found to be significantly higher in smokers when compared with nonsmokers (figure-7.13). Human lung microsomes obtained from smokers (2.67 ± 0.43 nmoles/min.mg protein) showed higher activity than lung microsomes obtained from nonsmokers (0.78 ± 0.36 nmoles/min.mg protein) (figure-7.14).
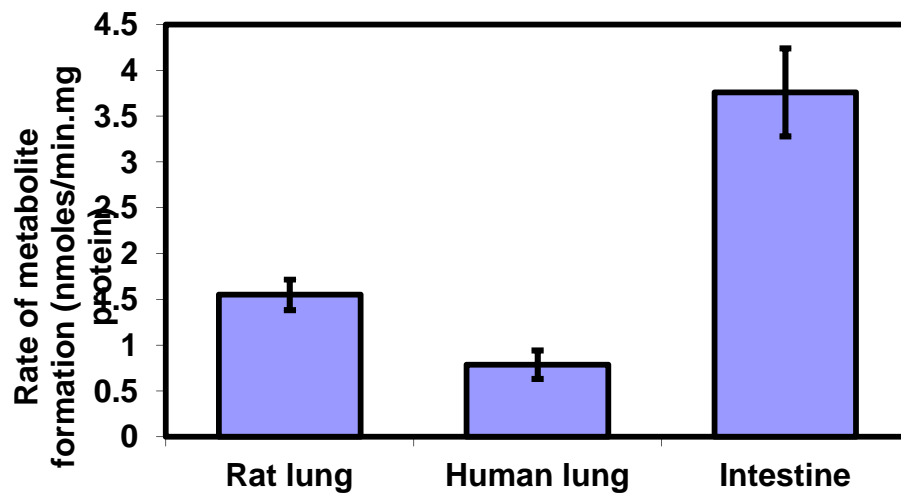
Figure 7.12   Rate of 6β-hydroxycortisone metabolite formation from cortisol in rat lung, human lung and human intestine microsomes
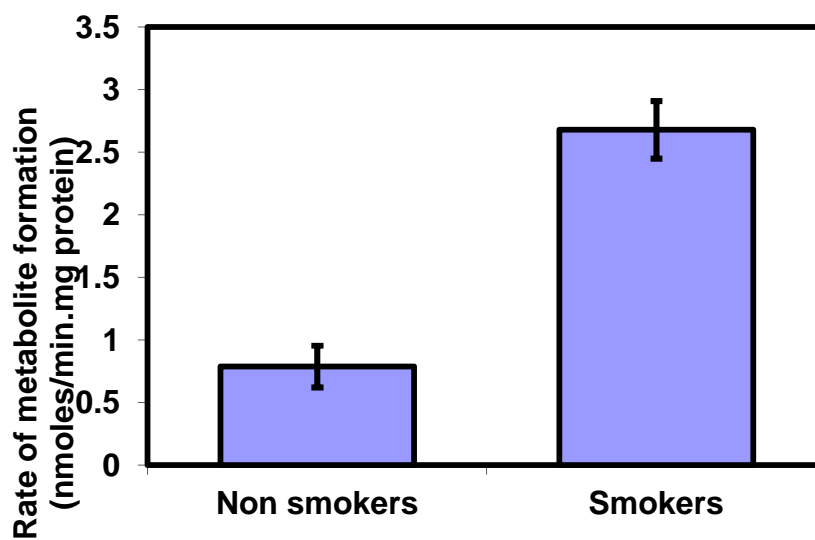
Figure 7.13   Rate of 6β-hydroxycortisone metabolite formation from cortisol in microsomes obtained from non-smokers and smokers

Therefore, these results clearly suggested upregulation of CYP3A4 mRNA and protein expression after treatment with nicotine. This upregulation was further confirmed by enhanced CYP3A4 activity. However, more studies have to be performed to delineate the difference in CYP3A4 and CYP3A5 activity and expression. Although, both CYP3A4 and CYP3A5 isoenzymes have same substrate specificity, they have different km values for substrates. To elucidate the mechanism and interplay between CYP3A4 and CYP3A5, expression and induction of gene regulatory elements for these isoenzymes need to be studied.
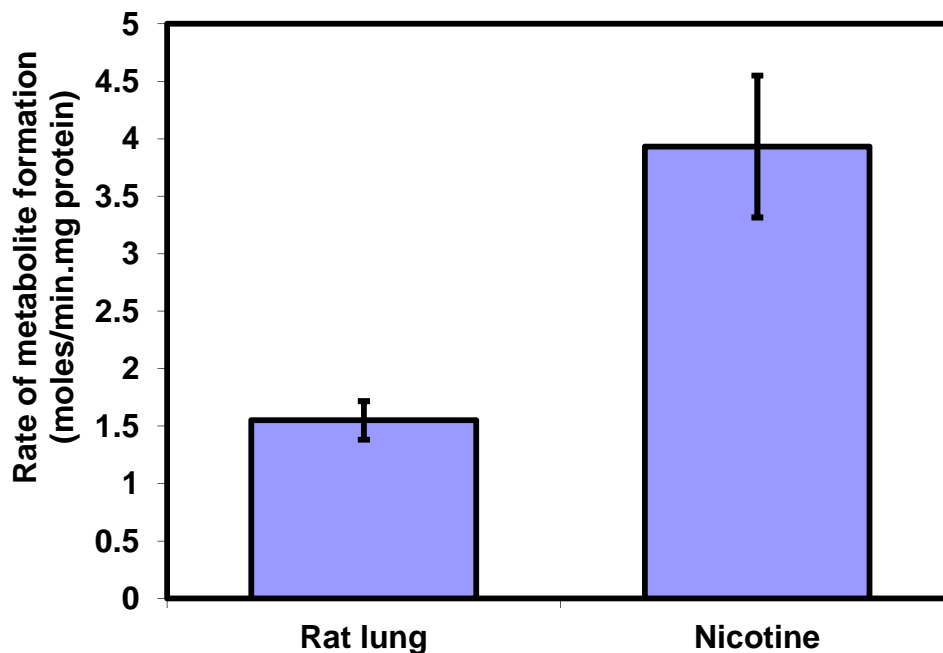
Figure 7.14 Rate of 6β-hydroxycortisone metabolite formation from cortisol in rat lung microsomes from control rats and nicotine treated rat lungs

Conclusions

Upregulation of MDR1, ABCG2 and CYP3A4/A5 mRNA levels was observed after exposing cells to nicotine. This may suggest the possibility of modulation of efflux transporters and metabolizing enzymes by inhaled xeniobiotics. Cigarette smoking can alter the permeability and metabolism of inhaled drugs and thereby, reduce their efficacy. Furthermore, nuclear receptor PXR activation by nicotine can play a significant role in the induction of metabolism in lungs.

Chapter-8

SUMMARY AND RECOMMENDATIONS

Summary

The objective of this study was to evaluate the effect of chronic treatment with morphine and nicotine on the expression of efflux transporters (MDR1, MRP2 and BCRP) and metabolizing enzymes (CYP3A4) in LS180, Caco-2 and HepG2 cells. Furthermore, the chronic effect of morphine and nicotine on the intracellular accumulation of model HIV protease inhibitors was determined.

In the third chapter, RT-PCR and Western blot studies were performed to quantify the expression of efflux transporters. Differential induction of MDR1, MRP2 and ABCG2 mRNA levels was observed when LS180 and Caco-2 cells were exposed to morphine and nicotine. Intracellular accumulation of the model radioactive substrates was reduced following treatment. Morphine and nicotine induced signaling of efflux transporter gene expression can act as significant stimulators of efflux function. Morphine and nicotine activation of PXR can explain the induction of these efflux transporters and metabolizing enzymes. Due to the enhanced efflux transporter and CYP3A4 gene expression observed after the chronic treatment with morphine and nicotine, there could be potential drug-drug interactions with HIV protease inhibitors that are substrates for the efflux transporters and that are metabolized via CYP3A4. There could be alternate pathways involved in the failure of clinical HIV therapy based on these

results. Regular monitoring of plasma concentrations of HIV protease inhibitors are recommended in chronic nicotine and morphine abusers.

Since nicotine is smoked through lungs, chronic effect of nicotine on the expression and activity of efflux transporters needs to be investigated. Lungs are one of the primary sanctuary sites for HIV viruses and opportunistic infections of the lungs are most commonly prevalent infections associated with HIV. Pulmonary drugs are widely prescribed along with anti HIV agents and these combinations can result in drug-drug interactions resulting in therapeutic failure. There is limited information known about the expression and functional activity of influx and efflux transporters in lungs

In the fourth chapter, expression and activity of folic acid carriers was investigated in human bronchial epithelial cell line, Calu-3. Our studies demonstrated the expression and functional activity of PCFT and FR-α receptor in Calu-3 cells. Also, the folic acid carriers were characterized and their functional activity was determined by employing [^{3}H] folic acid.

In the fifth chapter, efflux transporter MRP2 expression in Calu-3 cells was studied in Calu-3 cells. RT-PCR studies demonstrated the presence of MRP2 expression in Calu-3 cells. Model MRP2 substrates and HIV protease inhibitor [^{3}H]ritonavir was utilized to determine the functional activity of MRP2. Apical to basolateral and basolateral to apical transport studies confirmed the presence of MRP2 mediated efflux of ritonavir.

In the sixth chapter, BCRP was identified and characterized in Calu-3 cells. Uptake studies were performed using model substrates for BCRP, mitoxantrone and Hoechst 33342 dye.

In the seventh chapter, chronic effect of nicotine on MDR1, CYP3A4 and ABCG2 expression levels was studied after treating Calu-3 cells with nicotine. Nicotine induced MDR1, CYP3A4 and ABCG2 mRNA levels following treatment. Furthermore, nicotine induced cortisol metabolism in lung microsomes obtained from rats treated with nicotine when compared to control rats. Most of the inhaled compounds have a longer retention time resulting in significant contribution to efflux and CYP3A4 metabolism. Modulation of efflux transporters and CYP3A4 metabolism can play an important role in the absorption of inhaled rugs. These results conclude that chronic nicotine exposure can alter the disposition of inhaled drugs and there is a possibility of occurrence of drug-drug interactions in clinical setting.

Recommendations

Finding a better inexpensive *in vitro* drug interaction model is essential to study the mechanisms of drug-drug interactions mediated by efflux transporters and metabolizing enzymes. New drug entities are often screened for their potential to interact with efflux transporters and metabolizing enzymes. There is an obvious need to study the role of efflux transporters and metabolizing enzymes to establish their role in disposition of anti HIV drugs. There is a need to study the role of chronic nicotine treatment on the pulmonary drug absorption for local and systemic action. This information could be extremely valuable in the preclinical prediction of drug absorption at the target of action.

❖ Evidence of transporter mediated drug disposition in lungs *in vivo* needs to be investigated. There are no adequate *in vitro* and *in vivo* studies currently available to study the contribution of efflux transporters in lungs. Also, the lack of proper *in vivo* model and complexity of the current *in vivo* models pose several drawbacks in predicting the pulmonary bioavailability.

❖ Contribution of nicotine to the disposition of inhaled drugs needs to be studied *in vivo* because of stronger induction effect of nicotine on efflux transporters.

❖ More studies are to be designed to help us understand the *in vivo* modulation of efflux transporters in lungs and their contribution to physiology of pulmonary diseases.

❖ Induction results confirmed the role of PXR in MDR1 and CYP3A4 mediated induction. However, expression and activation of CAR, aryl hydrocarbon receptor

(AhR) needs to be investigated. Further, computer docking studies and functional activation assay of AhR can give a better understanding of higher BCRP induction by morphine and nicotine.

- ❖ Mechanism of drug interactions between drugs of abuse and HIV protease inhibitors should be studied in clinical settings and role of efflux transporters and nuclear receptors should be investigated.

- ❖ Finally, more studies should be designed to study the relation between induction of efflux transporters and nuclear receptors and their contribution to anti HIV drug resistance.

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VITA

Durga Kalyani Paturi was born in Guntur, Guntur district, Andhra Pradesh, India. She studied in local public schools at different cities and graduated from Gudlavalleru High School as class first ranker in 1994. She received her Bachelor of Pharmacy degree with first-rank in her class from the Jawaharlal Nehru Technological University, Hyderabad in 2001.

She joined the Master's Program at School of Pharmacy, University of Missouri-Kansas City in the Spring of 2003 to pursue her higher studies. Later she got admitted into the interdisciplinary Ph.D. program with Pharmaceutical Sciences as the major in August 2003.

During her graduate studies, Miss. Paturi has actively pursued her research goals and presented her work at various prestigious national meetings (AAPS Annual Meetings) and regional meetings (PGSRM). She also actively assumed various leadership positions. She served in the capacity of treasurer to the UMKC student chapter of American Association of Pharmaceutical Scientists. She also acted as secretary for the Pharmaceutical Graduate Students Association.

She is an active member of the American Association of Pharmaceutical Scientists

The following are the list of her professional achievements.

Publications

1. Functional characterization of folate transport proteins in Staten's Seruminstitut rabbit corneal epithelial cell line. Jwala J, Boddu SH, **Paturi DK**, Shah S, Smith SB, Pal D, Mitra AK. Current Eye Research. 2011; 36(5): 404-16.

2. Identification and characterization of breast cancer resistance protein in Calu-3 cells. **Durga Kalyani paturi**, Deep Kwatra, Hari Ananthula, Dhananjay Pal, Ashim K. Mitra. International Journal of Pharmaceutics. 2010; 384(1-2): 32-38.

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8. Nasal and pulmonary delivery of macromolecules-Pulmonary nanomedicine book. **Durga Paturi**, Mitesh Patel, Ranjana Mitra, Ashim K. Mitra. March 2013

9. Development of non-invasive delivery for proteins. Pradeep Karla, **Durga Paturi**, Nanda Mandava, Sulabh Patel, Ashim K. Mitra. Biomedical Engineering hand book- In review

Articles (In preparation)

1. Transport of folic acid across human bronchial epithelial cell line (Calu-3), facilitation by proton-coupled folate transporter and folic acid receptor-alpha. **Durga Paturi**, Ashim K. Mitra

2. Long term effects of morphine and nicotine on induction of efflux proteins. **Durga Paturi**, Ashim K. Mitra.

3. Interactions between antifungal and antiretroviral agents

4. Review article on patents in pulmonary delivery

Presentations

 Presented posters at American Association of Pharmaceutical Scientists annual conference (AAPS), Pharmaceutics Graduate Student Research Meeting (PGSRM) and Kansas City Life Sciences (KCLS) meetings

1. Poster at AAPS 2009 and poster at PGSRM 2010
2. Poster at AAPS 2008 and poster at PGSRM 2008
3. Poster at AAPS 2007 and poster at PGSRM 2007
4. Poster at AAPS 2006 and poster at PGSRM 2006
5. Poster at AAPS 2005; PGSRM 2005 and KCLS 2005
6. Poster at AAPS 2004 and poster at PGSRM 2004