Curriculum Vitae

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EDUCATION

PhD candidate in Pharmaceutical Sciences Sep 2007~Apr 2013

University of Maryland School of Pharmacy, Maryland, USA

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ACADEMIC EXPERIENCE

Research Assistant Aug 2007~Apr 2013

University of Maryland School of Pharmacy, Maryland, USA

- Established an innovative cell-based co-culture system to quantitatively monitor the metabolism of cyclophosphamide and its main active metabolite, 4-hydroxycyclophosphamide using LC-MS/MS. Tested this assay on mouse, rat and human primary hepatocytes. Monitored the formation of metabolite in a time- and dose-dependent manner with quantification.
- Established a 96-well plate based reporter assay and screened a 800-herbal medicine bank, identified several compounds of interest.
- Explored the role of Constitutive Androstane Receptor (CAR) in the induction of Drug Metabolizing Enzymes by a variety of drugs, drug candidates and other compounds. Confirmed CAR preferentially induces CYP2B6 over CYP3A4. Specific CAR activators may alter the metabolism profile of drugs involving these isozymes.
- Used a variety of biotechnological methods to test the induction profiles of different clinically applied drugs, drug candidates and other compounds on the P450s and other relevant phase I&II enzymes and transporters. Identified several compounds that have a preferential induction of CYP2B6 over CYP3A4 and may enhance cyclophosphamide bioactivation.

- Evaluated the toxicity of anti-cancer drugs on their target cells as well as hepatocytes and other cells/tissues both *in vitro* and *in vivo* using multiple end points and biomarkers. Observed different toxicity of the drugs on different cells/tissues, established an apoptosis-related mechanism.
- Conducted the construction of His-tagged, pEGFP-tagged, and GST-tagged protein expression plasmids, amplification and purification of these proteins with success.
- Conducted an *in vitro* GABA binding assay using radio labeled GABA and mouse primary neurons. Confirmed a mutation in GABA receptor may alter its affinity to substrate.
- Performed the construction and screening of a N1-Mouse Opioid Receptor expressing cell line based on transfected CHO cells. Successfully generated the target cell line.

Teaching Assistant Aug 2007~Jul 2008

University of Maryland School of Pharmacy, Maryland, USA

- Served as teaching assistant in pharmacology class, helped proctoring, grading exams and explaining results to over 120 students. Established good working and personal relationships with professors, colleagues and fellow students.

- Helped to identify and solve a design flaw of the electronic grade-posting system that randomly mismatched students' test score data which led to manual check and correction. Greatly enhanced the reliability and efficiency of the system when processing massive amount of data.

RESEARCH SKILL SETS

- Molecular biology: DNA transfection, cloning, Realtime-PCR, Western Blot, Reporter Assay,et al.
- Cell biology: Mid-throughput reporter assay, Human, mouse and rat primary hepatocyte isolation and culture, Human hepatic cell line and hematopoietic cell line culture, cryopreservation, cell viability assays, cell apoptosis assays, et al.
- In vivo: Experiment design, rodent tumor generation, isolation and analysis.
- Analytical techniques: LC-MS/MS, flow cytometry, platereader, confocal microscopy, automated cell counter, et al.
- Software: SigmaPlot, GraphPad Prism, Microsoft Office, Photoshop CS, et al.

PUBLICATIONS

- *Wang, D., Li, L.,Yang,H., Fuhrman, J., Ferguson, S. & Wang, H.*A novel in vitro model to investigate pro-drug activation and metabolism. ***Blood*** 2013
- *Wang, D.& Wang, H.* Oxazaphosphorine bioactivation and detoxification: The role of xenobiotic receptors.*Acta Pharmaceutica Sinica B* 2012
- *Wang, D., Li, L., Fuhrman, J., Ferguson, S. & Wang, H.* The Role of Constitutive Androstane Receptor in Oxazaphosphorine-Mediated Induction of Drug-Metabolizing Enzymes in Human Hepatocytes. *Pharm Res.* 2011

- *Hassan,H.,Myers,A.,Lee,I.,Mason,C.,Wang,D.,Sinz,M.,Wang,H.&Eddington,N.* Induction of Xenobiotic Receptors, Transporters and Drug Metabolizing Enzymes by Oxycodone. *Drug Metab Dispos.* 2013

ABSTRACTS

- *Wang, D., Li, L.,Yang,H., Fuhrman, J., Ferguson, S. & Wang, H.* Constitutive Androstane Receptor is a novel therapeutic target for leukemia (Abstract to 18th North American ISSX meeting) 2012
- *Wang, D., Li, L., Fuhrman, J., Ferguson, S. & Wang, H.* Different roles of CAR and PXR in oxazaphospharine-mediated induction of Drug-Metabolizing Enzymes (Abstract to GPEN2010 Meeting) 2010

SOCIAL ACTIVITIES

Executive Board Member of CBA (Chinese Biopharmaceutical Association)
2009~2010

- One of the only two graduate students who sat on the Chinese Biopharmaceutical Association (CBA, a Washington DC based pharmaceutical organization with more than 300 members global wise) executive board, in charge of bridging CBA and the academia.
- Programmed and advocated CBA events and presentations to professional groups; invited outstanding CBA members from academia and industry to present to students and scholars.

HONORS

Travel Award to 18th North American ISSX meeting 2012
Awarded to 8 out of 125 applicants worldwide to an international academic meeting

Departmental travel award to GPEN 2010 meeting 2010
Awarded to 2 out of 20 fellow graduate students to an international academic meeting

Membership of Rho Chi Honor Society, Chapter Omega, USA 2008
The membership represents national-wide outstanding academic performance at graduate level

Excellent Student Leader of Huazhong University of Science & Technology2006
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Photography Website: https://picasaweb.google.com/117382207160355270901

Abstract

Title of the dissertation: The Role of Constitutive Androstane Receptor in the Bioactivation of Oxazaphosphorines

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Dissertation directed by: Hongbing Wang, Ph.D
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Prodrugs are pharmaceutical substances that are administered in an inactive form and are subsequently converted to the active therapeutic moiety through bioactivation. Among them, oxazaphosphorines represent a major class of anti-cancer prodrugs, with cyclophosophamide (CPA) and ifosfamide (IFO) being the most widely used ones. Because of the increased polypharmacy in oxazaphosphorine-based chemotherapy, drug-drug interactions have been raising concerns. Hepatic CYP2B6 and CYP3A4 differentially contribute to the activation and inactivation of CPA and IFO and many clinically used drugs and environmental compounds can influence the expression of these enzymes. Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR)are important regulators of CYP2B6 and CYP3A4 expression. Activation of PXR induces both isozymes, while selective activation of CAR leads to preferential induction of CYP2B6 over CYP3A4 in human liver. Since CPA is predominantly bioactivated by

CYP2B6 while deactivated through CYP3A4, we hypothesized that a combination of CPA and CAR activators may enhance the therapeutic effect of CPA by preferential induction of CYP2B6 over 3A4.

The hypothesis was tested in an innovative hepatocyte-hematopoietic cell co-culture system that was demonstrated to be a useful *in vitro* model for studying the biotransformation and therapeutic effects of CPA and potentially other prodrugs in an environment that closely mimics *in vivo* conditions.Based on this system, CITCO was proved to preferentially induce CYP2B6 over CYP3A4 and subsequently enhance CPA anti-cancer activity. Similar trends were observed using rodent primary hepatocytes and corresponding CAR activators.

Because CITCO is not clinically available, a panel of FDA approved drugs together with 800 Chinese herbal medical products were screened for preferential induction profile of CYP2B6 over CYP3A4. Matrine (Figure S1), the major component of two Chinese medical products was found to selectively induce CYP2B6 over CYP3A4 and enhanced CPA therapeutic effect *in vitro* in the co-culture system.

The autoinduction profile and the underlying mechanism of CPA and IFO were also addressed. Both compounds induced CYP2B6 and CYP3A4 at mRNA and protein levels in human primary hepatocyte. While both drugs were proved to be PXR activators which indiscriminately induce CYP2B6 and CYP3A4, CPA but not IFO was demonstrated to be an indirect CAR activator.

The Role of Constitutive Androstane Receptor in the Bioactivation of Oxazaphosphorines

By
Duan Wang

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

Acknowledgments

I would like to express my most sincere gratitude to my advisor, Dr. Hongbing Wang. His continuous trust, support, guidance and encouragement were indispensable for my training and development from a fresh graduate to a scientist. He has always educated me with his experience and skills, guided me through difficulties with patience and supports, and inspired me with his scientific professionalism and vision. I cannot be more fortunate to have Dr.Wang as my Ph.D mentor during the last six years of study. I will forever be respectful and grateful to him.

I also would like to thank my Ph.D Committee members, Dr. Douglas Ross, Dr. James Polli, Dr. Yan Shu and Dr. Maureen Kane for their continuous assistance, invaluable advice and professional performance.

My tremendous appreciation to my present and previous lab mates: Dr. Linhao Li, Dr. Hui Yang, Dr. Tao Chen, Dr. Haishan Li, Dr. Leslie Tompkins, Dr. Antonia Tolson, Caitlin Lynch and Brandy Garzel for their help and support. Their kindness, generousness as well as the happy moments we shared together will be remembered forever.

Many thanks to my treasured friends and colleagues at school. Though their names cannot be exhaustively presented here, their help and friendship are among the most highly appreciated throughout my life.

I would like to give my deepest appreciation and love to my family, especially my grandmother Liangyu Liu, my parents Jun Long and Qiyue Wang, who bore me, raised

me, taught me, supported me and loved me unconditionally and endlessly. All words become pale to express my most sincere love for them. Together with all my other family members, they constitute a great part of the whole meaning of my life.

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List of Abbreviations

4-OH-CPA	4-hydroxy- cyclophosphamide
4-OH-IFO	4-hydroxy- ifosfamide
ADH	Alcohol dehydrogenase
AhR	Aryl hydrocarbon Receptor
ALDH	Aldehyde dehydrogenase
ALL	Acute lymphatic leukemia
AML	Acute myelogenous leukemia
BCRP	Breast cancer resistance protein
CAA	Chloroacetaldehyde
CAR	Constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo-[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime
CPA	Cyclophosphamide
DME	Drug metabolizing enzyme
GST	Glutathione-S-transferase
HPH	Human primary hepatocyte
IFO	Ifosfamide
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
PB	Phenobarbital
PBREM	Phenobarbital responsive enhance module

PXR	Pregnane X receptor
RIF	Rifampicin
RIT	Ritonavir
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immune deficient
TCL	T-cell lymphoma
TCPOBOP	1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene
UGT	UDP-glucuronosyltransferases
XREM	Xenobiotic-responsive enhancer module

CHAPTER ONE
OVERVIEW AND OBJECTIVE

1.1 Prodrugs and their bioactivation

In 1958, the term “prodrug” was introduced by Adrien Albert to describe a class of chemically modified drug moieties that require bioactivation to achieve pharmacological activity; it was expected that this strategy could be utilized to improve the efficiency of the drugs, or to decrease their off-target toxicity[1]. Since then, a series of names have been designated to describe this concept, such as “latentiated drugs,” “bioreversible derivatives,” and “congeners”, among which “prodrug” remains the most widely accepted term. The opposite term of prodrug is the so called “soft drug”, which refers to a class of compounds that are designed to exert their therapeutic effect readily without bioactivation, rather undergo metabolic inactivation afterwards to form nontoxic products. Another related concept is “hard drug”, which are compounds with built-in pharmacologically active structures and invulnerable to metabolic or chemical transformation, thus avoiding the formation of toxic byproducts and increasing human exposure to the drugs. In some other cases, a prodrug may consist of two pharmacologically active drugs that are conjugated to form a single molecule, which is called a “co-drug”. As of today, prodrugs constitute 5~7% of total approved drugs worldwide, or roughly account for 250~300 in total number of drugs[2].

The development of prodrugs has been well established as an effective approach to improve the physicochemical, biopharmaceutical or pharmacokinetic properties of drugs or potential drug candidates, enhancing their developability and pharmacological

performance. The driving forces that most commonly seen in the development of prodrugs include: improving patient acceptability[2] (decreasing pain or other unpleasant experience during administration); alteration of absorption, distribution, metabolism, and elimination properties[3]; improving chemical stability and solubility[4]; improving barrier penetration[5]; improving target specificity[6]; decreasing side effects and unwanted toxicity[7], etc. In essence, the development of prodrugs represents an important strategy in overall new drug design and development, as well as in improving products through generations.

Table 1.1 Classification of prodrugs by purpose of design.

Purpose	prodrug	Use of drug	Active moiety	Conjugated group	Mechanism of Activation
Improving oral bioavailability	Valaciclovir	Antiviral	Acyclovir	L-valine	Hepatic esterases
Improving lipophilicity	Dipivefrin	Anti- glaucoma	Epinephrine	Pivalic Acid	Enzymatic hydrolysis in the eye
Improving solubility	Clindamycin	Antibacterial	clindamycin	Phosphate group	Hydrolysis by phosphatases
Improving site specificity	Hexamine	Anti-urinary tract infections	formaldehyde	N/A	Disintegration at pH<5
Improving site specificity	Diethylstilbestrol diphosphate	Anti-cancer	Diethylostilbestrol	Phosphate group	Hydrolysis by enriched phosphatases in tumors
Improving stability	Hetacillin	Antibacterial	Ampicillin	N/A	Spontaneous hydrolysis
Sustained release	Azothioprine	Immunosuppressant	6-Mercaptopurine	N/A	Spontaneous ring formation
Controlled Toxicity	Cyclophosphamide (CPA)	Anti-cancer	4-OH-CPA	N/A	Hydroxylation by CYP2B6

There is no definite classification of prodrugs so far. Alternatively, these compounds can be classified based on different parameters, such as their purpose of design or mechanisms of activation. Above is a collection of prodrugs classified by their purpose of design, with one or two example for each class (Table 1.1). Though the examples

presented above are typical representative of each class, it is important to note that the classification criteria are not mutually exclusive. For instance, the design of CPAachieved controlled toxicity and prevented gut wall toxicity in oral administration through the removal of hydroxyl group from its activated form, 4-hydroxy-cyclophosphamide (4-OH-CPA). At the same time, significant enhancement of stability is achieved when a half-life of 4 minutes for 4-OH-CPA is compared with that of longer than 12 hours for CPA[8].

1.2 Anti-cancer prodrugs

Compared to other medicines, anti-cancer agents usually present significantly greater cytotoxicity and thus increased potential of off-target toxicity. To accommodate the needs of controlled toxicity, enhanced target specificity and better bioavailability, different prodrug-based therapies have been introduced in clinical application for the last decades. In fact, a great portion of the anti-cancer agents currently in use are prodrugs[5]. In the following content, major categories of anti-cancer prodrugs are classified by their mechanism of action.

1.2.1 Alkylating agents

Alkylating agents are the most classic, and most widely used family of anti-cancer drugs. They exert cytotoxicity through covalent binding to macromolecules such as DNA. Their high activity comes hand in hand with a lack of specificity, which led to the development of prodrugs and their analogues.

1.2.1.1 Nitrogen Mustards

Nitrogen mustards are alkylating agents which contain N,N-bis-(2-chloroethyl) amine as the major functional group. They react with DNA at the N^7 of guanine in most cases, while reaction with adenine has also been reported[9].DNA crosslinks are formed as a result of these reactions, which leads to DNA damage and ultimate cell death. However, the high activity of the functional group renders the drugs compromised specificity. Nitrogen mustards also react with other cellular components such as proteins and thiols, resulting in reduced therapeutic activity. In addition, the functional group lacking intrinsic DNA binding affinity produces a greater portion of genotoxic mono-adducts than the bi-adducts which are essential for the maximum therapeutic effect of the drugs[10]. To overcome these drawbacks, prodrugs such as CPA and ifosfamide (IFO) have been developed. Both prodrugs require hepatic P450 bioactivation to generate cell membrane-permeable intermediates that are ultimately transformed into DNA-alkylating agents. Detailed information regarding the ADME of these drugs as well as their derivatives will be discussed in following sections.

1.2.1.2 Nitrosoureas

Carmustine and lomustine represent another class of alkylating agents, namely nitrosoureas. Their anti-tumor activity depends on the chlorethylating activity and subsequent DNA cross-link formation[11]. Other activities such as carbamoylation, hydroxyethylation and vinylation contribute a small portion to the therapeutic effects but a significant portion to the side effects and toxicity of the drugs. Thus a series of prodrugs that retains the chlorethylating activity but not the other activities have been designed, among which cloretazine is a promising candidate to treat acute myelogenous leukemia

(AML)[12]. A new drug application was filed in 2009 for this compound and more clinical studies are being conducted upon FDA requests. In another approach, proline derivatives of nitrosoureas are designed to enhance the tissue specificity of the prodrugs since some cancer tissues possess higher prolidase activity than normal tissues[13].

1.2.1.3 Triazenes

Triazene compounds methylate the O^6 position ofguanine in DNA and represent another family of alkylating agents. Among this group of prodrugs, dacarbazine and temozolomide are the most widely used in clinical application[14], with their derivatives under active research and development.

1.2.2 Antimetabolites

Antimetabolites can interfere with nucleic acid synthesis, thus inhibit cell proliferation and tumor growth. Some of these compounds are analogues of pyrimidines and purines and thus compete with them in constructing DNA and RNA, others inhibit key enzymes in nucleic acid synthesis. These compounds usually suffer from poor oral bioavailability, tissue specificity and water solubility. Prodrugs have thus been developed to enhance these therapeutic indexes.

1.2.2.1 Pyrimidine Analogues

The earliest and one of the most widely used pyrimidine analogue is fluorouracil (5-FU). Two major metabolites, 5-FdUMP and 5-FUTP are generated upon bioactivation of 5-FU[15]. 5-FdUMP is a thymidylate synthase inhibitor which ultimately inhibits DNA synthesis, while the later metabolite, 5-FUTP disrupts RNA and protein synthesis. 5-FU is normally dosed by i.v. to avoid erratic oral administration. Capecitabine, an oral

formulation of 5-FU, was developed with enhanced anti-tumor specificity[16]. Another 5-Fu analogue with oral bioavailability is tegafur. Hepatic P450 activation is required for this prodrug to achieve controlled, prolonged release of 5-FU. More often, tegafur is combined with other substances in different formulations that modulate the metabolism of 5-FU for enhanced therapeutic effects, such as UFT[17] and TS-1[18]. Other commonly used pyrimidine analogues include but are not limited to cytarabine (ara-C) and its derivatives, which are used primarily in the treatment of hematological malignancies[19; 20]; and gemcitabine and its derivatives, more often serve as anti-cancer agents for solid tumors[21; 22; 23].

1.2.2.2 Purine Analogues

6-mercaptopurine (6-MP) is a potent anti-cancer agent to treat acute lymphatic leukemia (ALL). Different prodrugs have been designed and tested in rodent models to enhance its water solubility and specificity, such as PEG-6-MP[24] and S-(6-purinyl)-L-cysteine (PC)[6; 25]. 6-thioguanine (6-TG) is very similar to 6-MP in terms of mechanism of action, and a kidney-specific prodrug has been developed accordingly[26]. Fludarabine phosphate (Fludara) is essentially the antiviral agent vidarabine (ara-A) in a fluorinated form. The presence of phosphate ester enhances the water solubility of the compound[27].

1.2.3 Tubulin-binding agents

The formation of microtubules, which consist of tubulins, plays a vital part in a variety of cellular activities such as maintaining cell shape, cell division, cell signaling, intracellular transportation and cell migration. The discovery of natural compounds that exhibit tubulin-interfering capacity has led to the development of a series anti-cancer prodrugs.

Colchicine is the first known natural product that binds to tubulin and causes microtubule depolymerization. To regulate its significant cytotoxicity and enhance its tissue specificity, different prodrug approaches have been introduced, most of which involve the conjugation of colchicine with a targeting moiety[28; 29]. Combretastatin A-4 (CA-4) is another natural product that inhibits tubulin polymerization. Soluble phosphate CA-4 prodrugs are in clinical trials[30; 31]. Podophylotoxin and its derivatives represent another important class of anti-tubulin polymerization agents known as "etoposide-like" compounds[32].

On the other hand, the taxane class of compounds exerts their anti-tumor activity through binding to and strengthening microtubular network and decreasing its dynamicity, thus inducing apoptosis. Paclitaxel (PTX) is the first member of this class of compounds and is considered as one of the most important chemotherapeutic agents in cancer treatment[33; 34]. Other members of this family, such as docetaxel, abraxane, and polyglumex are also in active clinical application. Early attempts in paclitaxel prodrug development focused on improving its water solubility. The first group of such derivatives is paclitaxel C-2 or C-7 succinate, glutarate, sulfonate and amino acid esters[35; 36], followed by phosphate ester derivatives[37]. Protaxel, a 1,2-dihydroxylpropylcarbonate conjugate, readily releases paclitaxel through a pH-dependent mechanism[38]. Another approach to increase water solubility of paclitaxel and docetaxel involves the conjugation of sugar moieties[39; 40]. Alternatively, lipophilic compounds such as docosahexaenoic acid (DHA) have been conjugated to taxane prodrugs as well as other anti-cancer agents to promote their accumulation in tumor cells, as in the cases of paclitaxel, doxorubicin and etoposide[41; 42]. More recently, relatively sophisticated mechanisms have been employed to enhance the

therapeutic performance of taxane prodrugs. Some of them utilize specific enzymes that are highly expressed in target tissues[43; 44], or specific bioactivation mechanisms that feature in tumor cells such as hypoxia[45] , others introduce site-directed activation mechanisms like photodynamic therapy (PDT) to enhance tissue specificity[4]. To sum up, paclitaxel remains one of the most important prodrug targets with more and more novel approaches being developed to enhance its therapeutic indexes, as well as for other anti-cancer compounds.

1.2.4 Platinum analogues

Platinum analogues exert their anti-cancer effect through platination of DNA on the N^7-position of neighboring guanine bases to form interstrand crosslinks. Cisplatin, carboplatin oxaliplatin, and nedaplatin are the most important representatives of this family with good efficacy and broad spectrum of anti-cancer capacity. However, the mechanism of action of these drugs renders them intrinsic off-target cytotoxicity such as nephrotoxicity, neurotoxicity and myelotoxicity[46]. In addition, development of drug resistance has also been reported[47]. Photosensitizer-conjugated prodrugs have been developed to target specific tumor tissues utilizing a photoacitvation mechanism similar to the PDT described earlier[48; 49]. To overcome drug resistance, a glutathione-S-transferase (GST) inhibitor, ethacrynic acid is attached to a platinum complex to form ethacraplatin. The activation of the prodrug produces both cisplatin and the GST inhibitor which enhances cisplatin accumulation in the cells[50]. Novel drug delivery systems such as pH-sensitive polymers, nanotubes and nanocapsules have also been utilized to enhance the therapeutic index of platinum analogues[51; 52], in a prodrug-related or non-related manner.

1.2.5 Antineoplastic antibiotics

1.2.5.1 Anthracyclines

Doxorubicin (DOX) and daunorubicin are two of the most effective anti-cancer agents belonging to this group of drugs. Although the exact mechanism(s) of action for anthracyclines to exert their anti-tumor effects has not been fully understood, several hypotheses have been proposed with supportive experimental observations. The mechanisms might involve intercalation of DNA, induction of apoptosis, inhibition of RNA synthesis, etc. In the last decades, efforts have been made in the development of anthracycline prodrugs to enhance their efficacy and safety. Different prodrugs designed to target tumor markers or tumor-specific micro-environment have been tested with promising outcomes[7; 53; 54].

1.2.5.2 Non anthracyclinic antibiotics

Bleomycins (BLMs) and mytomycin C are the major components of this class. For BLMs, the anti-cancer capacity results from binding and oxidative degradation of cellular DNA. By modification of specific domains of BLMs, enhanced anti-tumor activity can be achieved[55]. The recent development for mytomycin C prodrugs mainly focuses on liposomes and polymeric conjugates for enhanced delivery.

1.2.6 DNA topoisomerase inhibitors

1.2.6.1 DNAtopoisomerase I inhibitors

Camptothecin (CPT) and its analogues interact with DNA topoisomerase I (TOP I), producing both single-stranded and double stranded breaks in DNA and causing collision of the DNA replication fork, which ultimately leads to cell death[56]. Camptothecins are

highly specific to TOP I that represents their only target. Besides, these compounds also have excellent permeability to mammalian cells and target TOP I within minutes of administration[57]. However, these characters also bring drawbacks that limit the therapeutic index of CPT. Firstly, DNA-TOP I complex must be maintained long enough to generate DNA breaks while CPT rapidly diffuse from the complex and thus requires a prolonged infusion to maintain its concentration at the site of action. Secondly, CPT causes side effects such as leucopaenia which limits its safety dose. Thirdly, CPT undergoes simultaneous carboxylation which leads to inactivation. Fourthly, CPT and its analogues are substrates of efflux transporters such as P-gp (P-glycoprotein) and BCRP (Breast Cancer Resistance Protein). To overcome these problems, different strategies have been explored including conjugation of carboxylic acid, amino acids, carbamates and peptide hormones[3; 58; 59]. Some of these candidates seem promising in on-going clinical trials.

1.2.6.2 DNAtopoisomerase II inhibitors

DNA topoisomerase II catalyzes reactions by introducing transient double-strand DNA breaks. The most important TOP II selective inhibitor is etoposide, which is used in treating breast cancer, leukemia and non-Hogkin lymphoma. Etoposide, mitoxantrone plus prednisone is one of the most widely used salvage chemotherapy for refractory or relapsed non-Hodgkin's lymphoma. Mitoxantrone is another important drug of this class. Among its prodrugs, AQ4N is activated under hypoxic conditions with the involvement of cytochrome P450 (CYP) enzymes to produce AQ4, a close derivative of mitoxantrone that exhibit enhanced cytotoxicity in cell lines and xenografted mouse tumor models[60; 61].

1.3 Cyclophosphamide and Oxazaphosphorines

Oxazaphosphorines are a class of bi-functional alkylating agents that have been extensively investigated in the past 50 years for their anticancer and immune-regulating activities, with the most successful representatives including CPA, IFO, and to a lesser extent trofosfamide [62; 63; 64; 65]. Most oxazaphosphorines are designed as prodrugs, which require CYP enzyme-mediated bioactivation to generate highly reactive alkylating nitrogen mustards that exert their chemotherapeutic effects by attacking specific nucleophilic groups of DNA molecules in target cancer cells [66; 67; 68; 69; 70; 71]. CPA is the first oxazaphosphorine agent that achieved great success in its clinical application in many cancer patients[72; 73; 74]. CPA has been clinically available for over a half century, and it continues to be amongst the front-line choices of chemotherapy for solid tumors, such as breast cancer, for which it is used as an important component of the CPA-methotrexate-fluorouracil (CMF) regimen [75; 76], and hematopoietic malignancies, such as non-Hodgkin lymphoma, for which it is applied as a critical constituent of the rituximab-CPA-doxorubicin-vincristine-prednisone (R-CHOP) multidrug regimen [77; 78]. Additionally, CPA has also been used at higher doses in the treatment of aplastic anemia and leukemia prior to bone marrow transplantation and as a therapeutic immunosuppressor for several autoimmune disorders[79; 80].

IFO, the second anticancer drug in the oxazaphosphorine class, was introduced to clinics in the early 1970s [65; 81]. Developed as an analogue of CPA, IFO only differs chemically from CPA by one chloroethyl group transpositioned from the mustard nitrogen to the ring nitrogen [82]. Like CPA, IFO also requires CYP-mediated metabolism to produce active alkylating moieties before manifesting its antitumor effects[83; 84]. Clinically, IFO has been

used in young adult and pediatric tumors along with other chemotherapeutics in adjuvant treatment. In a number of malignant diseases, IFO exhibits a higher therapeutic response rate, with less myelosuppression, in comparison with its parent analogue CPA [85; 86]. Trofosfamide is another derivative of CPA and an orally administered oxazaphosphorine prodrug with high bioavailability [87]. As a congener of CPA and IFO, the antitumor cytotoxicity of trofosfamide also relies on its metabolic activation by “ring” oxidation, using the hepatic mixed-function oxidase system [88; 89]. Trofosfamide is often used clinically in adult soft tissue sarcomas and non-Hodgkin lymphomas with relatively low toxicity profiles[90; 91; 92].

In addition to these traditional oxazaphosphorines, several new analogues of CPA and IFO such as mafosfamide and glufosfamide have been designed, aiming to achieve increased therapeutic selectivity and reduced off-target toxicity, in comparison with their ascendants[93; 94]. Unlike traditional oxazaphosphorines, mafosfamide and glufosfamide do not require hepatic oxidative enzyme-mediated bioactivation. For instance, mafosfamide is a 4-thioethane sulfonic acid salt of 4-OH–CPA, a key bioactive intermediate metabolite of CPA [8; 71]; while glufosfamide is a glucose conjugate of IFO, in which isophosphoramide mustard, the bioactive alkylating metabolite of IFO, is covalently linked to β-D-glucose [95; 96]. At present, several Phase I studies have shown favorable outcomes from intrathecal administration of mafosfamide in the treatment of meningeal malignancies, although further comprehensive clinical evaluation is needed[97; 98]. In the case of glufosfamide, the development of this oxazaphosphorine agent was based on the rationale that cancer cells are active in importing and utilizing glucose. Thus, the differential expression of transmembrane glucose transporters between cancer and normal

cells accounts for the target selectivity of glufosfamide [99; 100]. Recent clinical trials revealed beneficial effects of utilizing glufosfamide in the treatment of pancreatic adenocarcinoma, non-small cell lung cancer, as well as head and neck squamous cell carcinoma [101; 102; 103]. Together, these promising anticancer activities of mafosfamide and glufosfamide indicate that new generation of oxazaphosphorine agents, with better target selectivity and less unwanted cytotoxicity could be clinically available in the near future.

1.4 Metabolism and transport of oxazaphosphorines

1.4.1 Cytochrome P450

Cytochrome P450 forms a very large and diverse hemoprotein superfamily that catalyzes metabolic reactions of thousands of endogenous and exogenous compounds. They account for a great portion of drug metabolic reactions, detoxification reactions and biotransformation of many metabolic products, as well as the synthesis and metabolism of endogenous compounds such as hormones, cholesterol, vitamin D and bilirubin. CYP enzymes are present in most human tissues but predominantly and most importantly, expressed in the liver. At the cellular level, human P450s are primarily located in the endoplasmic reticulum and the inner membrane of mitochondria. On average, CYP3A, 2C, CYP1A2, CYP2A6 and CYP2D6 represent the most abundantly expressed P450s in adult human liver [104]. Together, they account for approximately 70% of the P450 content. However, in terms of drug metabolism, CYP3A4 alone is responsible for over 50% of the total reaction, followed by CYP2D6 and CYP2C family.

As will be detailed in the following paragraph, CYP2B6 and CYP3A4 play a pivotal role

in the metabolism of oxazaphospharines. CYP2B and CYP3A are highly inducible P450 enzymes that are predominantly expressed in the liver, and the drug-mediated expression of CYP2B and CYP3A is tightly controlled at the transcription level. Traditionally, human CYP2B6 was recognized as an isozyme of P450 with moderate function in pharmacology and toxicology. In recent years, however, accumulating evidence has revealed that CYP2B6 plays an important role in the metabolism and detoxification of an increasing number of clinically important drugs such as CPA, methadone, efavirenz, bupropion, nevaripine[105] ,selegiline, artemisinin and propofol[106; 107; 108].A 51-base-pair consensus sequence termed phenobarbital responsive enhancer module (PBREM) located in the promoter of CYP2B gene has been identified and functionally characterized in mouse, rat, and humans[109]. Sequence analysis of this element shows that it is composed of two nuclear receptor binding sites (NR1 and NR2) and a nuclear factor 1 binding site. NR1 is conserved among mammalian species and is critical for conferring phenobarbital response[110; 111].More recently, a far-distal xenobiotic-responsive enhancer module (XREM) has been identified around -8 kb in the human CYP2B6 promoter region. Together with the proximal PBREM, they are believed to be responsible for the maximal induction of the CYP2B6 gene[112].

As previously mentioned, CYP3A4 is the most abundant hepatic P450 isozyme that is involved in the metabolism of approximately 50~60% of all clinical used drugs[113; 114]. Other members of the human CYP3A subfamily include 3A5 and 3A7, which account for a minor portion and exhibit polymorphic features in the population. CYP3A4 promoter contains a basal transcription element, AP-3 binding site, a p53 binding motif, a hepatocyte nuclear factor-4 element, two hepatocyte nuclear factor-5 elements, a

glucocoticoid response element, a pregnane X receptor element and an oestrogen response element. Similar as CYP2B6, an XREM is discovered in human CYP3A4 promoter and it is essential for the maximal induction of enzyme expression with the involvement of PXR (Pregnane X Receptor) [114].

Prototypical oxazaphosphorine cytostatics such as CPA and IFO are chemically and pharmacologically inactive forms of alkylating nitrogen mustards that are biotransformed to their active forms predominantly in the liver[115; 116]. As one of the key mechanisms of action, hepatic metabolism of CPA has been extensively studied during the past several decades, utilizing human and animal liver microsomes, primary hepatocytes, recombinant CYP enzymes, CYP-selective chemical and antibody inhibitors, as well as whole animal models [106; 116; 117]. Upon administration, CPA undergoes hepatic oxidation to form the pharmacologically active intermediate metabolite 4-OH-CPA, which enters the blood circulation and is transported to target tissues by binding to erythrocytes[118; 119; 120; 121]. 4-OH-CPA is further tautomerized to aldophosphamide, followed by spontaneous β-elimination to release the phosphoramide mustard as the final DNA-cross-linking metabolite[71; 122]. Notably, hydroxylation of CPA at the 4-carbon position is the rate limiting step of its bioactivation, and blood concentration of 4-OH-CPA has often been used as a biomarker monitoring the efficacy of CPA therapeutics[123; 124; 125]. Multiple CYP isozymes are involved in the hydroxylation of CPA, including CYP2A6, CYP2B6, CYP3A4, CYP3A5, CYP2C9, CYP2C18, and CYP2C19 [106; 117; 126; 127], with CYP2B6 being the primary player, which contributes approximately 45% of CPA bioactivation [106; 116; 117]. To a lesser extent, CYP3A4 and CYP2C9 also contribute around 25% and 12% of CPA 4-hydroxylation, respectively[106; 117]. Alternatively, CPA is subject to significant side-

chain oxidation, primarily N-dechloroethylation, by a number of P450s to generate the inactive dechloroethyl-CPA and the toxic byproduct chloroacetaldehyde (CAA) [88; 106; 125; 127] (Figure 1.1). The predominant CYP enzyme responsible for the *N*-dechloroethylation of CPA is CYP3A4, which was reported to be responsible for up to 95% of this reaction, followed by CYP3A7 and CYP3A5 [88; 106; 120; 127]. On the other hand, CYP2B6 only provides negligible contribution to this non-therapeutic biotransformation of CPA [127].

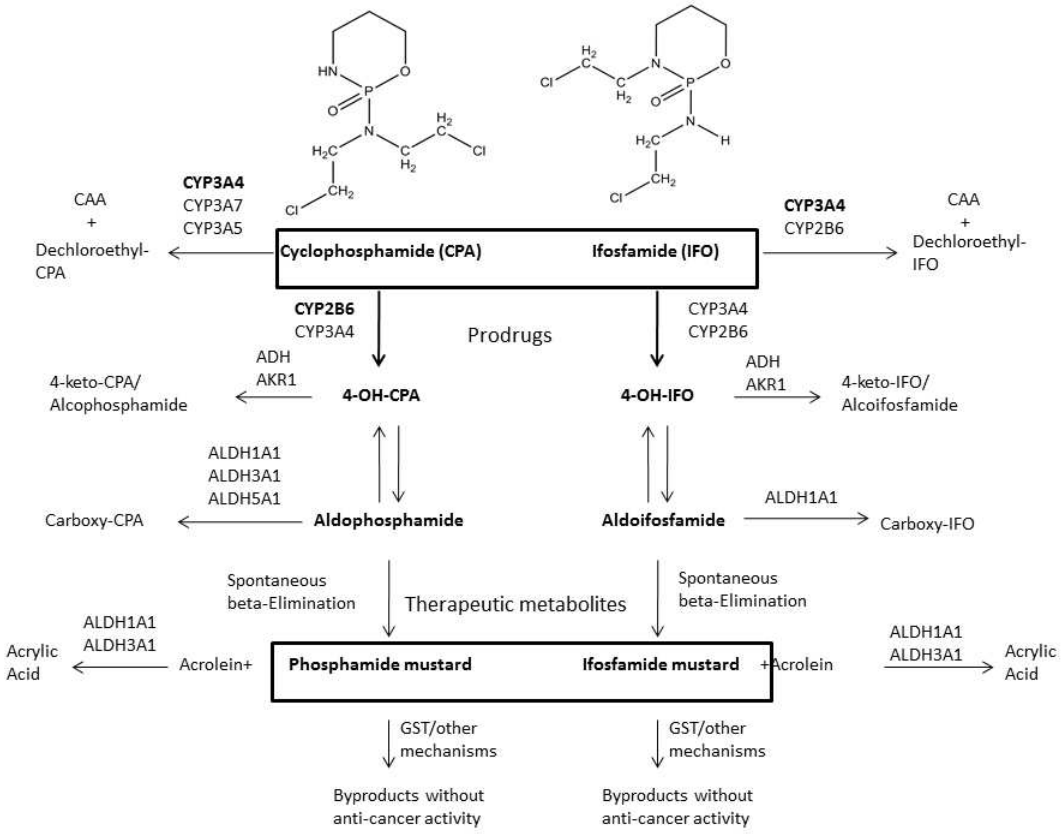


Figure 1.1 Schematic summary of CPA and IFO metabolism. The prodrugs CPA and IFO are biotransformed through a group of CYP and non-CYP drug-metabolizing enzymes to form their therapeutically active DNA-crosslinking mustards, as well as non-therapeutic metabolic byproducts (modified from Wang et al., 2011, Pharmaceutical Res[128]).

Metabolism of IFO shares a generally CYP-based pathway with that of CPA, but exhibits differential CYP affinity and metabolism rates [8; 84]. Akin to CPA, IFO is bioactivated by CYP3A4 and CYP2B6 to form the 4-hydroxy-ifosfamide (4-OH-IFO), which subsequently goes through a series of biochemical reactions to yield the ultimate therapeutic alkylating agent, ifosfamide mustard [116; 129]. More detailed characterization, however, has revealed that CYP3A4 plays a major role in the 4-hydroxylation of IFO with CYP2B6 as a supplementary isozyme [123; 130]. Moreover, these two CYP enzymes also control the *N*-dechloroethylation of IFO forming the neurotoxic CAA [106; 127], with CYP3A4 contributing approximately 70% of liver microsomal CAA formation and CYP2B6 accounting for roughly 25% [8; 106]. Additionally, CYP3A5 was also reported to be involved in the dechloroethylation of IFO. Polymorphic mutations of CYP3A5 can affect the rate of CAA formation as well [131].

In comparing the biotransformation of CPA and IFO, only 10% of CPA is subject to *N*-dechloroethylation, whereas approximately 25%-60% of IFO undergoes this metabolic pathway, generating more toxic byproducts [125; 132; 133]. This rather distinct profile of metabolism also contributes to the clinically observed side-effects, in which CAA-mediated neurotoxicity occurs in ~20% of IFO-treated patients, while this is quite rare in CPA-treated patients[134; 135].Importantly, CYP enzymes are involved differentially in the biotransformation of these oxazaphosphorines; for instance, CYP2B6 selectively activates CPA over IFO and only exhibits a negligible effect on CPA-*N*-dechloroethylation. Therefore, it might be possible to design novel CPA-based therapeutic regimens by modulating these metabolic pathways to achieve greater bioactivation without concurrent augmentation of unwanted cytotoxicity.

1.4.2 Other Drug-Metabolizing Enzymes

Following CYP-mediated 4-hydroxylation, both CPA and IFO can be further activated to their corresponding therapeutic mustards or inactivated to different byproducts through sequential metabolic processes mediated by other non-CYP drug-metabolizing enzymes [8; 87]. First, 4-OH-CPA quickly reaches equilibrium with its acyclic form, aldophosphamide, which can be spontaneously decomposed through β-elimination to form the ultimate active alkylating product phosphoramide mustard and a urotoxic byproduct acrolein, which is commonly associated with clinically important hemorrhagic cystitis [8; 64; 71]. Intracellular phosphoramide mustard then attacks host DNA to exert expected cytotoxicity. Alternatively, phosphoramide mustard can undergo detoxification by glutathione S-transferase (GST)-mediated conjugation, hydrolysis of the chloroethyl side chain to form alcohols, or cleavage of the phosphorus-nitrogen bond to release 3-(2-chloroethyl)-1,3-oxazolidin-2-one , which are all metabolic byproducts without antitumor activity [136]. The acrolein, meanwhile, is converted to acrylic acid by aldehyde dehydrogenases (ALDH), ALDH1A1 and ALDH3A1[137; 138]. An important detoxification pathway for 4-OH-CPA is the conversion of its tautomer, aldophosphamide, to the less toxic carboxyphosphamide , which represents a major stable non-therapeutic metabolite of CPA found in clinical samples [139; 140]. This oxidative reaction is primarily catalyzed by ALDH1A1 [141; 142], and to a lesser extent by ALDH3A1 and ALDH5A1 [143]. Alternatively, aldophosphamide can be oxidized to form alcophosphamide by alcohol dehydrogenase (ADH) and aldo-keto reductase (AKR1)[144] (Figure 1.1). An additional detoxification pathway occurs through reversible dehydration to form imminocyclophosphamide, which is further conjugated with glutathione mediated by GSTA1, GSTA2, GSTM1, and GSTP1,

and eventually generates the nontoxic 4-glutathionylcyclophosphamide[145; 146].

The major difference regarding the metabolism of CPA and IFO happens in the CYP-mediated 4-hydroxylation and *N*-dechloroethylation. Metabolic destinations of these oxazaphosphorines thereafter are highly comparable. As with 4-OH-CPA, 4-OH-IFO exists in equilibrium with its tautomer aldoifosfamide, which decomposes through β-elimination to yield ifosforamide mustard and acrolein. Ifosforamide mustard is also subject to further degradation, forming the inactive metabolite CNM and chloroethylamine [147]. Similarly, 4-OH-IFO can also be biotransformed to carboxyifosfamide by ALDH1A1, to 4-keto-IFO by AKR1, or to alcoifosfamide by ADH and AKR1[8; 84]. Glutathione conjugation represents another important detoxification mechanism of ifosforamide mustard[148].

Collectively, it is evident now that both CYP and non-CYP drug-metabolizing enzymes can contribute to the bioactivation and detoxification of CPA and IFO. Although the liver contains the most abundant drug-metabolizing enzymes and plays predominant roles in the biotransformation of oxazaphosphorines, extrahepatic expression of these enzymes also contribute to the targeted “selective cytotoxicity” which is one of the leading motive in developing safe and effective chemotherapeutics.

1.4.3 Drug Transporters

It is believed that all oxazaphosphorine prodrugs are highly hydrophilic and thus are not easily diffused across cell membranes. Mounting clinical and experimental evidence, however, has agreed that both CPA and IFO can be readily administered orally or intravenously with high bioavailability and sufficient intracellular concentrations[149; 150;

[151]. These phenomena suggest that active uptake transporters may contribute to the absorption of these oxazaphosphorines though direct scientific evidence is limited thus far. Conversely, the circular proactive metabolites of these oxazaphosphorines, 4-OH-CPA,aldophosphamide and 4-OH-IFO, can easily cross the lipid bilayer membranes of many cells through passive diffusion [8]. In contrast to uptake, more research efforts have centered on the efflux transportation of these alkylating agents from cancer cells, which is pivotal in multidrug resistance of cancer chemotherapy. In this regard, a number of ATP-binding cassette transporters have been identified as transmembrane modulators associated with exporting CPA, IFO, and their metabolites [8; 152; 153].

In vitro studies utilizing HepG2 cells stably transfected with multidrug resistance-associated protein 4 (MRP4, ABCC4) expression vector, have clearly established that CPA and IFO are substrates of this efflux transporter. Overexpression of MRP4 in HepG2 cells led to increased resistance to CPA- and IFO-induced cytotoxicity, while inhibition of this transporter by diclofenac or celecoxib, two known inhibitors of MRP4, significantly sensitized the MRP4-HepG2 cells to CPA and IFO [153; 154]. Notably, glutathione, the most abundant cellular redox molecule, plays an important role in the function of MRP4 and depletion of intracellular glutathione can significantly affect the export of cAMP by MRP4[155; 156]. Since glutathione is pivotal in detoxification of phosphamide and ifosforamide mustards, it was speculated that MRP4-mediated resistance to CPA and IFO might be glutathione-dependent. Indeed, addition of buthionine sulfoximine, a glutathione synthesis inhibitor, considerably reversed MRP4-mediated resistance to CPA and IFO in MRP4-HepG2 cells [154].

The multidrug resistance-associated protein 2 (MRP2, ABCC2) has been reported to

export a detoxified CPA metabolite, 4-glutathionylcyclophosphamide, from hepatocytes into the bile in rats; this biliary excretion appears to compete with the bioactivation pathway that generates the active alkylating agent [157]. In addition, clinical studies have shown that multidrug resistance-associated protein 1 (MRP1, ABCC1) and the breast cancer resistance protein (BCRP, ABCG2) are involved in the resistance to chemotherapy in breast cancer patients receiving CMF regimen[158; 159; 160]. However, whether CPA and/or its metabolites are substrates of BCRP and/or MRP1 requires further investigation, given that methotrexate and 5-fluorouracil in the CMF regimen are substrates of BCRP [161; 162].

MRP1, MRP2, and BCRP are all expressed in the apical (canalicular) membrane of hepatocytes and are in charge of hepatic biliary excretion of many drugs and endobiotics into the bile. Conversely, MRP4 is localized in the basolateral (sinusoidal) membrane of hepatocytes and in the apical (luminal) membrane of kidney proximal tubules [163]. The fact that approximately 70% of CPA is excreted in urine and only a small portion through the bile, may positively reflect the importance of MRP4 in CPA transport[84; 164].

1.5 Xenobiotic receptor and oxazaphosphorine metabolism

1.5.1 Pregnane X receptor

As one of the important components of the body's adaptive defense mechanism against xenobiotics, PXR represents the most promiscuous xenosensor among all xenobiotic receptors and can be activated by a broad spectrum of ligands including prescription drugs, herbal medicines, environmental pollutants, and endobiotic derivatives [165; 166; 167; 168]. The structural diversity of PXR ligands stems mainly from the unusually large,

spherical, and flexible ligand binding pocket of the receptor[169; 170]. Drug-mediated activation of PXR is associated with the inductive expression of many target genes including drug-metabolizing enzymes, such as CYP3A4, CYP2B6, CYP2Cs, and UDP-glucuronosyltransferases (UGT); and drug transporters, such as the multidrug resistance 1 (MDR1, ABCB1), and MRPs by recognizing and binding to specific xenobiotic response elements located in the promoters of these genes [165; 171; 172]. Among others, CYP3A4 and CYP2B6 are highly inducible PXR target genes, which exhibit marked inter- and intra-individual variations in their expression[173; 174]. As such, many clinically used drugs as PXR activators can influence the pharmacokinetics of CPA and IFO when co-administered in multidrug regimens. Additionally, accumulating evidence suggests that hepatic bioactivation of CPA and IFO is auto-inducible upon repeated application of these oxazaphosphorines [129; 175; 176].

Both CPA and IFO have been identified as human PXR agonists that contribute significantly to the observed autoinduction of CYP2B6 and CYP3A4 by which their own metabolism and clearance are increased [167; 168; 177]. In this process, CPA and IFO bind to the ligand binding domain of PXR that leads to the release of PXR-bound corepressors, such as the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor 1 (NCOR1), and the recruitment of its heterodimer partner, the retinoic X receptor (RXR), and other coactivators, such as steroid receptor 1 [178]. The PXR/RXR heterodimer directly interacts with specific promoter sequences of CYP2B6 and CYP3A4 genes and stimulates their transcription [179]. Additionally, Harmsen et al., recently reported that CPA and IFO can also induce the expression of MDR1 through PXR transactivation [180181]. Although MDR1 represents one of the major

mechanisms associated with the multidrug resistant phenotype in response to many chemotherapeutics, CPA, IFO, and their proactive metabolites 4-OH-CPA and 4-OH-IFO, are not typical substrates of MDR1 [8]; thus, this induction may not directly affect the intracellular levels of these oxazaphosphorines and their active metabolites.

In addition to drug-induced activation, genetic polymorphisms of PXR may also affect the metabolism and pharmacokinetics of CPA and IFO in different patients. The presence of PXR variants was investigated by Hustert et al., in two Caucasian and African ethnic groups[182]; three PXR protein variants (V140M, D163G, and A370T) were identified to be functionally associated with altered basal and/or induced transactivation of CYP3A promoter reporter genes. In a separate study, Lim et al., reported that the Q158K variant of PXR, found in Chinese populations, impairs drug-mediated induction of CYP3A4 by altering ligand-dependent PXR interaction with the steroid receptor coactivator-1[183]. Although autoinduction of CYP3A4 by CPA and IFO was not directly investigated in these two studies, CPA and IFO are known activators of PXR, and inducers of CYP3A4 that enhance their own metabolism and clearance. Thus, these naturally occurring PXR genetic variants may play a role in the observed interindividual variability of CYP3A4 expression and therefore, influence the varied bioactivation of chemotherapeutic prodrugs including CPA and IFO.

1.5.2 Constitutive Androstane Receptor

The constitutive androstane receptor, also denoted as the constitutively activated receptor (CAR; NR1I3), is the closest relative of PXR in the nuclear receptor superfamily and shares with PXR a panel of overlapping target genes, including a number of Phase I and II drug-metabolizing enzymes, as well as drug transporters that are involved in the

metabolism and clearance of the oxazaphosphorines [171; 184]. PXR and CAR also share many xenobiotic activators, such as the sedative phenobarbital, the anti-malaria artemisinin, the synthetic opioid methadone, as well as the oxazaphosphorine CPA but not IFO [185; 186; 187; 188; 189]. As such, the extensive cross-talk between PXR and CAR may form a compensatory biological safety net that ensures comprehensive protection against various exogenous and endogenous chemicals. On the other hand, CAR also holds several unique features that separate itself from PXR and many other nuclear receptors. First, in line with its designated name, CAR is constitutively activated and spontaneously localized in the nucleus of nearly all immortalized cell lines independent of chemical stimulation [167; 174; 190]. Secondly, unlike activation of PXR that is prototypically ligand-dependent, CAR could be transactivated by either direct binding to ligands such as the human CAR selective agonist 6-(4-chlorophenyl)imidazo-[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime (CITCO, Figure S1) or ligand-independent indirect mechanisms such as the prototypical CYP2B inducer, phenobarbital [191; 192]. In fact, the majority of CAR activators identified thus far is actually phenobarbital-type of indirect activators without binding to the receptor. Last but not least, differing from the situation in immortalized cells, CAR is predominantly localized in the cytoplasm of hepatocytes cultured *in vitro* or in the liver *in vivo* without activation and only translocates to the nucleus upon chemical stimulation[191; 193]. Together, these features of CAR make the identification of its activators more challenging, particularly towards a high-throughput format *in vitro*. As a result, only a limited number of CAR activators thus far have been reported, in comparison to the numerous drugs and environmental toxicants documented as agonistic ligands of PXR.

Recent studies from our laboratory have shown that CPA but not IFO is a phenobarbital-like activator of CAR. Treatment of human primary hepatocytes at clinically relevant concentration of CPA but not IFO resulted in significant nuclear accumulation of adenovirus-expressing enhanced yellow fluorescent protein-tagged human CAR[185]. Supported by additional *in vitro* data, it appears that while CPA can activate both PXR and CAR, IFO primarily transactivates PXR. Assuming this selective autoinduction also occurs *in vivo*, these findings may potentially be of clinical importance where drug-mediated manipulation of hCAR activity could alter the autoinduction and pharmacokinetics of selective oxazaphosphorines.

Similar to that of PXR, accumulating evidence suggests that both polymorphism and alternative splicing of human CAR play an important role in the modulation of its target gene expression [194; 195; 196]. To date, approximately 30 single nucleotide polymorphisms (SNPs) of the human CAR gene have been identified, residing in the 5'-flanking regulatory regions, coding exons, and non-coding introns [197]. Functional analysis of four SNPs, localized in the ligand binding domain of CAR, (His246Arg, Leu308Pro, Asn323Ser, and Val133Gly) has revealed that His246Arg is associated with decreased CAR activation by CITCO, while Leu308Pro affect basal but not chemically stimulated CAR activation in cell-based reporter assays [196; 197]. Recently, a number of naturally occurring alternative splicing variants of human CAR have been identified[198; 199; 200]. Functional characterization of these spliced CAR transcripts has revealed that some are associated with altered expression, cellular localization, and chemical response of the receptor [201; 202; 203; 204]. For instance, unlike its constitutively active reference form, a splicing variant of human CAR, termed hCAR3, which contains an insertion of five

amino acids (ALPYT) in the ligand-binding domain, exhibits low basal but xenobiotic-inducible activities in immortalized cell lines [201; 205]. Another splicing variant, hCAR2, with an insertion of four amino acids (SPTV) in a different region of the ligand-binding domain displays unique profile of xenobiotic-mediated activation that differs from the reference human CAR [206]. Together, these genetic variation of human CARmay differentially affect the basal and inductive expression of many drug-metabolizing enzymes that eventually influence the disposition of CPA and IFO in clinical practice.

Notably, both human CAR and PXR also exhibit significant species-specific differences in comparison with their rodent counterparts[167; 190]. In the context of "cross-talk" between these two receptors, while most data imply symmetrical cross-regulation of their target genes by rodent PXR and CAR, studies in our lab revealed that human CAR but not PXR asymmetrically cross-regulate the inductive expression of CYP2B6 and CYP3A4, in that human CAR exhibits preferential induction of CYP2B6 over CYP3A4, while human PXR mediates the expression of both genes with little discrimination [205; 207] (Figure 1.2). Given that CPA undergoes 4-hydroxylation to a therapeutically active metabolite primarily by CYP2B6 and *N*-dechloroethylation to a non-therapeutic neurotoxic metabolite by CYP3A4, the preference of hCAR for CYP2B6 over CYP3A4 may have clinical relevance in developing novel therapeutic regimens. Concurrent administration of CPA with a selective hCAR activator may facilitate the enhanced production of its beneficial metabolites without simultaneously increasing formation of its toxic metabolites.

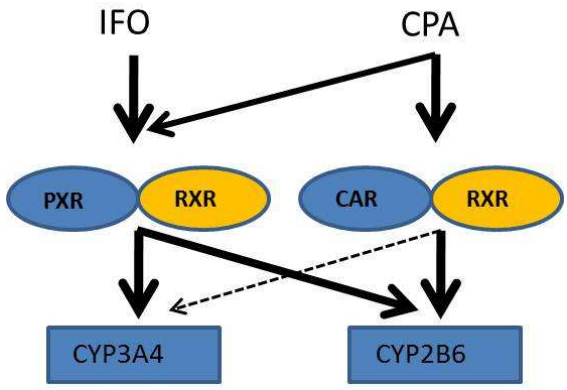


Figure 1.2 Schematic illustration of cross-talk between PXR and CAR in the regulation of CYP2B6 and CYP3A4 genes. Activation of human CAR preferentially induces the expression of CYP2B6 over CYP3A4, while activation of human PXR increases the expression of both CYP genes with little discrimination. IFO is a selective activator of human PXR, while CPA can activate both receptors (modified from Faucette et al., 2006, JPET[205]).

1.5.3 Aryl hydrocarbon Receptor (AhR)

Functioning as another xenosensor dictating the inductive expression of many drug-metabolizing enzymes, AhR is actually classified into the basic helix-loop-helix protein of the PER-ARNT-SIM (PAS) family not to the nuclear receptor superfamily[208]. Nevertheless, AhR shares a number of comparable characteristics with CAR and PXR, which are important in modulating the toxicity and biological functions of many environmental aromatic hydrocarbons and clinically used drugs [209; 210; 211]. Upon activation, ligand-bound AhR dissociates with its cytoplasmic chaperon partners and translocates to the nucleus. There, it forms a heterodimer with the aryl hydrocarbon nuclear translocator and stimulates the expression of its target genes [211; 212]. Along with the ever growing list of AhR activators, transactivation of AhR is associated with altered expression of many genes including but not limited to CYP1A1, CYP1A2, CYP1B1, UGT1As, GST, ADH, ALDH3A1, and BCRP [213; 214143; 215].

Although AhR-modulated CYP and UGT1A enzymes, and efflux transporter BCRP only have moderate effects on the bioactivation and clearance of CPA and IFO, other AhR target genes such as ADH and ALDH3A1, are proved enzymes that play critical roles in the detoxification of these two oxazaphosphorines (Figure 1.1). To date, a number of studies have shown that the therapeutic outcomes of CPA- and IFO-based chemotherapy are inversely related to the intracellular levels of ALDH1A1 and ALDH3A1.The proactive metabolites of CPA and IFO were oxidized by these enzymes to form the nontoxic 4-keto- and carboxyl-byproducts in competition with generation of the therapeutically active alkylating mustards by β-elimination [143; 216; 217]. Indeed, cancer cells with higher expression of ALDH, such as the breast cancer stem-like cells, demonstrate increased resistance to chemotherapy[218]. In addition to chemical-stimulated induction, these ALDH enzymes demonstrate tissue specific distribution and developmental changes, and are also over-expressed in certain types of cancer cells[219; 220]. Thereby, manipulating tissue-specific expression of ALDH may alter the cellular sensitivity to oxazaphosphorines. Notably, recent studies revealed that other than the prevailing mechanisms of AhR activation stimulated by exogenous ligands, elevation of intracellular second messenger cAMP could also lead to the nuclear translocation of AhR[221]. Nevertheless, cAMP-mediated translocation of AhR acts as a repressor in lieu of an activator of AhR, which leads to repression of AhR target genes including ALDH[221; 222]. As such, drugs and endogenous signaling molecules differentially modulating the function of AhR may affect the expression of ALDH enzymes one way or the other and eventually influence the clinical responses to CPA- and IFO-containing regimens.

1.6 Conclusion

The oxazaphosphorines CPA and IFO represent the most widely used chemotherapeutic alkylating agents with a history of clinical application for more than 50 years. To date, extensive studies have elucidated the general pharmacology, metabolism, pharmacokinetics and cytotoxicity of these oxazaphosphorines. However, because of the increased polypharmacy in general and in oxazaphosphorine-based chemotherapy in particular, drug-drug interactions associated with CPA and IFO multidrug regimens have become rising concerns in clinical practice. Accumulating evidence thus far has established clearly that hepatic CYP2B6 and CYP3A4 differentially contribute to the 4-hydroxylation and *N*-dechloroethylation of CPA and IFO and many clinical used drugs and environmental compounds can stimulate the inductive expression of these two CYP enzymes. Notably, it is only in the past ten years that marked progress has been achieved in our understanding of the transcriptional regulation of CYP2B6 and CYP3A4, which are controlled by a group of transcription factors, particularly, the CAR and PXR. Importantly, although activation of PXR induces both CYP3A4 and CYP2B6, selective activation of CAR leads to preferential induction of CYP2B6 over CYP3A4 in the liver[207]. This notion might be clinically attractive in directed modulation of CPA-based chemotherapy, given the fact that CPA is predominantly bioactivated by CYP2B6 while deactivated through CYP3A4.

Selective cytotoxicity towards tumor but not normal cells is the ultimate goal for all chemotherapeutic agents to achieve. Realizing the specific role of CYP2B6 in the bioactivation of CPA, Waxman and colleagues have reported that locally delivery of adenovirus- or retrovirus-encoding CYP2B expression cassette into tumor cells resulted

in increased intracellular CPA 4-hydroxylation and cytotoxicity [223; 224; 225]. Thereafter, a number of studies have demonstrated that such strategy could be successful in cell cultures *in vitro*, tumor xenografts in animal, and to a certain extent in initial clinical trials [223; 225; 226; 227]. The reality, however, is that clinically used CPA and IFO rely predominantly on hepatic CYP-mediated biotransformation and the activated metabolites are transported by erythrocytes to tumors and normal tissues via blood circulation. Moreover, unlike localized solid tumors, systemic chemotherapy is necessary for hematopoietic malignancies such as lymphoma and leukemia, in which CPA continues to be used among the first-line R-COUP regimen. Therefore, understanding the role of xenobiotic receptors in the regulation of key drug-metabolizing enzymes in the liver involving in the bioactivation and deactivation of oxazaphosphorine agents is of both scientific significance and clinical importance. In addition, this knowledge will help to interpret observed as well as predict potential drug-drug interactions involving oxazaphosphorines in multi-drug regimens, which are commonly used in chemotherapy.

1.7 Research Objective

This dissertation research was to investigate the role of CAR in the metabolism of oxzaphosphorines, with a focus on the regulation of relevant CYP enzymes and its potential clinical implications. Importantly, a strategy to enhance CPA therapeutic effect through preferential induction of CYP2B over CYP3A was proposed and tested in an innovative co-culture system that closely mimic the *in vivo* condition. Clinically used compounds were screened for potential co-treatment with CPA to enhance its anti-cancer effects.

In Chapter 2, the role of CAR in the metabolism of CPA was investigated. The hypothesis that selective activation of CAR may enhance CPA therapeutic effect was proposed based on previous discoveries that CAR preferentially induces CYP2B6 over CYP3A4, which are majorly responsible for the activation and inactivation of CPA, respectively. To test the hypothesis and evaluate its potential clinical value, an innovative Human Primary Hepatocyte (HPH)-leukemia cell co-culture system was established. Specific human CAR activator CITCO was evaluated at multiple levels and endpoints as the model compound with respect to its influence on the metabolism of CPA using the co-culture system. In addition, the clinical relevance of the proposed therapeutic strategy was evaluated in an *in vivo* study using specific mouse CAR activator TCPOBOP and an established xenograft mouse tumor model, given the similarity of CPA metabolism pathways between human and mice.

The clinical feasibility of the above strategy depends not only on the selective enhancement in CPA therapeutic over side-toxic effects that the compounds can deliver, but also on the clinical relevance and availability of these hCAR activators. Since neither CITCO nor TCPOBOP is used in clinical application, Chapter 3 focused on the screening and identification of potential drug candidates for preferential induction of CYP2B over CYP3A from a pool of FDA-approved drugs and clinically used Chinese herbal medicines using different approaches. Luciferase assay, Real-time PCR and western blot assays were applied to different cell models including HepG2 and HPH to examine the activation of PXR and CAR, and subsequent induction of relevant P450 genes.

In additional to the potential benefit of CAR activation in CPA metabolism, oxazaphosphorines exhibit autoinduction in which process their own metabolism is

promoted. Chapter 4 further investigated the role of CAR in oxazaphosphorine-mediated autoinduction of drug-metabolizing enzymes. Both CAR and PXR activation are studied with respect to the regulation of relevant DMEs in CPA and IFO metabolism. Results were compared between different nuclear receptors as well as different drugs.

CHAPTER TWO
EXPLORATION OF THE ROLE OF CAR IN THE METABOLISM OF CYCLOPHOSPHAMIDE

2.1 Introduction

Hematopoietic malignancies are a group of heterogeneous disorders associated with considerably variable prognoses, depending in large part on the specific diagnosis and available treatment options. CPA, as introduced previously, has been widely used in combination with other antineoplastics in the adjuvant treatment of various cancers, including hematological disorders such as lymphoma and leukemia.[75; 77; 78] For instance, CPA constitutes a critical component in the front-line chemotherapy for two common lymphoid malignancies, non-Hodgkin lymphoma and chronic lymphocytic leukemia which are often treated with the R-CHOP multidrug regimen and rituximab in combination with fludarabine and CPA (FCR), respectively.[78; 228] Nevertheless, a significant number of patients succumb to their disease despite standard therapeutic regimens.[229] The need for further optimization of current treatment paradigms is evident. Given that CPA is a prodrug requiring metabolic activation by hepatic drug-metabolizing enzymes, CPA-based chemotherapy could be improved by selectively enhancing the metabolic conversion of CPA to the pharmacologically active metabolite but not to the non-therapeutic byproducts.

Upon administration, CPA undergoes hepatic oxidation to form the therapeutically active intermediate metabolite, 4-OH-CPA, primarily mediated by CYP2B6 and to a lesser extent by CYP3A4 and CYP2C9.[106; 117] The 4-OH-CPA is further tautomerized to aldophosphamide, followed by spontaneous β-elimination to release the phosphoramide

mustard that exerts chemotherapeutic effects by attacking specific nucleophilic groups of DNA molecules in target cancer cells.[122] Alternatively, CPA is subject to significant side-chain oxidation, primarily *N*-dechloroethylation to generate the inactive dechloroethyl-CPA and the toxic byproduct chloroacetaldehyde predominantly by CYP3A4.[127] In addition, 4-OH-CPA and aldophosphamide can be further deactivated by ALDHs such as ALDH1A1, ALDH3A1, and ALDH5A1 (Figure 2.1).[141] Notably, hydroxylation of CPA at the 4-carbon position represents the rate limiting step of its bioactivation and, blood concentration of 4-OH-CPA has often been used as a biomarker monitoring the efficacy of CPA-based chemotherapy.[124; 125]

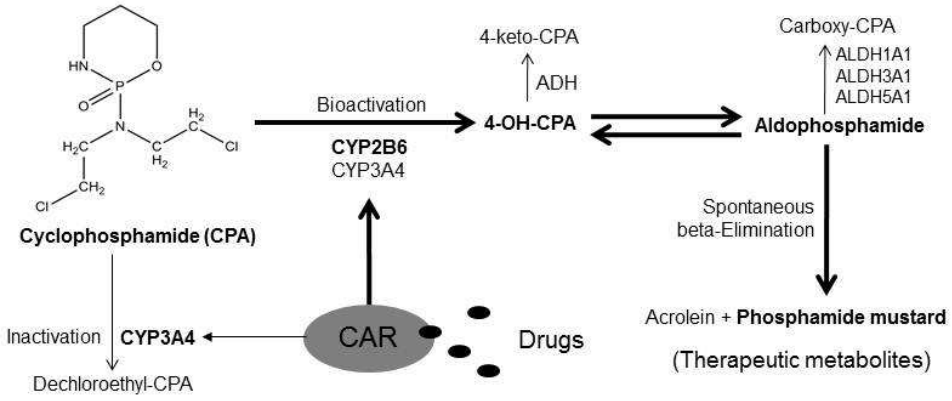


Figure 2.1Schematic illustration of CPA metabolism and the proposed role of CAR in CPA bioactivation.

Because multiple drug-metabolizing enzymes are involved in the biotransformation of CPA, drugs and environmental chemicals that influence the expression and activity of these enzymes could significantly alter the therapeutic efficacy as well as the side effects

of CPA.[8; 229; 230] It is worth noting that although induction of CYP expression generally increases the elimination of drugs and leads to therapeutic failures, in the case of CPA, increasing CYP-mediated biotransformation can generate more cytotoxic intermediate metabolites with or without therapeutic potentials, which may lead to comprehensive clinical ramifications. Currently, nuclear receptors CAR and PXR are recognized as the primary regulators of drug-induced expression of CYP2B6 and CYP3A4 in the liver.[171; 231] Although activation of human (h) PXR induces both CYP3A4 and CYP2B6 with less discernible differences, recent evidence from our laboratory has demonstrates that selective activation of hCAR leads to marked preferential induction of CYP2B6 over CYP3A4 in the liver.[207] Based on the fact that hCAR asymmetrically cross-regulates the inductive expression of CYP2B6 and CYP3A4, we hypothesize that selective activation of hCAR can enhance the efficacy of CPA-based chemotherapy without simultaneously increasing off-target cytotoxicity.

Recognizing the specific role of CYP2B6 in the bioactivation of CPA, a number of studies have shown that local delivery of adenovirus- or retrovirus-encoding CYP2B expression cassette into tumor tissues can lead to enhanced intracellular CPA 4-hydroxylation and cytotoxicity.[223; 224] Although this strategy appears to be attractive in CPA-based treatment of localized solid tumors, it may not be applicable to hematopoietic malignancies such as leukemia and lymphoma, in which systemic chemotherapy is necessary. Inasmuch as liver represents the primary organ responsible for metabolism and detoxification of the majority of endogenous and exogenous chemicals, drugs affecting hepatic expression of CYP2B6 may profoundly influence the systemic exposure of CPA metabolites, as well as the efficacy and safety of CPA in the treatment of leukemia and

lymphoma.

In the current study, we presented a novel HPH-suspension cell co-culture model, which represented an excellent *in vitro* system mimicking the human *in vivo* situation, where hepatic drug metabolism and extrahepatic anticancer activity can be investigated simultaneously. The human promyelocytic leukemia cells (HL-60) were used as a model target for CPA in the current study. By employing gene expression assays, target and off-target cytotoxicity experiments, cellular apoptosis analyses, and LC-MS/MS-based measurement of 4-OH-CPA formation, we provided experimental evidence demonstrating that selective activation of hCAR heightens the efficacy of CPA-mediated leukemia treatment without concomitant increment of off-target cytotoxicity.

In addition, a pilot *in vivo* study was conducted in a Severe Combined Immune Deficient (SCID) mice model with on xenografted B-cell lymphoma. Although there are species differences between human and rodent CAR in terms of ligands, mechanism of activation as well as target genes[171], mouse CAR retains similar gene regulation functions as its human counterpart. Particularly, mCAR activates CYP2B10, the homologue of CYP2B6 in human and CYP3A13, the homologue of CYP3A4 and CYP3A5 in human. Likewise, CPA undergoes bio-activation in mice predominantly by CYP2B10 and to a less extent by CYP3A13, while the inactivation pathway is governed by CYP3A13[232]. In the pilot study, a mCAR specific activator, 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene (TCPOBOP) was co-injected with CPA to the lymphoma-bearing mice. An inhibition of tumor growth was expected from the co-treatment group versus the CPA alone group.

2.2 Material and Methods

2.2.1 Chemicals and biological reagents

CPA, phenobarbital (PB), rifampicin (RIF), Hoechst 33342, semicarbazide hydrochloride (SCZ), and hexamethylphosphoramide (HMP) were purchased from Sigma-Aldrich (St. Louis, MO).CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). 4-hydroperoxycyclophosphamide was purchased from Enoresearch (Hillsborough, NC). Oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). Matrigel, insulin and ITS^+ culture supplements were from BD Biosciences (Bedford, MA). Other cell culture and molecular reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

2.2.2 Culture and treatment of Human primary hepatocytes

Human liver tissues were obtained following surgical resection by pathology staff after diagnostic criteria were met and with prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously,[233] or obtained from Life Technologies Corporation (Durham, NC). Sandwich cultures of hepatocytes were maintained in 6-well or 12-well collagen-coated plates as described earlier.[193] Briefly, four hours after attachment at 37°C in a humidified atmosphere of 5% CO2, hepatocytes were overlaid with Matrigel (0.25 mg/mL) in Williams' E medium supplemented with ITS^+, 0.1 μM dexamethasone, 100 U/mL penicillin, and 100 μg/mL streptomycin. Thirty-six hours after seeding, hepatocytes were treated with vehicle control (0.1% DMSO), CITCO (1 μM), or RIF (10 μM) for another

24 h or 72 h before the detection of mRNA and protein, respectively. Cell culture medium was replaced on a daily base.

2.2.3 Human primary hepatocyte-HL-60 cell co-culture model

HPHs were cultured in collagen-coated 6-well plates as described above, 3.0 μm polycarbonate membrane inserts from a 24 mm Transwell plate (Corning, Lowell, MA) were placed in the HPH-containing plates. Approximately 0.6~1.5 × 10^6 leukemia (HL-60) cells, obtained from American Type Culture Collection (Manassas, VA), were transferred into each insert-chamber, and a total of 4 mL of complete Williams' E medium as aforementioned was shared by HPHs and HL-60 cells in each well. To evaluate the effects of hepatic metabolism on the anticancer activity of CPA in HL-60 cells, the co-cultured cells were pre-incubated with vehicle control (0.1% DMSO) or CITCO (1 μM) for 24 h followed by co-exposure of CPA at designated concentrations for up to 72 h depending upon experimental requirements.

2.2.4 Quantitative PCR analysis

Total RNA was isolated from control and treated hepatocytes or leukemia cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instruction. Expression of CYP2B6, CYP3A4, ADH1B, ADH1C, ALDH1A1, ALDH3A1, ALDH5A1, and CAR mRNA was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences for real-time PCR assays were acquired either from the PrimerBank

(http://pga.mgh.harvard.edu/primerbank)22 or as reported previously[207], please refer to Supplemental Table 1 for detailed information. Fold induction values were calculated according to the equation: fold over control = $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between the target gene and GAPDH, and ΔΔCt represents the relative change in these differences between control and treatment groups.

2.2.5 Western blotting analyses

Homogenate proteins (20 μg) from each treated HPHs or HL-60 cells were resolved on SDS-polyacrylamide gels, and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes. Subsequently, membranes were incubated with specific antibodies against CYP2B6, CYP3A4 (Millipore-Chemicon, Temecula, CA) or Cleaved Caspase-3 (Cell Signaling Technology, Danvers, MA) diluted 1:4000, 1:5000, and 1:1000, respectively. Beta-actin was used for normalization of protein loading. After incubating with horseradish peroxidase goat anti-rabbit IgG antibody diluted 1:4000, membranes were developed using ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ).

2.2.6 Cell viability assay

After pre-incubation with vehicle control (0.1% DMSO) or CITCO (1 μM) for 24 h, HPH-HL-60 co-culture was treated with 250, 500, or 1000 μM of CPA in the presence or absence of CITCO (1 μM), and 20 μL of HL-60 cells from each chamber were harvested at 12, 24, 36, and 48 h. Prototypical trypan blue exclusion assays were carried out to determine cell viability using Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA)

2.2.7 LC-MS/MS determination of 4-OH-CPA formation

Cell culture medium (200 μL) from each treatment group was mixed immediately with 20 μL of semicarbazide (SCZ, 2 M) to derivatize 4-OH-CPA into 4-OH-CPA-SCZ. After vortexing for 10 min at room temperature, 2 μM HMP was added as an internal standard and 200 μL acetonitrile was used for protein precipitation. After centrifugation, the supernatant was extracted twice with ethyl acetate. The organic phase was evaporated to dryness and reconstituted with mobile phase (water-acetonitrile, 50:50, v/v). The LC separation was performed on an Aquasil C18 column (50 mmX2.1mm ID, particle size 3 μm) with water–acetonitrile (90:10, v/v) as the starting gradient, at a flow-rate of 0.4 mL/min with a total run time of 6.5 min. Mass transitions were monitored at m/z: 261→ 140 for CPA, m/z: 334→221 for 4-OH-CPA-SCZ, and m/z: 180→135 for HMP under the positive multiple reaction monitoring mode.

2.2.8 Hoechst 33342 staining

Treated HL-60 cells from the co-culture system were fixed with 2% paraformaldehyde for 10 min at 4°C. After washing with PBS, HL-60 cells were stained with Hoechst 33342 and visualized under a fluorescence microscope (Nikon Eclipse TE-2000E). Cells that displayed intensely condensed and/or fragment nuclei staining were considered as apoptotic cells. The percentage of apoptosis was calculated from 200 cells randomly chosen from each treatment group.

2.2.9 Flow Cytometry assay

HL-60 cells were harvested and processed with Annexin V staining kit (eBioscience, San

Diego, CA) according to the manufacturer's instruction. Briefly, HL-60 cells were washed once with PBS and the binding buffer, before suspended in 100 µL of binding buffer at the density of 5×10^6 cells/mL. Subsequently, cells were stained with fluorochrome-conjugated Annexin V for 15 min at room temperature. After another round of washing, 200 µL of cells were further stained with Propidium Iodide and analyzed by MoFlo Legacy (Beckman Coulter, Miami, FL).

2.2.10 DNA fragmentation

Genomic DNA from HL-60 cells was extracted and purified using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. From each treatment group, 4 µg of DNA were electrophoresed on a 2% agarose gel containing 0.375 µg/mL ethidium bromide in TAE buffer at 100 V for 1 h as detailed in previous publication[234]. Images were acquired with ChemiDoc MP System (BIO-RAD, Berkley, CA) to visualize the "DNA ladder" formation.

2.2.11 Hepatocyte toxicity assays

A typical MTT assay was applied to measure potential cytotoxicity in HPHs. In brief, HPHs were seeded in a collagen-coated 96-well plate at a density of 7.5×10^4 cells/well. After a 24 h incubation, cells were treated with various concentrations of CPA in the presence and absence of CITCO (1 µM) for 48 h. An aliquot (20 µL) of MTT solution (5.0 mg/mL) was added to each well followed by 4 h incubation, and the resulting crystals were dissolved in 150 µL of DMSO. Absorbance was analyzed at 580 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA). In a separate experiment, the morphology of HPHs in the co-culture plates was monitored. Toxic concentration of CPA (4000 µM) was applied as a positive control.

2.2.12 Animal Model

Male SCID mice aged 6 weeks (Harlan Laboratories, Frederick, MD) were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. SUDHL-6 cells (3×10^6) were resuspended in 300 μlmedium with 33% of Matrigel. The mixture was subcutaneously injected into the right dorsal flanks of the mice. The weight of the mice and tumor volume was monitored every other day. Tumors were measured three times a week with calipers, and tumor volumes were calculated by the formula $½\times r_1^2\times r_2$ ($r_1< r_2$). When the tumor reached the size of 200 mm^3, the mice were then sorted into 4 groups with 4 mice each so that the mean tumor volumes were similar. Then, the treatments were administered intraperitoneally on day 1,4,7 and 10 for CPA and day 1 and 8 for TCPOBOP. CPA was freshly prepared in PBS for final doses as indicated below (Table 2.1). TCPOBOP was prepared in corn oil for a final dose of 3 mg/kg. Injection of the vehicle alone was used as a control. At the end of experiment, liver, kidney and 200 μL blood were harvested from each mouse for further studies.

Table 2.1 *In vivo* experiment design

Group	Dose CPA (mg/kg) q4dx3	TCBOPOP (mg/kg) q7dx2
1	Vehicle	
2	30	
3	60	
4	30	3

2.2.13 Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± S.D. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test or Student's t test where appropriate. The statistical significance was set at p values < 0.05 (*), or < 0.01 (**).

2.3 Results

2.3.1 Activation of CAR preferentially induces hepatic expression of CYP2B6 over CYP3A4, while exhibiting minor effects on the expression of ADHs and ALDHs

To investigate the effects of CAR on the expression of the major drug-metabolizing enzymes responsible for CPA biotransformation, HPHs were treated with the prototypical hCAR activator CITCO, or hPXR activator RIF as described under "Methods". In four different preparations of human hepatocytes, CITCO (1 µM) induced the expression of CYP2B6 mRNA by 14- to 34-fold, while CYP3A4 by 2- to 2.5-fold, respectively. On the other hand, RIF (10 µM) induced CYP2B6 mRNA by 7- to 18-fold and CYP3A4 mRNA by 4- to 8-fold in these HPH cultures (Figure 2.2A and 2.2B). The robust and preferential induction of CYP2B6 by CITCO was further confirmed at the protein level in HPHs prepared from three different donors (Figure 2.2C). Clearly, activation of hCAR exhibits preferential induction of CYP2B6 over CYP3A4, while hPXR activation induced both CYP3A4 and CYP2B6 with less discernible differences.

In addition to CYP2B6 and CYP3A4, several other drug-metabolizing enzymes such as ADH1B, ADH1C, ALDH1A1, ALDH3A1, and ALDH5A1 are also involved in the

biotransformation and deactivation of CPA.[142; 143] Notably, activation of hCAR and hPXR by CITCO and RIF only exhibits modest influence on the expression of ADH1B, ADH1C, ALDH1A1, ALDH3A1, and ALDH5A1 in treated HPHs (Fig.2.3). Together, these results indicate that selective activation of hCAR results in robust and selective induction of CYP2B6 that favor bioactivation of CPA, while exhibiting moderate effects on CYP3A4 and other enzymes associated with generation of non-therapeutic CPA metabolites.

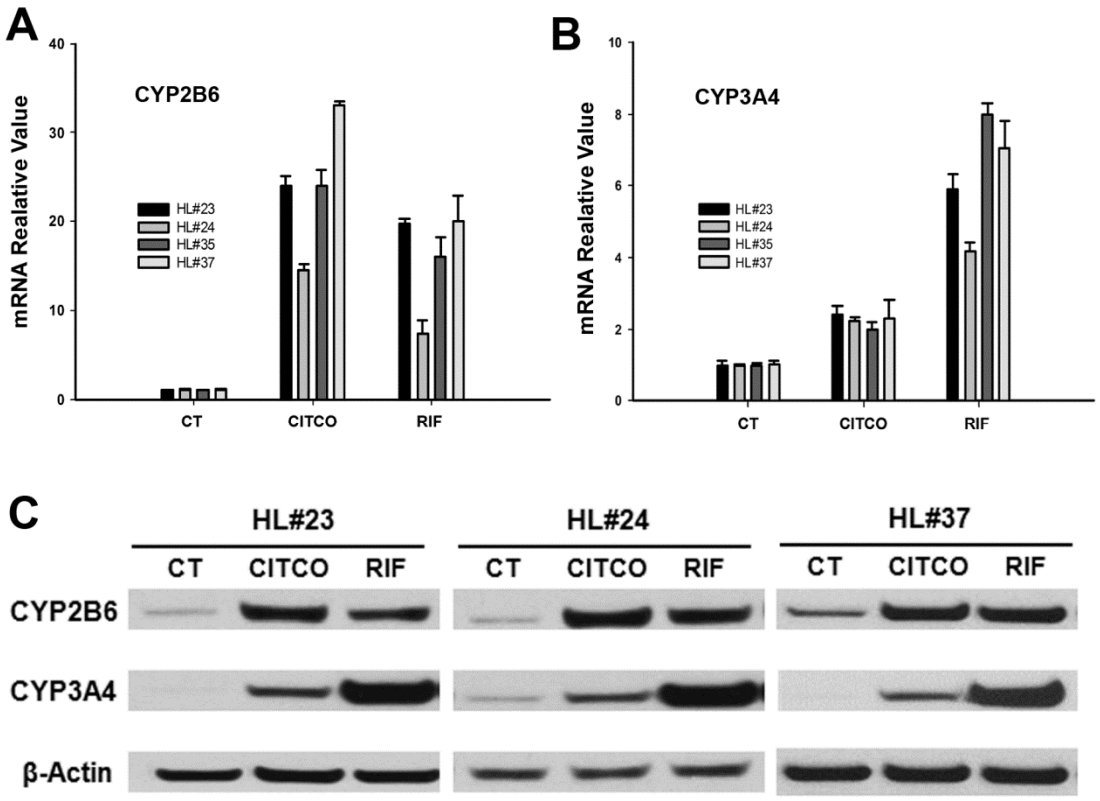


Figure 2.2CAR-mediated induction of CYP2B6 and CYP3A4 in human primary hepatocytes.CYP2B6 and CYP3A4 mRNA and protein were measured in human primary hepatocytes prepared from four donors treated with CITCO (1 μM), RIF (10 μM), or vehicle control (0.1% DMSO) as outlined in the "Methods". (A and B) Induction of CYP2B6 and

CYP3A4 mRNA analyzed with RT-PCR in HPHs from liver donors (HL#23, HL#24, HL#35 and HL#37). (C) Representative immunoblots of CYP2B6 and CYP3A4 proteins in HPHs from liver donors (HL#23, HL#24, and HL#37). RT-PCR data obtained from three independent experiments were expressed as mean ± SD normalized against vehicle control.

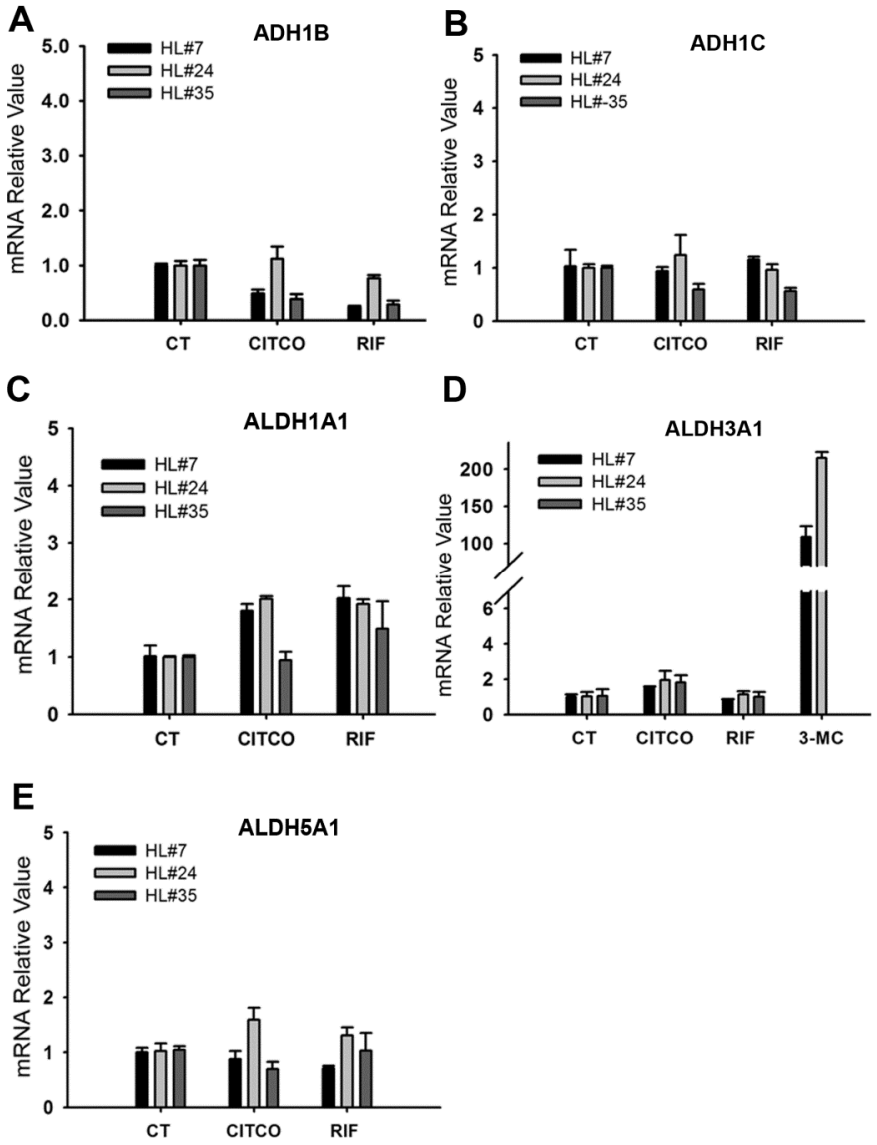


Figure 2.3PXR and CAR-mediated induction of ADHs and ALDHs in human primary hepatocytes.ADHs and ALDHs mRNA were measured in human primary hepatocytes prepared from three liver donors (HL#7, HL#24 and HL#35) treated with CITCO (1 µM), RIF (10 µM), or vehicle control (0.1% DMSO) as outlined in the "Methods". Induction of mRNA was analyzed

with RT-PCR. Data obtained from three independent experiments were expressed as mean ± SD normalized against vehicle control.

2.3.2 Endogenous expression of drug-metabolizing enzymes associated with CPA metabolism in hematopoietic cancer cells

Given that in situ biotransformation of CPA in cancer cells could profoundly influence its therapeutic efficacy, we further analyzed the expression profile of genes that are associated with CPA metabolism in a diverse panel of leukemia and lymphoma cells, including HL-60, REH, K562, SU-DHL-4, SU-DHL-6, OCI-LY-3, Farage, as well as primary lymphoma and leukemia cells obtained from four patients. The relevant gene expression levels in these cancer cells were compared with that in HPHs by real-time PCR analysis. As shown in Figure 2.4A, endogenous expression of CYP2B6, CYP3A4, ADH1B, ADH1C, ALDH3A1, and CAR in all leukemia and lymphoma cells is negligible in comparison with that in HPHs, whereas expression of ALDH5A1 is relatively consistent across all tested cells. It is noteworthy that in contrast to other tested cancer cell lines, K562 cells express relatively high levels of ALDH1A1 that are comparable to that of HPHs, indicating that K562 may not be a CPA-sensitive target. Subsequently, we examined whether expression of the CYP2B6 and CYP3A4 genes was inducible in HL-60 and primary lymphoma/leukemia cells by CITCO (1 μM) or RIF (10 μM). As expected, expression of CYP2B6 and CYP3A4 mRNA remains negligible upon these treatments in HL-60 and the primary cancer cells (Figure 2.4B, and 2.4C). Collectively, these observations suggest that endogenous and inductive expressions of CPA-metabolizing enzymes are insignificant in most leukemia and lymphoma cells, and bioactivation of CPA for the treatment of hematopoietic malignancies relies

predominantly on hepatic metabolism.

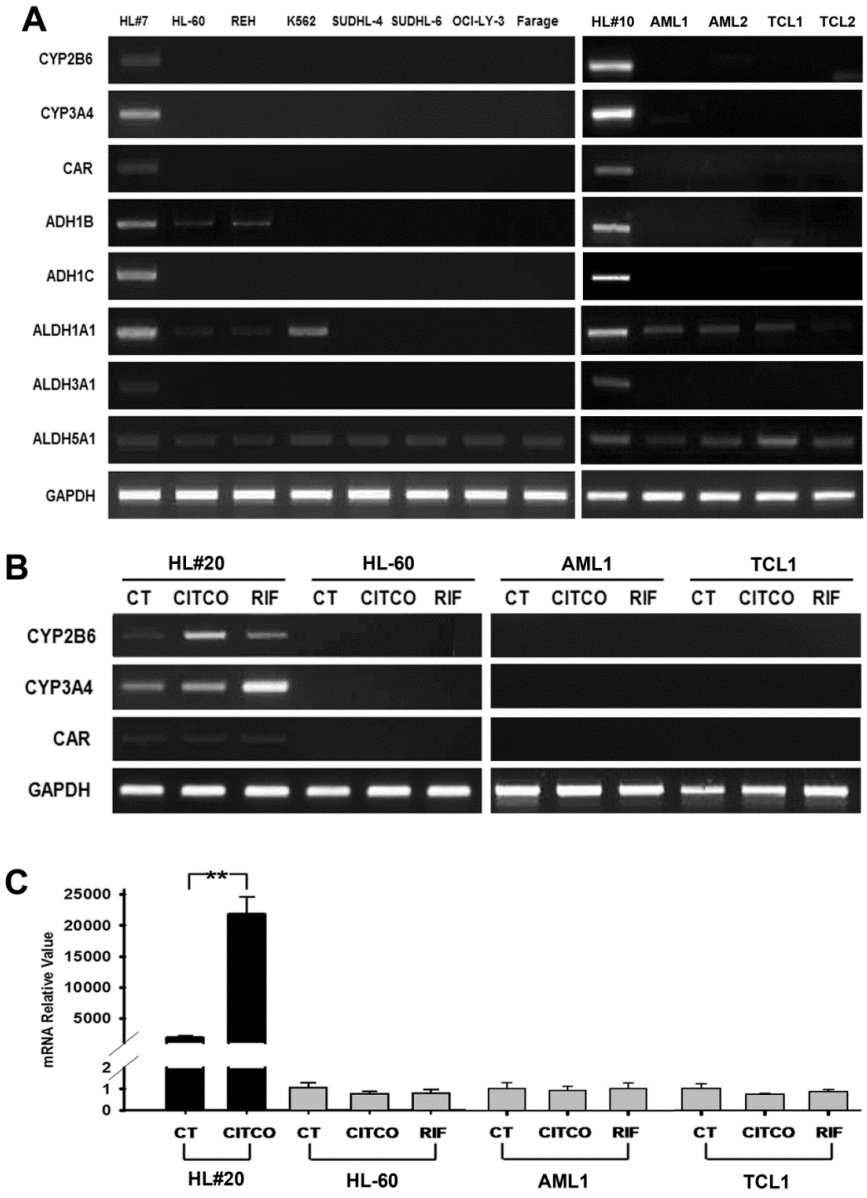


Figure 2.4Basal and induced expression of CYP2B6, CYP3A4, ADHs, ALDHs, and CAR in hematopoietic cancer cells. Total RNA was extracted from 7 hematopoietic cancer cell lines, namely, HL-60, REH, K562, SUDHL-4, SUDHL-6, OCI-LY-3, and Farage, and primary lymphoma and leukemia cells from 4 patients [T-cell lymphoma (TCL1, and TCL2), and acute myeloid leukemia (AML1, and AML2)]. (A) The relevant gene abundance of CYP2B6, CYP3A4,

ADH1B, ADH1C, ALDH1A1, ALDH3A1, ALDH5A1 and CAR in these cells was analyzed using RT-PCR in comparison with that in HPHs from donor HL#7 and HL#10, as detailed in the "Methods". (B and C) HL-60 cells, TCL1, AML1, and HPHs (HL#20) were treated with CITCO (1 μM), RIF (10 μM) or vehicle control (0.1% DMSO) for 24 h. Subsequently, inductive expression of CYP2B6, CYP3A4, and CAR in these cells was measured by RT-PCR. RT-PCR data obtained from three independent experiments were expressed as mean ± SD normalized against vehicle control. **, $p < 0.01$.

2.3.3Activation of CAR enhances CPA anticancer activity in the HPH-HL-60 co-culture system through promoting CPA bioactivation

The HPH-HL-60 co-culture model was established as depicted in Figure 2.5A. HPHs were pre-incubated with vehicle control (0.1% DMSO) or CITCO (1 μM) for 24 h to allow gene induction. Different concentrations of CPA were then applied to the co-cultures with the presence of vehicle control or CITCO in the medium. CITCO at the concentration of 1 μM produces optimal hCAR activation and CYP2B6 induction in HPHs.[192; 235] Therefore, unless otherwise indicated, CITCO was used at 1 μM in the current studies. Results from the co-cultures with HPHs prepared from a representative liver donor indicate that CPA at concentrations bracketing its pharmacologically relevant levels markedly decreased the viability of HL-60 cells in a concentration-dependent manner (Figure 2.5B). Notably, co-treatment with CITCO significantly enhanced CPA-mediated cytotoxicity in HL-60 cells, in that 250 μM of CPA + CITCO could achieve equal to greater magnitudes of anticancer activity than CPA alone at the concentration of 1000 μM. The temporal effects of CPA in the presence and absence of CITCO were also evaluated in parallel experiments. As shown in Figure 2.5C, CPA-induced cytotoxicity in HL-60 cells is time-dependent and the presence of CITCO can significantly enhance

CPA-induced cytotoxicity as early as 24 h after co-treatment. On the other hand, CITCO alone did not exhibit noticeable cytotoxicity in the co-cultured HL-60 cells (Figure 2.6).

To further evaluate the effects of CAR activation on the biotransformation of CPA in the HPH-HL-60 co-culture system, culture medium from each treatment group as described above was collected and subjected to LC-MS/MS quantification of 4-OH-CPA as outlined in “Methods”. The multiple-reaction monitoring chromatogram demonstrates clear separation and retention of 4-OH-CPA, CPA, and the internal standard (Figure 2.5D). As shown in Figure 2.5E, formation of 4-OH-CPA in the co-culture system was increased in a concentration-dependent manner upon CPA treatment; importantly, this trend was further enhanced in the presence of CITCO. Likewise, at a fixed concentration of CPA (500 μM), 4-OH-CPA formation in the co-culture medium increased through a time window from 2 to 24 h, and CITCO markedly enhanced the 4-OH-CPA concentrations at all the time points measured (Figure 2.5F). These results indicate that the observed enhancement in the chemotherapeutic activity of CPA in HL-60 cells was attributed to CAR-mediated augmentation of 4-OH-CPA formation in the liver.

Figure 2.5Activation of CAR enhances CPA anticancer activity in the human primary hepatocyte-HL-60 cell co-culture system. The anticancer activity of CPA was analyzed in a unique hepatocyte-cancer cell co-culture model. (A) Illustration of the HPH-HL-60 co-culture model and experimental scheme. (B) Effects of CAR activation on the concentration-dependent anticancer activity of CPA in HL-60 cells. As detailed in the “Methods”, the HPH-HL-60 co-cultures were treated with vehicle control (0.1% DMSO) or CPA (250, 500, and 1000 μM) in the presence and absence of CITCO (1 μM). Cell viability was analyzed 36 h after the co-treatment. (C) Effects of CAR activation on the temporal changes of CPA-mediated anticancer activity in

HL-60 cells. In the co-cultures, cells were treated with vehicle control (0.1% DMSO) or CPA (500 µM) with or without CITCO (1 µM). Cell viability was measured at 0, 12, 24, 36, and 48 h after the co-treatment. All viability data represent mean ± SD from three independent experiments and are expressed as percent viability of vehicle control. **, $p < 0.01$. (D) The multiple-reaction monitoring chromatogram demonstrates separation and retention of 4-OH-CPA, CPA, and the internal standard from LC-MS/MS detection. (E) Effects of CAR activation on the concentration-dependent formation of 4-OH-CPA in the co-culture under the treatment as outlined in (B). (F) Effects of CAR activation on the temporal changes of 4-OH-CPA formation in co-cultures under the treatment described in (C). 4-OH-CPA concentrations represent mean ± SD of three LC-MS measurements. **, $p < 0.01$.

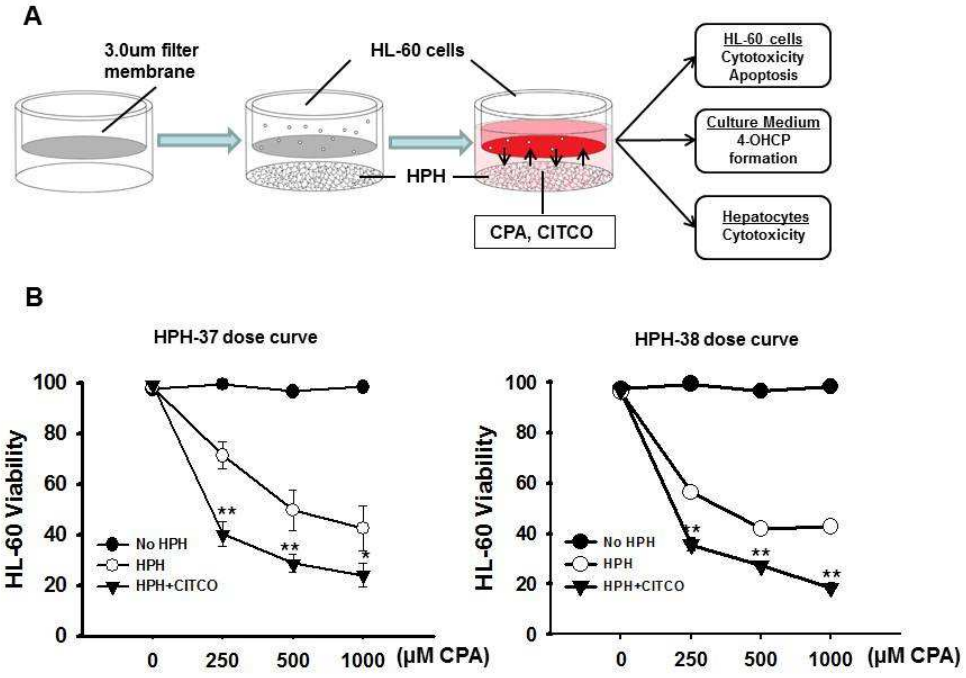
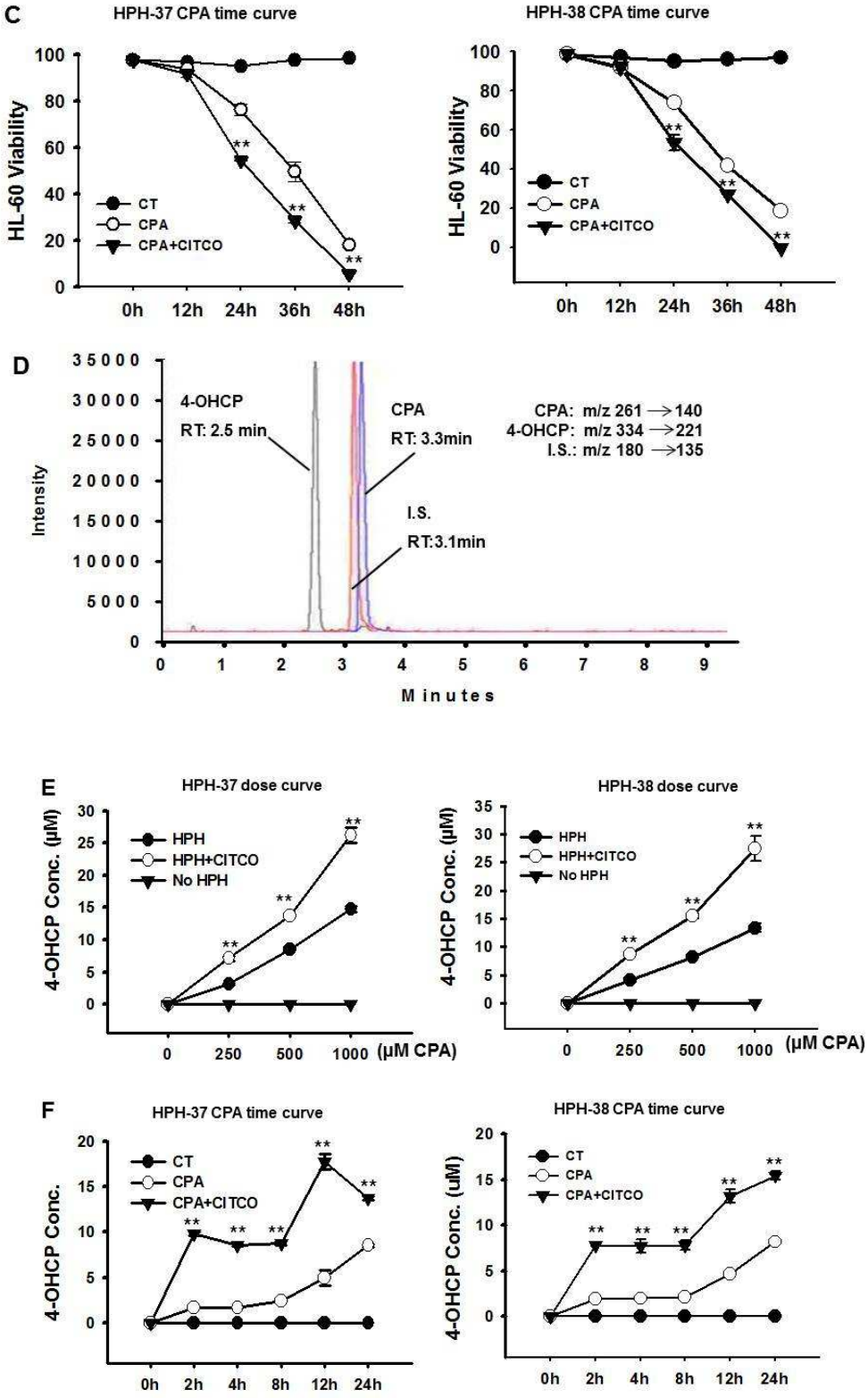


Figure 2.5 Continued



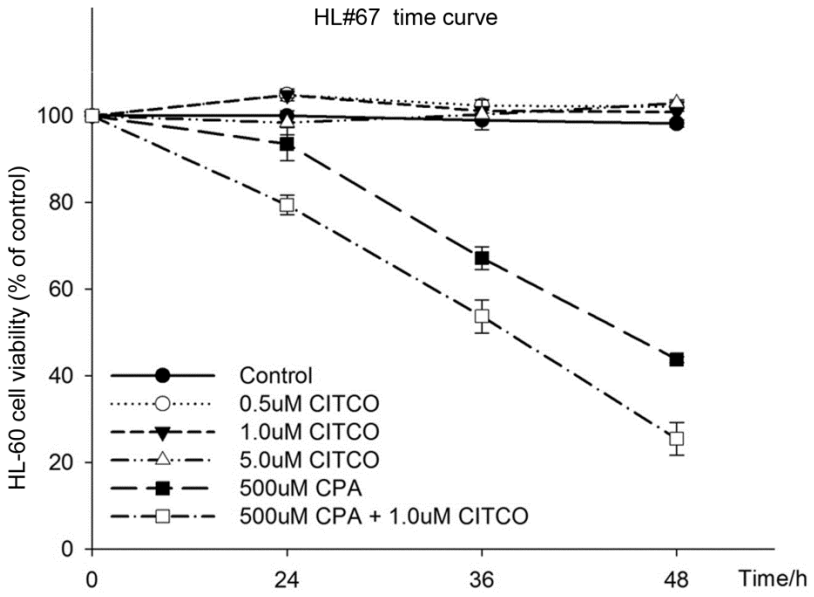


Figure 2.6 CITCO alone does not inhibit or promote the proliferation of HL-60 cells. As detailed in the "Methods", the HPH-HL-60 co-cultures were treated with vehicle control (0.1% DMSO), CITCO alone (0.5, 1, and 5 μM) or CPA (500 μM) in the presence and absence of CITCO (1 μM). Cell viability was measured at 0, 24, 36, and 48 h after the co-treatment.

2.3.4 Activation of CAR promotes CPA-mediated apoptosis of HL-60 cells in the HPH-HL-60 co-culture system

Following CYP-mediated hydroxylation, CPA went through sequential metabolic processes and was converted to the ultimate active alkylating nitrogen mustard that attacks specific nucleophilic groups of DNA molecules in cancer cells which often leads to cell apoptosis.[236; 237] To evaluate the effects of CAR activation on CPA-induced DNA damage in target cancer cells, a series of apoptosis experiments was carried out in the HPH-HL-60 co-culture system.

It is well-known that formation of apoptotic bodies from chromatin condensation and nuclear fragmentation is one of the prototypical features of cell apoptosis.[238] Examples of

normal nuclei (arrow head) and apoptotic bodies (arrow) were demonstrated in Hoechst 33342 stained HL-60 cells under different treatments as indicated (Figure 2.7A). To quantify the accumulation of apoptotic bodies induced by CPA, 200 HL-60 cells from each treatment group were calculated under fluorescence microscopy. As shown in Figure 2.7B, CPA at the concentration of 250, 500, and 1000 μM resulted in 11 to 14%, 55 to 66%, and 82 to 90% of HL-60 apoptosis, respectively. When co-treated with CITCO, a significant increase of the apoptosis rate was observed at all three concentrations of CPA treatment. Particularly, in the presence of CITCO, 250 μM of CPA treatment resulted in approximately 90% of apoptotic HL-60 cells, a rate comparable to that induced by CPA at 1000 μM alone. In parallel experiments, DNA ladder resulted from CPA induced DNA fragmentation was also examined in HL-60 cells after treatment with CPA and CITCO as described above. DNA fragmentation was clearly observed in HL-60 cells when treated with CPA at 500 and 1000 μM in the co-culture system (Figure 2.7C). Consistent with the above-mentioned chromatin condensation assays, CPA at 250 μM alone only resulted in moderate DNA damage, while inclusion of CITCO has strongly potentiated CPA-mediated DNA fragmentation.

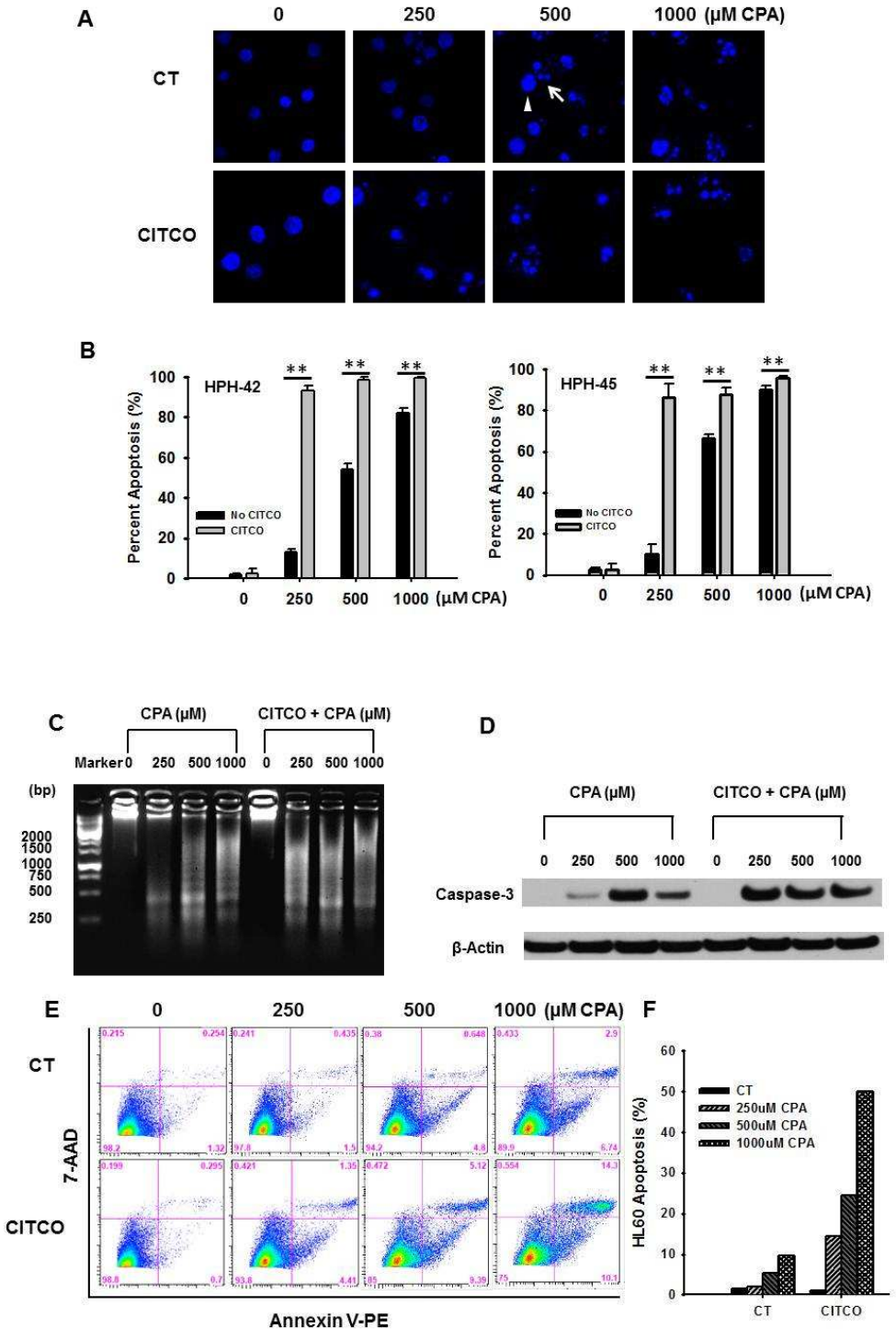


Figure 2.7Activation of CAR promotes CPA-mediated apoptosis of HL-60 cells in the HPH-HL-60 co-culture model. In the co-culture system, cells were treated with vehicle control (0.1%

DMSO) or CPA (250, 500, and 1000 μM) in the presence and absence of CITCO (1 μM) as outlined in the "Methods". CPA-mediated apoptosis in HL-60 cells was analyzed with different assays. (A) Representative visualization of HL-60 cells stained with Hoechst 33342. Arrow head and arrow depict normal nuclei and apoptotic bodies, respectively. (B) Quantitation of apoptotic bodies induced by CPA with/without CITCO. Two-hundred HL-60 cells from each treatment group were calculated under fluorescent microscopy. Percent of apoptotic cells were expressed as mean ± SD obtained from three independent experiments. **, $p < 0.01$. (C) DNA extracted from treated cells was loaded on an agarose gel to illustrate CPA-induced DNA fragmentation. (D) Caspase 3 activity was analyzed with western blotting to detect the large fragment (17/19 kDa) of activated caspase in HL-60 cells. Beta-actin was used to normalize protein loading. (E) Effects of CAR activation on CPA-mediated membrane translocation of phosphatidylserine during apoptosis were analyzed using flow cytometry as detailed in the "Methods". (F) Reorganized data from E in a bar chart.

Sequential activation of caspases plays a central role in cell apoptosis. Caspase 3 is considered to be one of the most important executive caspases in apoptosis pathways.[239] To better understand the relationship between CAR activation and CPA-mediated apoptosis, we conducted Western blotting analysis to detect the large fragment (17/19 kDa) of activated caspase 3 in HL-60 cells treated with the same combination of CPA and CITCO as described in "*Methods*". Inductive expression of capsase 3 protein was observed in all CPA treated groups, where 500 μM of CPA resulted in the strongest induction and 250 μM of CPA exhibited moderate enhancement of caspase 3 activation (Figure 2.7D). Similarly, when co-treated with CITCO, CPA (250 μM)-induced caspase-3 expression was extensively augmented. Interestingly, activation of caspase 3 by CPA appears saturated at 500 μM and further increment of CPA concentration did not result in

additional expression of this cysteine protease.

It has been reported that apoptotic cells exhibit increased externalization of phosphatidylserine as a cell surface modification.[240] Next, we examined whether CAR activation affects CPA-mediated translocation of phosphatidylserine during apoptosis by flow cytometry analysis. As shown in Figure 2.7E, CPA induced HL-60 apoptosis in a concentration-dependent manner. With CITCO co-treatment, the same trend but significantly higher apoptosis rates were achieved at all three CPA concentrations. Taken together, results from these apoptosis analyses clearly establish that CITCO promotes CPA therapeutic effect in HL-60 cells by enhancing multiple apoptotic pathways.

2.3.5 Cytotoxicity in human primary hepatocytes

As demonstrated above, co-treatment of CITCO with CPA can efficiently induce cancer cell death in the HPH-HL-60 co-culture system. It is equally important to investigate whether severe hepatotoxicity occurs when CITCO and CPA were co-administered. Prototypical MTT assays were performed in HPHs after 48 h incubation of CPA at a wide range of concentrations, in the presence or absence of CITCO. As shown in Figure 2.8A, the viability of HPHs remained above 90% at CPA concentrations up to 1 mM, which represents the highest concentration used in all above mentioned studies and, co-treatment with CITCO didn't increase hepatotoxicity within this concentration (0.125-1 mM) scope. However, when CPA concentration reaches 2 mM and beyond, significant hepatotoxicity was observed in HPHs and this toxicity was further enhanced by CITCO (Figure 2.8A). Additionally, we have monitored the morphological changes of HPHs in the co-culture models. Consistent with the MTT assay results, CPA exhibits no hepatotoxicity at the concentration of 250, 500, and 1000 μM regardless the presence or

absence of CITCO (Figure 2.8B). CPA at 4000 µM was applied as a positive control by which remarkable morphology changes and hepatocyte damage were observed, as expected. These results indicate that CPA at concentrations applied in the current studies (250 to 1000 µM) is non-toxic to the non-target hepatocytes with or without the presence of CITCO.

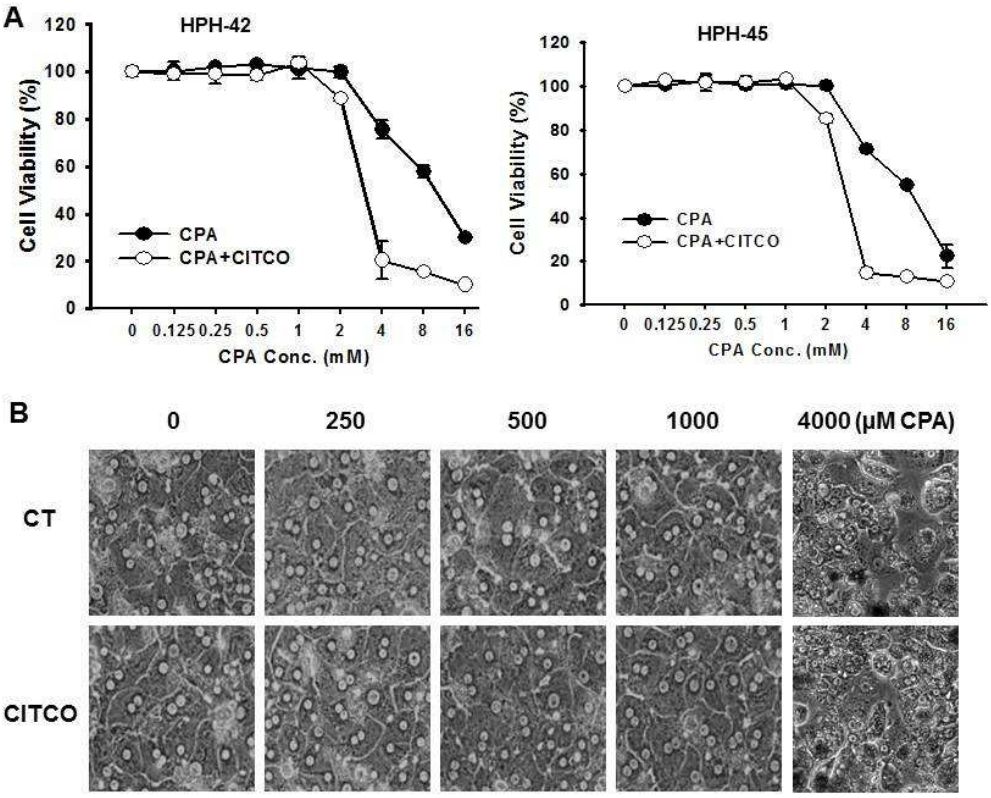


Figure 2.8 Cytotoxicity of CPA and CITCO in human primary hepatocytes.(A) HPHs from donors (HL#42 and HL#45) in 96-well coated plate were treated with CPA at the wide range of concentrations in the presence and absence of CITCO (1 µM). Prototypical MTT assays were performed 48 h after the treatments as described in the "Methods". Cell viability data represent mean ± SD from three independent measurements and are expressed as percent of vehicle control. (B) Morphological changes of HPHs in the co-culture model were monitored under microscopy

after various treatments. CPA at 4000 μM was used as a positive control demonstrating toxic morphology damages to the HPHs.

2.3.6 Application of co-culture model in different cell lines and species

As above proved, the innovative HPH-HL-60 co-culture system served as an excellent model in mimicking the *in vivo* condition of CPA metabolism and exploring potential therapeutic strategy to promote anti-leukemia effect of the drug. As CPA is also used as a first-line treatment for B-cell lymphoma[75; 77; 78], two representative cell lines of the disease, SUDHL-6 and OCI-LY3 were introduced to the co-culture model. Similar to that in HPH-HL-60 co-culture system, results from the co-cultures with aforementioned lymphoma cell lines indicated that CPA at concentrations bracketing its pharmacologically relevant levels markedly decreased the viability of lymphoma cells in a concentration-dependent manner (Figure 2.9A). Again, co-treatment with CITCO significantly enhanced CPA-mediated cytotoxicity in those cells, in that 250 μM of CPA + CITCO could achieve equal to greater magnitudes of anticancer activity than CPA alone at the concentration of 1000 μM. The temporal effects of CPA in the presence and absence of CITCO were also evaluated in parallel experiments. As shown in Figure 2.9B, CPA-induced cytotoxicity in lymphoma cells is time-dependent and the presence of CITCO can significantly enhance CPA-induced cytotoxicity as early as 24 h after co-treatment.Again, CPA induced HL-60 apoptosis in a concentration-dependent manner. With CITCO co-treatment, the same trend but significantly higher apoptosis rates were achieved at all three CPA concentrations(Figure 2.9C).

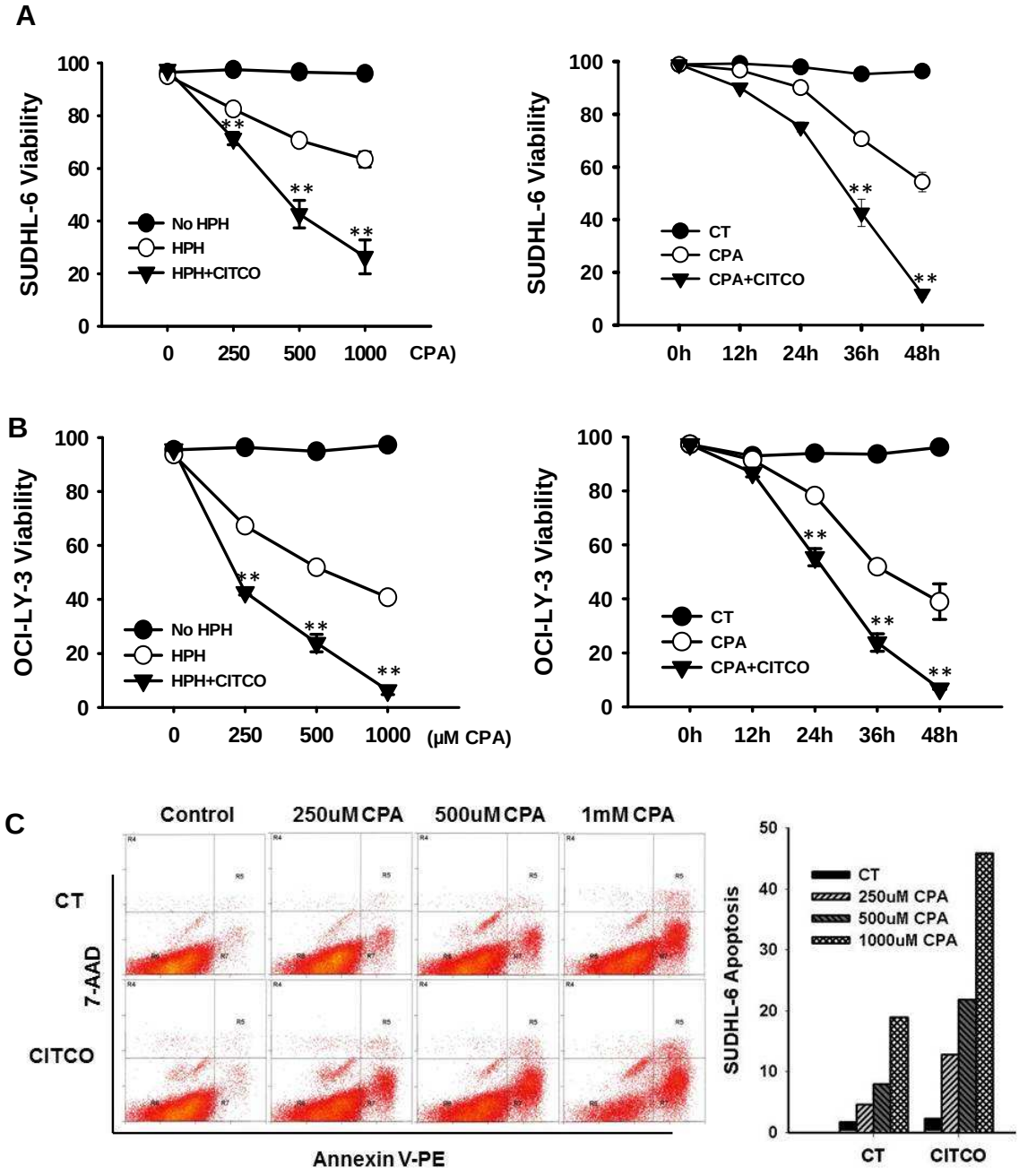


Figure 2.9 Activation of CAR enhances CPA anticancer activity in the human primary hepatocyte-lymphoma cell co-culture systems. The anticancer activity of CPA was analyzed in a unique hepatocyte-cancer cell co-culture model. (A) Effects of CAR activation on the concentration-dependent anticancer activity of CPA in SUDHL-6 cells and OCI-LY-3 cells. As detailed in the "Methods", the co-cultures were treated with vehicle control (0.1% DMSO) or

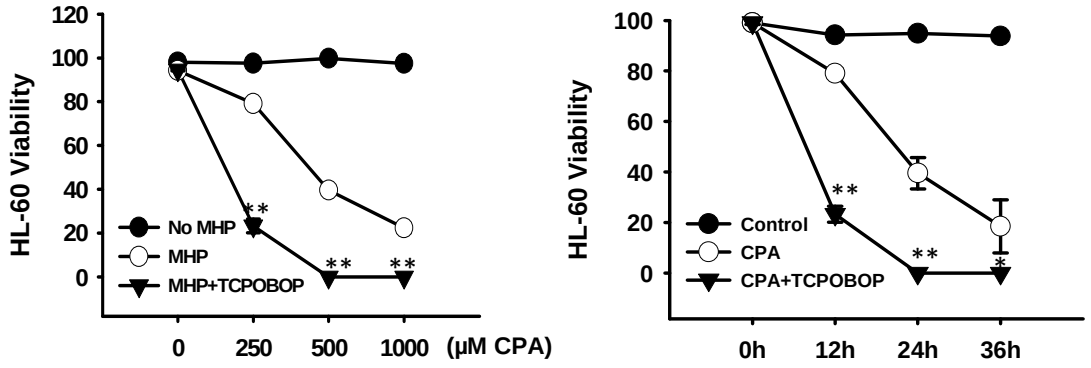
CPA (250, 500, and 1000 µM) in the presence and absence of CITCO (1 µM). Cell viability was analyzed 36 h after the co-treatment. (B) Effects of CAR activation on the temporal changes of CPA-mediated anticancer activity in SUDHL-6 cells and OCI-LY-3 cells. In the co-cultures, cells were treated with vehicle control (0.1% DMSO) or CPA (500 µM) with or without CITCO (1 µM). Cell viability was measured at 0, 12, 24, 36, and 48 h after the co-treatment. (C) Effects of CAR activation on CPA-mediated membrane translocation of phosphatidylserine during apoptosis were analyzed using flow cytometry as detailed in the "Methods".All viability data represent mean ± SD from three independent experiments and are expressed as percent viability of vehicle control. **, $p < 0.01$.

Moreover, the co-culture system was applied to mouse and rat primary hepatocytes to compensate the scarcity of human liver tissues and to serve as a bridge from *in vitro* metabolism manipulation to *in vivo* pilot study of the proposed therapeutic strategy. Similar results were observed in both mouse and rat hepatocytes as that in HPH. Co-treatment of CPA and CAR activators led to significant decrease of HL-60 viability in a dose and time dependent manner (Figure 2.10).

Figure 2.10 Activation of CAR enhances CPA anticancer activity in the mouse and rat primary hepatocyte-HL-60 cell co-culture systems.(A) Effects of mCAR activation on the concentration-dependent and time-dependent anticancer activity of CPA in HL-60 cells. As detailed in the "Methods", the co-cultures were treated with vehicle control (0.1% DMSO) or CPA (250, 500, and 1000 µM) in the presence and absence of TCPOBOP (250 nM). Cell viability was analyzed 36 h after the co-treatment with various concentrations of CPA (left) or with 500 µM CPA treatments at various time points (right). (B) Effects of rCAR activation on the concentration-dependent and time-dependent anticancer activity of CPA in HL-60 cells. As

detailed in the “Methods”, the co-cultures were treated with vehicle control (0.1% DMSO) or CPA (500, and 1000 μM) in the presence and absence of PB (10 μM). Cell viability was analyzed 36 h after the co-treatment with various concentrations of CPA (left) or with 500 μM CPA treatments at various time points (right). All viability data represent mean ± SD from three independent experiments and are expressed as percent viability of vehicle control. *, p<0.1;**, p < 0.01.

A



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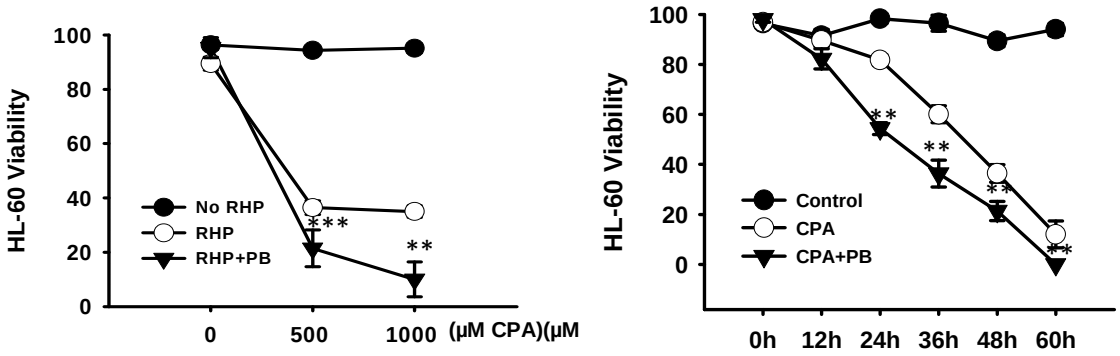


2.3.7 *In vivo* study with SUDHL-6 in SCID mice

TCPOBOP, a selective mCAR activator has been proved to significantly decrease SUDHL-6 viability *in vitro* when co-treated with CPA, as demonstrated above in the co-culture model. To further explore the feasibility of the proposed therapeutic strategy in a

whole animal setting, a pilot *in vivo* study was carried out. Firstly, SCID mice were inoculated with 1, 3 or 5 million SUDHL-6 cells each at left or right dorsal flank. The volume of tumor was monitored continuously to determine the adequate amount of cells and site of injection to deliver an appropriate rate of tumor growth with which the difference between drug treatment groups would be best evaluated. As shown in Figure 2.11, right flank injection of 3 million cells was determined to be an adequate condition in terms of tumor growth rate and measurement accuracy.

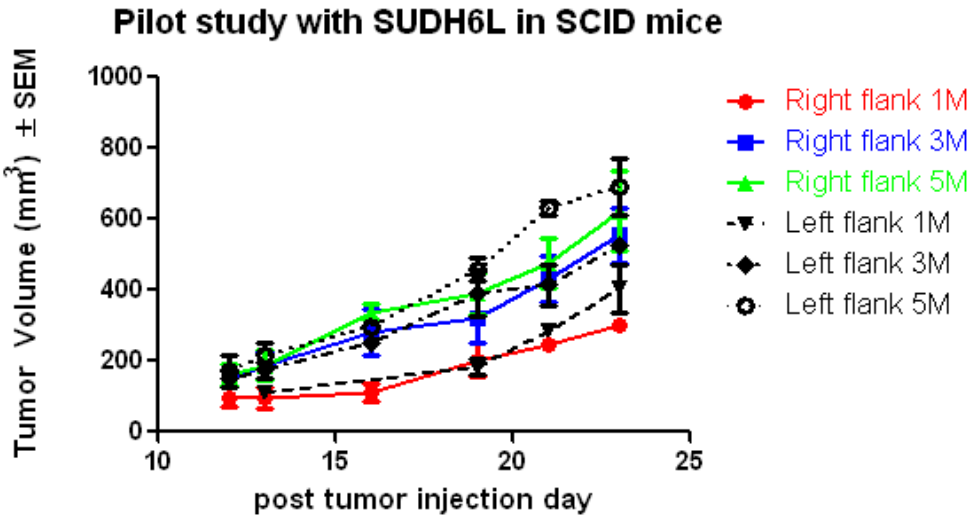


Figure 2.11 SUDHL-6 cell concentration and inoculation site in relationship to tumor growth.

Secondly, the dose and treatment schedule of CPA and TCPOBOP were determined based on relevant studies[241; 242; 243]. A tolerability study was carried out subsequently with 3 mice in each treatment group. In addition, another group with 60 mg/kg CPA+ 3mg/mg TCPOBOP was included to further probe for potential toxicity that may undermine the *in vivo* study. Results confirmed that none of the above drug treatments had statistically

significant change of mouse weight or behavior over the length of study (data not shown). Liver and kidney tissues were harvested and subject to HE staining for potential hepatic and renal damages. No significant histological or pathological changes were observed in the tissues upon the above mentioned drug treatments (Figure 2.12).

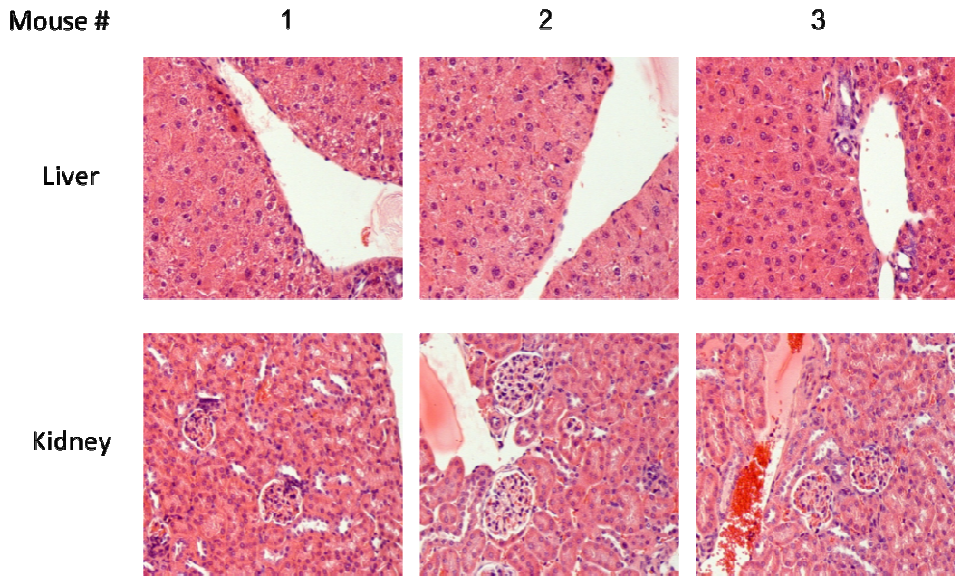


Figure 2.12 HE staining of mouse liver and kidney sections from group: 60 mg/kg CPA+3mg/mg TCPOBOP.

The formal *in vivo* study was conducted as described in "*Methods*". By day 9 of the experiment, mice treated with vehicle control (corn oil) had an average tumor size growth from 200mm^3 to 500 mm^3. When treated with 30mg/kg CPA, the growth of tumor decreased to an average of 400 mm^3. By the same time, 60mg/kg of CPA was able to inhibit the growth and decrease the size of tumor to less than 200 mm^3,which was the starting point. By the last day of experiment (Day16), no tumor was visible or measurable

from this treatment group. Compared to 30mg/kg CPA alone, co-treatment of TCPOBOP delivered a modest decrease of tumor growth from an average of 400 mm^3 to around 350 mm^3 by day 9. These results suggested that TCPOBOP had a marginal enhancement in tumor growth inhibition when co-treated with 30mg/kg CPA, but the effect was not comparable to that by doubling the dose of CPA (Figure 2.13).

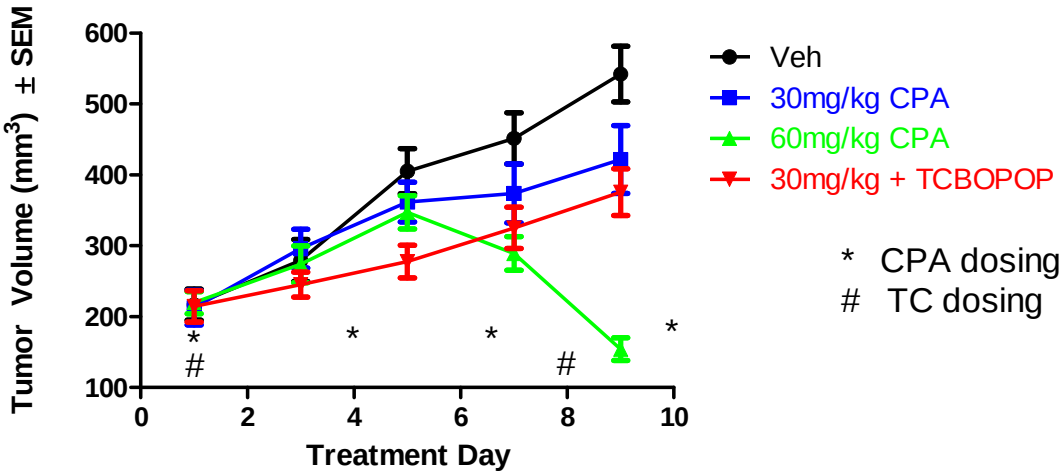


Figure 2.13 SUDHL-6 tumor growths under CPA and TCPOBOP treatment

2.4 Discussion

CPA belongs to the oxazaphosphorine-type of alkylating agents that holds a broad spectrum of antineoplastic activity against various types of cancers, including both solid tumors such as breast cancers, and hematopoietic malignancies such as leukemia and lymphoma.[75; 78] Designed as a prodrug, the therapeutic efficacy of CPA largely relies on drug-metabolizing enzyme-mediated bioactivation to generate the DNA-crosslinking metabolite. Among others, CYP2B6 enzyme is predominantly responsible for the hydroxylation of CPA at the 4-carbon position as the initial and rate-limiting step of CPA bioactivation.[88] Therefore, selective increase of the expression and activity of CYP2B6 in

cancer patients may benefit CPA-based chemotherapy. In the current study, we showed that CAR is a novel therapeutic target that can facilitate CPA-based anticancer activity in leukemia cells. Activation of CAR preferentially induces hepatic expression of CYP2B6 and formation of the pharmacologically active 4-OH-CPA. Moreover, we have established a unique HPH-leukemia cell co-culture model, by which the anticancer activity of CPA in leukemia cells could be directly evaluated in an *in vitro* cellular environment where hepatic metabolism is well maintained. Importantly, activation of CAR selectively potentiated CPA-mediated cytotoxicity in the targeted HL-60 cells but not in the co-cultured HPHs.

As one of the key mechanisms of action, biotransformation of CPA has been extensively studied and many drug-metabolizing enzymes that differentially contribute to the bioactivation and deactivation of CPA have been identified.[8; 229] Specifically, the CYP2B6 enzyme contributes primarily to the bioactivation of CPA, while CYP3A4, the major hepatic CYP isoform, was responsible for approximately 95% of CPA *N*-dechloroethylation, generating the inactive dechloroethyl-CPA and the neurotoxic chloroacetaldehyde.[88; 106] Additionally, several ALDHs convert an active intermediate metabolite of CPA, the aldophosphamide, to the carboxyphosphamide which represents a major stable non-therapeutic metabolite of CPA.[137; 143] Notably, although liver is often not the target for CPA-based chemotherapeutic treatment, biotransformation of CPA to its active forms occurs predominantly in this metabolic organ before reaching the targeted cancer cells.[64; 115] In accord with this concept, our results revealed that endogenous expression of the major drug-metabolizing enzymes responsible for CPA metabolism is negligible in a number of leukemia and lymphoma cells compared with that in HPHs,

suggesting that *in situ* bioactivation of CPA in these cancer cells is generally insignificant. However, it is noteworthy that expression of ALDH1A1 in K562 cells is comparable to that in HPHs, and is markedly greater than the expression in all other cancer cells tested. Given the importance of ALDH1A1 in the deactivation of CPA, this result indicates that K562 cells might be less sensitive to CPA than other leukemia cells. Indeed, previous studies showed that cancer stem cells expressing higher levels of ALDH1A1 are resistant to CPA treatment;[244] and selective knockdown of ALDH1A1 expression in K562 cells sensitized their response to CPA.[245]

To date, it has been widely accepted that inductive expression of CYP2B6, the rate-limiting enzyme for CPA bioactivation, is primarily regulated at the transcriptional level by the nuclear receptors CAR and PXR in a tissue specific manner.[188; 190; 231] However, many drugs induce both CYP2B6 and CYP3A4 expression with less discernible differences in HPHs through activation of PXR only (e.g. RIF, hyperforin, and SR12813), activation of both PXR and CAR (e.g. PB, and artemisinin), or activation of PXR but deactivation of CAR (e.g. clotrimazole, ethynyl estradiol, and PK11195).[235; 246; 247; 248] On the other hand, in line with our previous findings,[207] we showed that selective activation of human CAR by CITCO leads to preferential induction of CYP2B6 over CYP3A4 in HPHs but not in leukemia and lymphoma cells. Given the favorable effects of CYP2B6 in CPA bioactivation, these observations support our hypothesis that selective activation of CAR may benefit CPA-based chemotherapy of hematopoietic malignancies, in which CPA continues to be an important component of the front line regimens.

Tumor-specific expression of drug-metabolizing enzymes, particularly CYP2B6, would significantly contribute to the “selective cytotoxicity” of CPA and its therapeutic efficacy.

In this effort, a number of studies have shown that utilizing retroviral, replicating herpes viral, and adenoviral vectors, regional delivery of CYP2B expression cassette into tumor tissues significantly increased cancer cell cytotoxicity and intracellular 4-OH-CPA formation.[223; 224; 225; 249] Whereas this strategy may benefit patients with localized solid tumors, in the case of hematological malignancies such as leukemia and lymphoma, systemic treatment is required and bioactivation of CPA primarily relies on hepatic drug-metabolizing enzymes. Our HPH-leukemia cell co-culture model represents an excellent *in vitro* system mimicking human *in vivo* condition that allows simultaneous investigation of hepatic metabolism and extrahepatic anti-cancer activity under a shared cellular environment. We found that co-culture of HPHs with HL-60 cells markedly increased the cytotoxicity of CPA in HL-60 cells, as well as the formation of 4-OH-CPA in the shared culture medium in concentration- and time-dependent manners. Of importance, activation of CAR by CITCO synergistically enhanced the anti-cancer activity of CPA in HL-60 cells, particularly, 250 µM of CPA in the presence of CITCO (1 µM) led to an increased anti-cancer activity that could challenge what was achieved by 1000 µM of CPA alone. On the other hand, inclusion of CITCO did not alter the cytotoxicity in co-cultured HPHs upon the treatment of CPA up to 1000 µM. This selective toxicity of 4-OH-CPA in HL-60 cells over HPHs may be attributable to the quiescent nature of HPHs, as well as the fact that HPHs express abundant enzymes associated with both activation and deactivation of CPA. Given that chemotherapeutics are generally more toxic than other drugs, including CAR activators, it is reasonable to speculate that concurrent administration of a selective hCAR activator with a lower dose of CPA would benefit cancer patients receiving CPA-based chemotherapy.

Many chemotherapeutics trigger necrosis or apoptosis by attacking cellular macromolecules in actively proliferating cancer cells. In the case of CPA, formation of the active DNA-crosslinking metabolite, the phosphoramide mustard, primarily provokes cancer cell apoptosis.[236] We found that hepatic activation of CPA resulted in multifaceted apoptotic reactions in the targeted HL-60 cells, including the formation of apoptotic bodies, DNA fragmentation, cleavage of caspase 3, and plasma membrane blebbing. In line with aforementioned cytotoxicity results in HL-60 cells, activation of hCAR markedly enhanced CPA-mediated apoptosis in these cells. In particular, a synergistic response was observed between CITCO and the lower concentration of CPA, further signifying the potentially plausible effects of CAR activation in CPA treatment.

As mentioned previously, our innovative co-culture system closely mimics the *in vivo* condition and serves as an excellent *in vitro* platform to investigate and manipulate the metabolism of prodrugs in relationship to their therapeutic outcomes. The demonstrated flexibility of accommodating different sources of hepatocytes across species as well as different target cells across diseases renders the system greater value than we originally proposed. It is noticed that a few hepatocyte co-culture systems have been reported or patented[250; 251]. However, our system is the first one reported that focuses on simultaneous monitoring of drug metabolism and its therapeutic effects, especially with regard to hematopoietic diseases. Further development of the system may include the accommodation of: 1. primary hematopoietic cells which may allow even closer mimic of the pathologic environment and more precise forecast of the therapeutic effects; 2.hepatocytes from P450 or transporter-specific knock-out mice, which may provide more relevant information of specific gene function with regard to prodrug metabolism

and transportation.

It was the first time in report that CPA and TCPOBOP were co-treated in a lymphoma xenografted mouse model to evaluate potential enhancement of CPA therapeutic effect in light of our *in vitro* studies. Previous publications explored the methodology of tumor formation and dose ranges of TCPOBOP and CPA for different experimental purposes. Given the fact that CPA was used for both acute and chronic treatments in clinical application and that the current *in vivo* study was a pilot, concept-proving exploration, the experiment was designed majorly to accommodate the time frame between the up-regulation of P450 enzymes upon TCPOBOP administration and the exertion of CPA's therapeutic effect on the tumor. Since the induction of P450 protein expression by TCPOBOP takes up to 72h to peak and lasts for approximately one week, the dosing scheme was expected to catch the induction effect and its influence on CPA's therapeutic effect, if there was any. 3 million SUDHL-6 cells were injected into each mouse to allow quick development of tumor, compared to 1.2 million cells in a previous publication[252]. The amount was proved to be adequate and effective as demonstrated in the pilot study. Different doses of TCPOBOP ranging from 0.33mg/kg to 3mg/kg have been applied in relevant *in vivo* studies[253; 254]. 1 mg/kg TCPOBOP was determined to be an adequate dose since it is able to effectively induce mouse P450s *in vivo*[255] without introducing side effects such as enlarged liver, as seen with 3mg/kg treatment[256]. In line with those observations, our study did not show any liver enlargement in TCPOBOP treated groups (data not shown).

The current experimental design was successful in illustrating the different therapeutic outcomes between treatment groups in a sensitive and reliable manner. As mentioned in

the result section, 30 mg/kg of CPA alone was able to significantly retard the growth of implanted tumor, while 60 mg/kg of CPA further shrink the tumor in the same length of time. Interestingly, co-treatment of TCPOBOP with CPA only delivered a marginal enhancement in its therapeutic effect in comparison to CPA alone. However, previous observations in co-culture system suggested significant enhancement of CPA therapeutic effect by TCPOBOP. Although this dramatic discrepancy between *in vitro* and *in vivo* results of TCPOBOP is rather disappointing, it is important to note that TCPOBOP is an established cell proliferator and potent tumor promoter in mice[257; 258]. Though TCPOBOP alone did not promote or inhibit the proliferation of SUDHL-6 *in vitro*(data not shown), nor did it significantly change the morphology or weight of mouse liver, it is possible that the compound itself stimulated the growth of tumor which compensates metabolism-based therapeutic beneficial. Importantly, utilizing a humanized CAR transgenic mouse model, recent preliminary data from our laboratory indicated that different from its rodent counterpart, hCAR activator CITCO doesn't stimulate cell proliferation, while maintains its capability in the induction of CYP2B10 in this animal model. Future xenograft studies using this transgenic model are warranted to provide pivotal information regarding the potential clinical benefit of hCAR activation in CPA-based chemotherapy.

2.5 Conclusion

In summary, our results demonstrate that selective activation of hCAR can facilitate CPA-based antineoplastic activity in leukemia and lymphoma cells by preferentially promoting CYP2B6-mediated CPA bioactivation, without increasing cytotoxicity in co-cultured hepatocytes. Moreover, we have established an innovative hepatocyte-

hematopoietic cell co-culture system and demonstrated it to be a useful *in vitro* model for studying the biotransformation and therapeutic effects of prodrugs in an environment that closely mimics *in vivo* conditions. Our findings thus far suggest a provocative notion that inclusion of a selective hCAR activator in CPA-based regimen may benefit cancer patients with hematological malignancies. Conversely, we are aware that the current discoveries are still at the preclinical stage and selective hCAR activators identified thus far from clinically used drugs are extremely limited. It is interesting to note that previous studies have shown that administration of PB (activator of both hCAR and hPXR) was associated with acceleration of CPA biotransformation, but with little impact on drug efficacy.[259; 260] Notably, while PB is a potent tumor promoter by virtue of stimulating cell proliferation, such effect was not associated with CITCO (data not shown), emphasizing a need to identify novel and selective hCAR modulators with eliminated undesirable effects from a therapeutic perspective. Given that improving the therapeutic index of CPA continues to have high clinical relevance, future *in vivo* and clinical studies are warranted.

CHAPTER THREE
IDENTIFICATION OF POTENTIAL DRUG CANDIDATES FOR PREFERENTIAL INDUCTION OF CYP2B OVER CYP3A

3.1 Introduction

As described in the previous chapters, preferential induction of CYP2B6 over CYP3A4 leads to promoted CPA activation and enhanced therapeutic effects, without a significant influence on the cytotoxicity in co-cultured HPHs. However, since neither hCAR specific activator CITCO nor mCAR specific activator TCPOBOP used in previous experiments are clinically available, the lack of clinical relevance renders our discovery compromised clinical importance. In fact, selective hCAR activators identified from clinically used drugs are extremely limited to date. Thus, identifying novel drug candidates that preferentially induces CYP2B6 over CYP3A4 through selective activation of hCAR would greatly enhance the potential clinical impact of the current research. In this chapter, the goal was approached in three different ways.

Firstly, although there is very limited selective hCAR activator identified thus far, a panel of FDA-approved drugs has been reported as agonists of hCAR with less selectivity[192; 205; 261]. Specifically, these compounds were evaluated in HPH for their efficacy and potency of induction of CYP2B6 and CYP3A4. Compounds possess selective induction property of CYP2B6 over CYP3A4 were considered potential drug candidates that might fit the proposed therapeutic strategy.

Secondly, selective inhibition of CYP3A4 might also benefit the proposed strategy. Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV

infection and AIDS. It was reported that ritonavir can induce the expression of CYP2B6 at mRNA, protein and enzyme activity levels[262]. On the other hand, ritonavir binds to the active center of CYP3A4[263] and inhibits CYP3A4 enzymatic activity even though it induces the mRNA and protein content of this enzyme[264]. As such, ritonavir may represent a unique chemical tool in facilitating CPA's therapeutic bioactivation. In this chapter, Ritonavir was evaluated for its enhancement of CPA therapeutic effect with the co-culture system.

Thirdly, traditional Chinese herbal medicines have long been used in clinical practice in eastern Asia. Notably, a number of them have been increasingly used by cancer patients during chemotherapy[265; 266]. Despite their complicated chemical composition, their pharmacology and toxicity have been more and more intensely studied to accommodate the need of scientific understanding. Nonetheless, limited publications up to date have scoped these herbal mixtures with respect to their interaction with P450s. In the current study, a collection of 800 Chinese herbal medicines was subject to luciferase assays to identify potential hCAR activators. Candidates that exhibited hCAR activation capability greater that CITCO were then tested for hPXR activation capacity to address selectivity. Subsequently, these compounds were examined for their induction profile of CYP2B6 and CYP3A4, as well as their therapeutic effects in HPH based assays.

3.2 Material and Methods

3.2.1 Chemicals and biological reagents

Carbamazepine (CMZ), efavirenz (EFV), nevirapine (NVP), artemisinin (ART),

phenytoin (PHN), chlorpromazine (CPZ), RIF, ritonavir (RIT) were purchased from Sigma-Aldrich (St. Louis, MO).CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Eight hundreds Chinese herbal medical productswere generously gifted by Dr. Yuan Luo (NIH, Bethesda, MD). Oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). Matrigel, insulin and ITS^+ culture supplements were from BD Biosciences (Bedford, MA). Other cell culture and molecular reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

3.2.2 Culture and treatment of Human primary hepatocytes

Human liver tissues were obtained following surgical resection by pathology staff after diagnostic criteria were met and with prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously,[233] or obtained from Life Technologies Corporation (Durham, NC). Sandwich cultures of hepatocytes were maintained in 6-well or 12-well collagen-coated plates as described earlier.[193] Briefly, four hours after attachment at 37°C in a humidified atmosphere of 5% CO2, hepatocytes were overlaid with Matrigel (0.25 mg/mL) in Williams' E medium supplemented with ITS^+, 0.1 μM dexamethasone, 100 U/mL penicillin, and 100 μg/mL streptomycin. Thirty-six hours after seeding, hepatocytes were treated with vehicle control (0.1% DMSO), CITCO (1 μM), CMZ (50 μM), NVP (50 μM), EFV (10 μM), CPZ (25 μM), ART(50 μM), or PHN (50 μM) for another 24 h or 72 h before the detection of mRNA and protein, respectively. Cell culture medium was replaced on a daily base.

3.2.3 Human primary hepatocyte-HL-60 cell co-culture model

HPHs were cultured in collagen-coated 6-well plates as described above, 3.0 µm polycarbonate membrane inserts from a 24 mm Transwell plate (Corning, Lowell, MA) were placed in the HPH-containing plates. Approximately 0.6~1.5 × 10^6 leukemia (HL-60) cells, obtained from American Type Culture Collection (Manassas, VA), were transferred into each insert-chamber, and a total of 4 mL of complete Williams' E medium as aforementioned was shared by HPHs and SUDHL-6 cells in each well. To evaluate the effects of hepatic metabolism on the anticancer activity of CPA in SUDHL-6 cells, the co-cultured cells were pre-incubated with vehicle control (0.1% DMSO) or RIT (1 µM or 10 µM) for 24 h followed by co-exposure of CPA at designated concentrations for up to 72 h depending upon experimental requirements.

3.2.4 Quantitative PCR analysis

Total RNA was isolated from control and treated hepatocytes or leukemia cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instruction. Expression of CYP2B6 and CYP3A4R mRNA was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences for real-time PCR assays were reported previously[128],please refer to Supplemental Table 1 for detailed information. Fold induction values were calculated according to the equation: fold over control = $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between the target gene

and GAPDH, and ΔΔCt represents the relative change in these differences between control and treatment groups.

3.2.5 Western blotting analyses

Homogenate proteins (20 µg) from each treated HPHs or HL-60 cells were resolved on SDS-polyacrylamide gels, and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes. Subsequently, membranes were incubated with specific antibodies against CYP2B6(Millipore-Chemicon, Temecula, CA) diluted 1:4000. Beta-actin was used for normalization of protein loading. After incubating with horseradish peroxidase goat anti-rabbit IgG antibody diluted 1:4000, membranes were developed using ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ).

3.2.6 Cell viability assay

After pre-incubation with vehicle control (0.1% DMSO) or RIT (1 µM or 10 µM) for 24 h, HPH-HL-60 co-culture was treated with 250, 500, or 1000 µM of CPA in the presence or absence of RIT (1 µM or 10 µM), and 20 µL of HL-60 cells from each chamber were harvested at 12, 24, 36, and 48 h. Prototypical trypan blue exclusion assays were carried out to determine cell viability using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA)

3.2.7 Hepatocyte toxicity assays

A typical MTT assay was applied to measure potential cytotoxicity in HPHs. In brief, HPHs were seeded in a collagen-coated 96-well plate at a density of 7.5×10^4 cells/well. After 24 h incubation, cells were treated with various concentrations of CPA in the

presence and absence of RIT (1 μM or 10 μM) for 48 h. An aliquot (20 μL) of MTT solution (5.0 mg/mL) was added to each well followed by 4 h incubation, and the resulting crystals were dissolved in 150 μL of DMSO. Absorbance was analyzed at 580 nm using an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA).

3.2.8 Plasmids and generation of adenovirus-EYFP tagged hCAR

The pSG5-hPXR expression vector was obtained from Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). The pCR3-hCAR1+A expression vector and the CYP2B6 reporter construct, containing both phenobarbital-responsive enhancer module and the distal XREM (CYP2B6–2.2kb), were generated as described previously[112; 204].The pRL-TK Renilla reniformis luciferase plasmid used to normalize firefly luciferase activities was obtained from Promega. As reported previously, the CYP2B6-2.2kb firefly luciferase construct is composed of the PBREM containing 1.8 kb of the native promoter, and the 400 bp distal XREM region[112]. The pRL-TK Renilla luciferase plasmids used to normalize firefly luciferase activities were from Promega. hCAR1+A was generated by Tao Chen in our lab by introducing nucleotides corresponding to the alanine residue at site 270 of hCAR3 into the pCR3-hCAR1 expression vector by use of the QuikChange Site-directed Mutagenesis System (Stratagene, La Jolla, CA).

3.2.9 Transfection of cell lines

HepG2 cells in 96-well plates were transfected with hCAR1+A or hPXR expression vector, and CYP2B6-2.2kb reporter construct using Lipofectamine 2000 Transfection Kit (Invitrogen, NY) following the manufacturer's instruction. 24 hours after transfection, cells were treated with solvent (0.1% DMSO) or test compounds at 100mg/mL.24 hours

later, cell lysates were assayed for firefly activities normalized against the activities of cotransfected Renilla luciferase using Dual-Luciferase Kit (Promega, WI) by Lmax Microplate Luminomter (Molecular Devices, Sunnyvale, CA). Data were represented as mean±S.D. of three individual transfections.

3.2.10 Statistical analysis

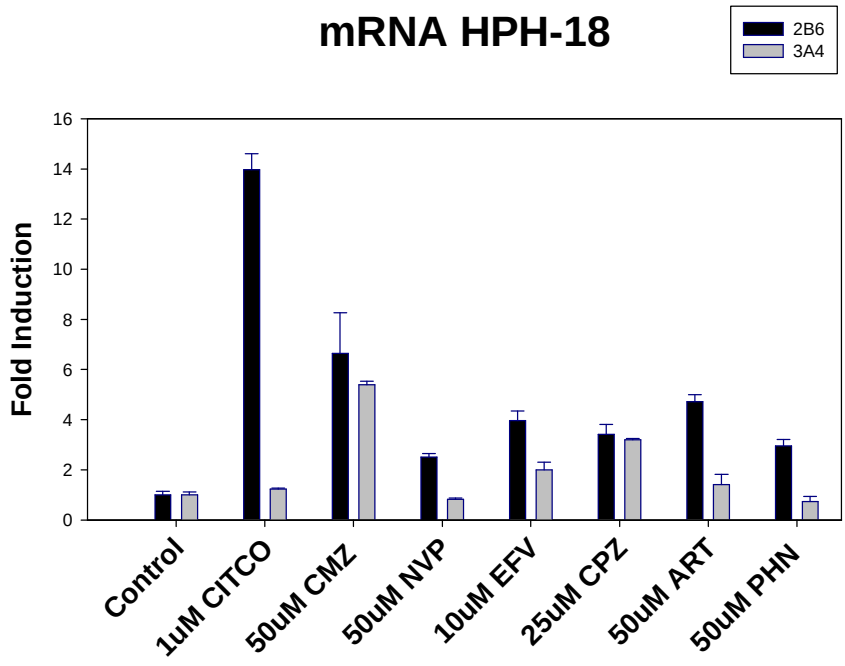
All data represent at least three independent experiments and are expressed as the mean ± S.D. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test or Student's t test where appropriate. The statistical significance was set at p values < 0.05 (*), or < 0.01 (**).

3.3 Results

3.3.1 hCAR activators from FDA-approved drugs exhibited limited preferential induction of CYP2B6 over CYP3A4 *in vitro*

A panel of FDA-approved drugs that have been reported to be hCAR activators was introduced to HPH for their induction profile of mRNA and protein of P450. NVP, EFV, ART and PHN exhibited moderate preferential induction of CYP2B6 mRNA over CYP3A4 (Figure 3.1A), though neither the absolute fold of induction of CYP2B6 or the magnitude of preference was only a fraction of that induced by CITCO. In terms of CYP2B6 protein expression, only EVF and ART exhibited slight induction compared to control (Figure 3.1B). NVP and PHN, which induced CYP2B6 mRNA, decreased its expression at protein level. Taken together, this panel of hCAR activators was proved to exhibit only limited preferential induction of CYP2B6 over CYP3A4.

A



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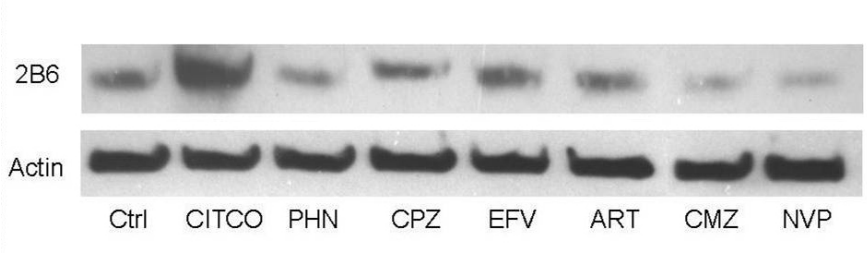


Figure 3.1 Induction profiles of hCAR activators from FDA-approved drugs. Human hepatocytes from liver donors (HL#18) were treated with CITCO,CMZ,NVP,EFV,CPZ,ART,PHN or vehicle control (0.1% DMSO) at indicated concentrations for 24 h (mRNA) and 72 h (protein), respectively. The expression of mRNA for CYP2B6 and CYP3A4 was analyzed using RT-PCR; related protein expression was detected using immunoblotting analysis as outlined in "Materials and Methods." RT-PCR data are expressed as mean ± SD (n=3).

3.3.2 Ritonavir did not enhance CPA therapeutic effect *in vitro*

Ritonavir was introduced to HPH-SUDHL-6 co-culture assay at two concentrations of

clinical relevance[267]. However, no statistically significant difference in SUDHL-6 viability was observed between treatment groups at either a fixed CPA concentration through time or a fixed time point with different concentrations of CPA (Figure 3.2A, B). In agreement with these observations, ritonavir did not lead to extra cytotoxicity to hepatocyte in MTT assay at either concentration (Figure 3.2C). Similar results from another batch of hepatocytes confirmed the inability of this drug in enhancing CPA therapeutic effect *in vitro* (data not shown).

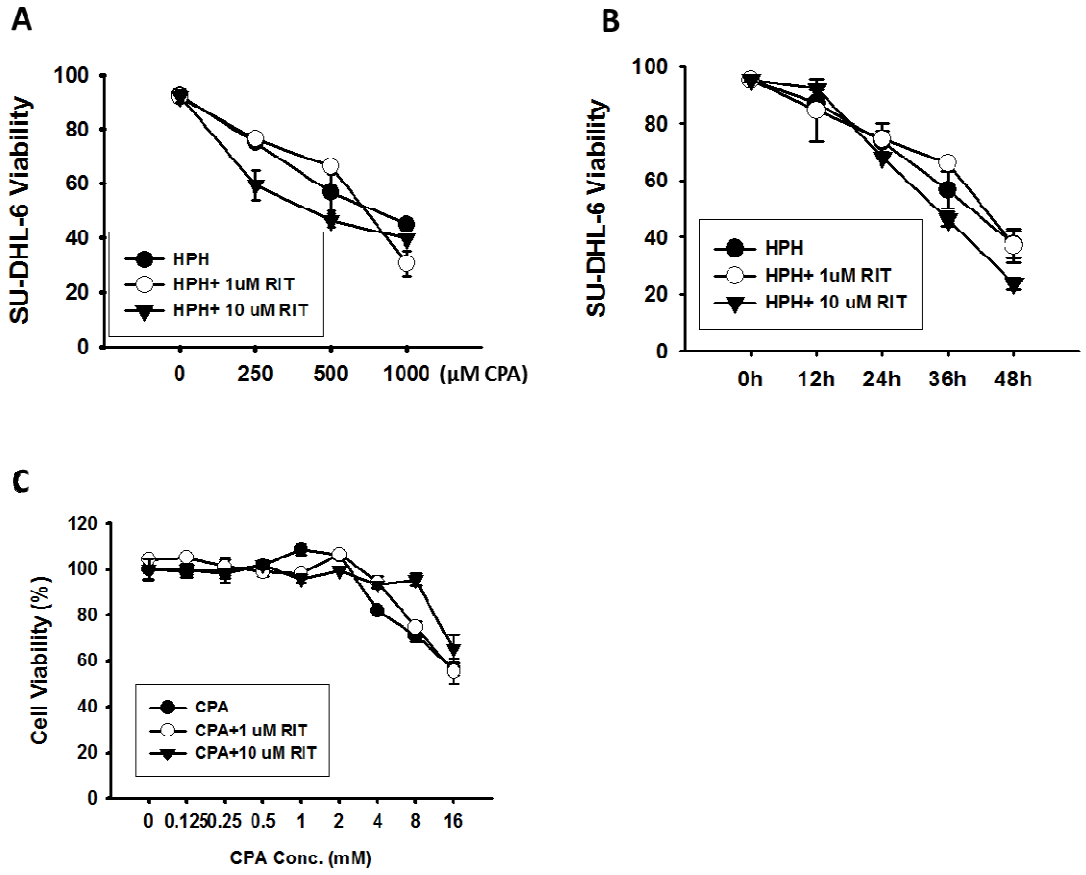


Figure 3.2 Ritonavir did not enhance CPA therapeutic effects *in vitro*. (A) Effects of CAR activation on the concentration-dependent anticancer activity of CPA in SUDHL-6 cells. As detailed in the "Methods", the HPH-SUDHL-6-cocultures were treated with vehicle control (0.1% DMSO) or CPA (250, 500, and 1000 µM) in the presence and absence of RIT (1 µM,10 µM). Cell

viability was analyzed 36 h after the co-treatment. (B) Effects of CAR activation on the temporal changes of CPA-mediated anticancer activity in SUDHL-6 cells. In the co-cultures, cells were treated with vehicle control (0.1% DMSO) or CPA (500 µM) with or without RIT (1 µM,10 µM). Cell viability was measured at 0, 12, 24, 36, and 48 h after the co-treatment. All viability data represent mean ± SD from three independent experiments and are expressed as percent viability of vehicle control. (C) HPHs from donor (HL#54) in 96-well coated plate were treated with CPA at the wide range of concentrations in the presence and absence of RIT (1 µM, 10 µM). Prototypical MTT assays were performed 48 h after the treatments as described in the "Methods". Cell viability data represent mean ± SD from three independent measurements and are expressed as percent of vehicle control.

3.3.3 Twenty candidates out of 800 Chinese herbal medical products identified as potent CAR activators by reporter assay

As the first step to identify potential compounds that preferentially induces CYP2B6 over CYP3A4 with acceptable accuracy and efficiency, a mid-throughput reporter assay system was introduced to process the 800 Chinese herbal medical products. As positive control, CITCO consistently induced the luciferase activity by an average of 4 fold over negative control with acceptable variation. Hence, the functionality and stability of the 96-well plate based reporter assay was confirmed. Samples were subject to assay in 27 batches with 30 samples tested in each batch at a fixed concentration of 100µg/mL. Those exhibited equivalent or stronger activation of CAR than CITCO were selected for further study. In total, 20 samples met the aforementioned criterion, as shown in an example result below (Figure 3.3).

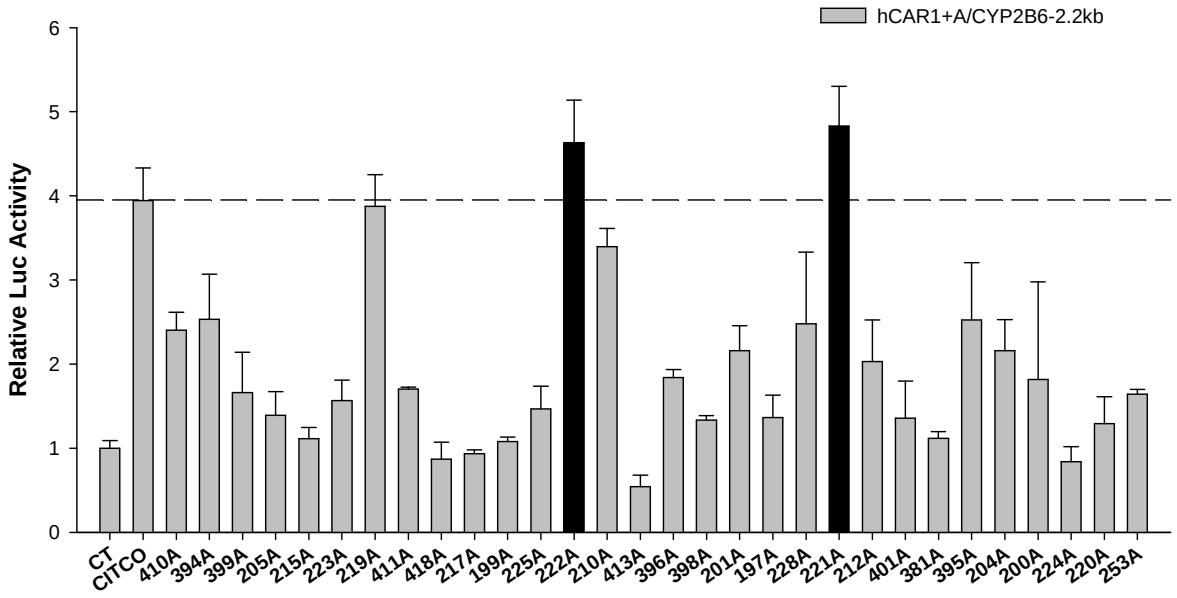


Figure 3.3 An example result of 96-well based reporter assay. HepG2 cells were transfected with hCAR1 expression vector in the presence of CYP2B6-2.2 kb reporter construct and the pRL-TK control vector. Transfected cells were treated for 24 h with Control (0.1% DMSO), CITCO (1 μM) or 100 μg/mL samples. Candidates that exhibited equivalent or stronger signal than CITCO were labeled in black.

3.3.4 Eight candidates out of 20 selected samples identified as weak/non PXR activator

Given the goal of identifying potential candidate that preferentially induces CYP2B6 over CYP3A4, and the fact that PXR induces both enzymes with almost the same potency, it is reasonable to conduct a PXR activation assay to exclude potential strong PXR activators. The 20 candidates selected from the last round of screening were subject to a 96-well plate based PXR reporter assay. Samples that induced a signal lower than 60% of that induced by the positive control RIF were considered as weak/non PXR activators.

In total, 8 samples met this criterion and were subject to further study (Figure 3.4).

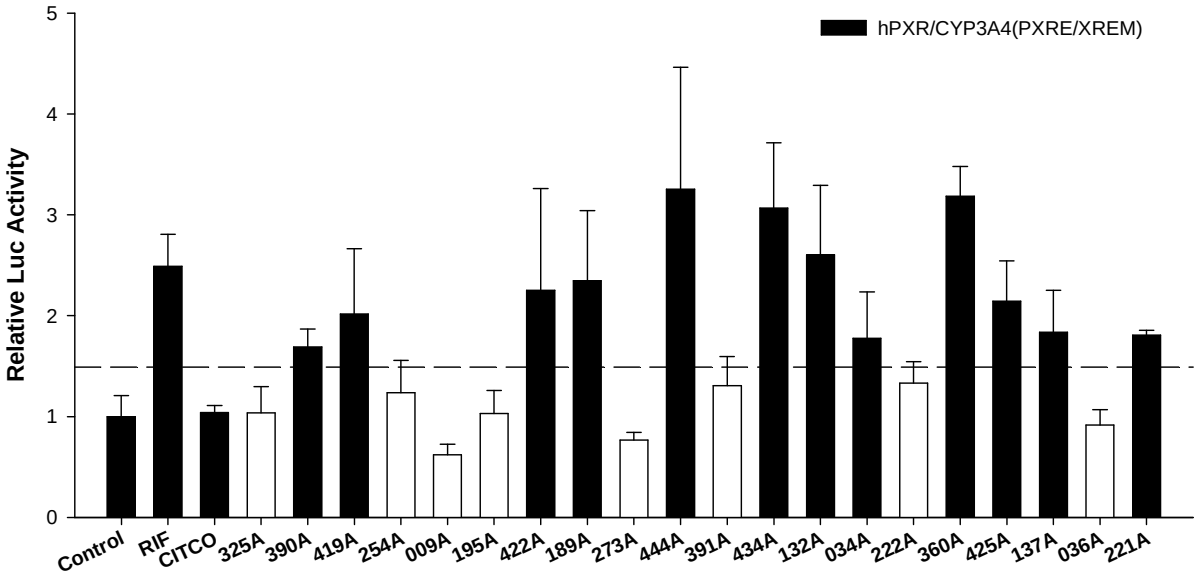


Figure 3.4 8 samples exhibited weak/non activation of PXR. .HepG2 cells were transfected with hPXR expression vector in the presence of CYP3A4 (PXRE/XREM) reporter construct and the pRL-TK control vector. Transfected cells were treated for 24 h with Control (0.1% DMSO), RIF (10 μM) or 100ug/mL samples. Candidates that exhibited 60% or less signal than RIF were labeled in white.

3.3.5 Preferential activation of CAR over PXR by the candidates

The 20 candidates were summarized by their activation of CAR, PXR and relative preference of CAR over PXR as shown in the following table (Table 3.1). The top 8 candidates with the strongest preference were subject to another round of CAR activation assay to address their activation potency (Km) and efficacy (Emax). Despite of their chemical reality of mixtures, these samples varied from roughly 200% to 600% of 1 μM CITCO in efficacy and 18μg/mL to 150μg/mL in potency (Figure 3.5). It is important to

note that the Km and Emax calculations were not exact, especially when a plateau of response was not reached.

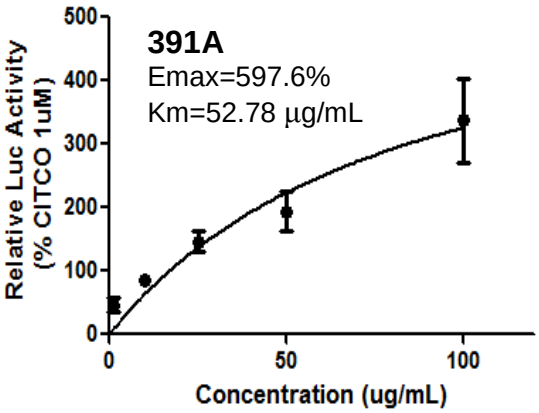
Table 3.1 Comparison of induction profile of 20 candidates in reporter assays.

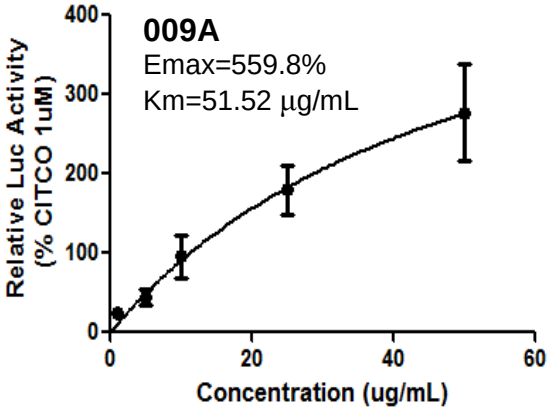
Sample	English Name	Fold over CITCO	Fold over RIF	F CITCO/F RIF
PBC-419A	Kalopanacis Cortex	1.10	0.81	1.36
PBC-425A	Elscholtziae Herba	1.06	0.86	1.23
PBC-434A	Nepetae Spica	1.21	1.23	0.98
PBC-422A	Cyperi Rhizoma	1.58	0.91	1.74
PBC-390A	Biotae Orientalis Folium	2.05	0.68	3.01
PBC-391A	Biotae Orientalis Folium	2.47	0.53	4.66
PBC-360A	Atractylodis Rhizoma	2.89	1.28	2.26
PBC-195A	Sophorae Subprostratae Radix	1.20	0.41	2.93
PBC-325A	Peucedani Radix	1.16	0.42	2.76
PBC-273A	Linderae Radix	1.03	0.31	3.32
PBC-034A	Ligustici sinensis Rhizoma et Radix	1.21	0.71	1.70
PBC-036A	sophorae Radix	1.45	0.37	3.92
PBC-009A	Nardostachys Rhizoma	1.13	0.25	4.52
PBC-444A	Cartami Semen	1.03	1.31	0.79
PBC-222A	Acori Graminei Rhizoma	1.17	0.53	2.21
PBC-221A	Acori Graminei Rhizoma	1.22	0.73	1.67
PBC-254A	Ponciri Fructus	1.00	0.50	2.00
PBC-137A	Peucedani Japonici Radix	1.15	0.74	1.55
PBC-132A	Potulacae Grandiflorae Herba	1.06	1.05	1.01
PBC-189A	Belamcandae Rhizoma	1.7	0.94	1.81

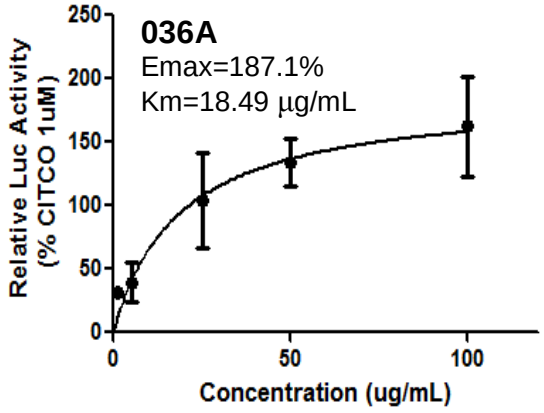
Blue: top 8 CAR activators as defined by fold induction of CAR reporter gene over CITCO. Green: Bottom 8 PXR activators as defined by fold induction of PXR reporter gene over RIF. Yellow: Top 8 candidates ranked by fold induction of CAR reporter gene over CITCO divided by fold induction of PXR reporter gene over RIF.

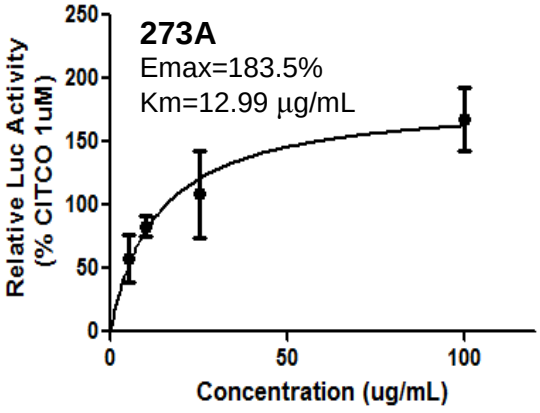
Figure 3.5 CAR activation dose curves of the top 8 candidates with strongest preferential activation of CAR over PXR. .HepG2 cells were transfected with hCAR 1+A expression vector in the presence of CY2B6-2.2kb reporter construct and the pRL-TK control vector. Transfected cells were treated for 24 h with Control (0.1% DMSO), CITCO (1 μM) or samples at 6 concentrations in gradient. Data were fitted to Michaelis-Menten equation using

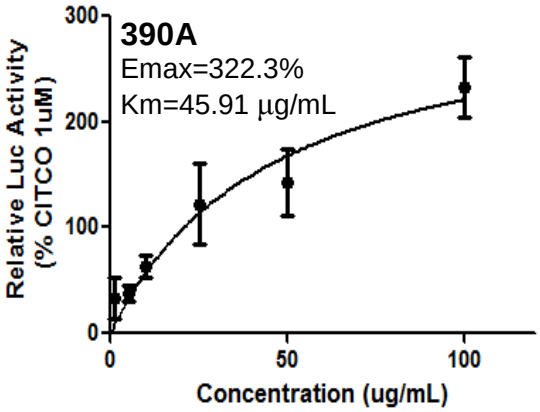
GraphPad Prism.











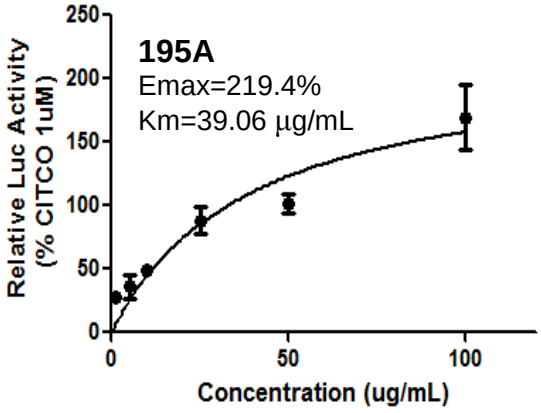
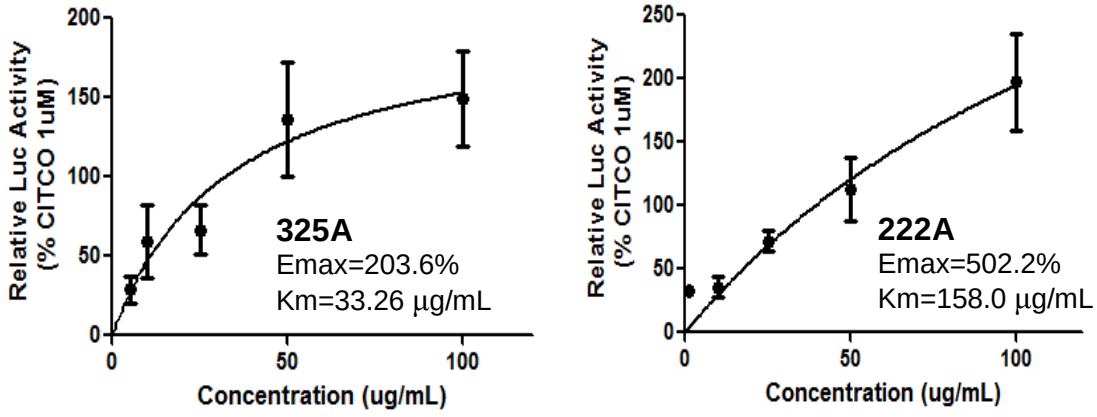


Figure 3.5 Continued



3.3.6 Induction of CYP2B6 and CYP3A4 mRNA by 20 candidates in human primary hepatocytes

Since mRNA is a more direct indicator of enzyme expression than nuclear receptor-based reporter assay results, and the sample volume of 20 was reasonably small for primary hepatocyte-based assessments, the induction profile of these samples were tested with respect to CYP2B6 and CYP3A4 in two batch of hepatocyte from different donors. In agreement with reporter assay results, most of these samples exhibited potent induction of both CYP2B6 and CYP3A4 mRNA (Figure 3.6). The results from reporter assays and HPH-based mRNA assays were further organized and compared (Table 3.2). In general, these results were in consistency in selecting the samples with preferential induction of CYP2B6 over 3A4, noting that most samples selected from mRNA assay were among the 8 candidates screened from reporter assays. However, 422A and 390A were two exceptions.

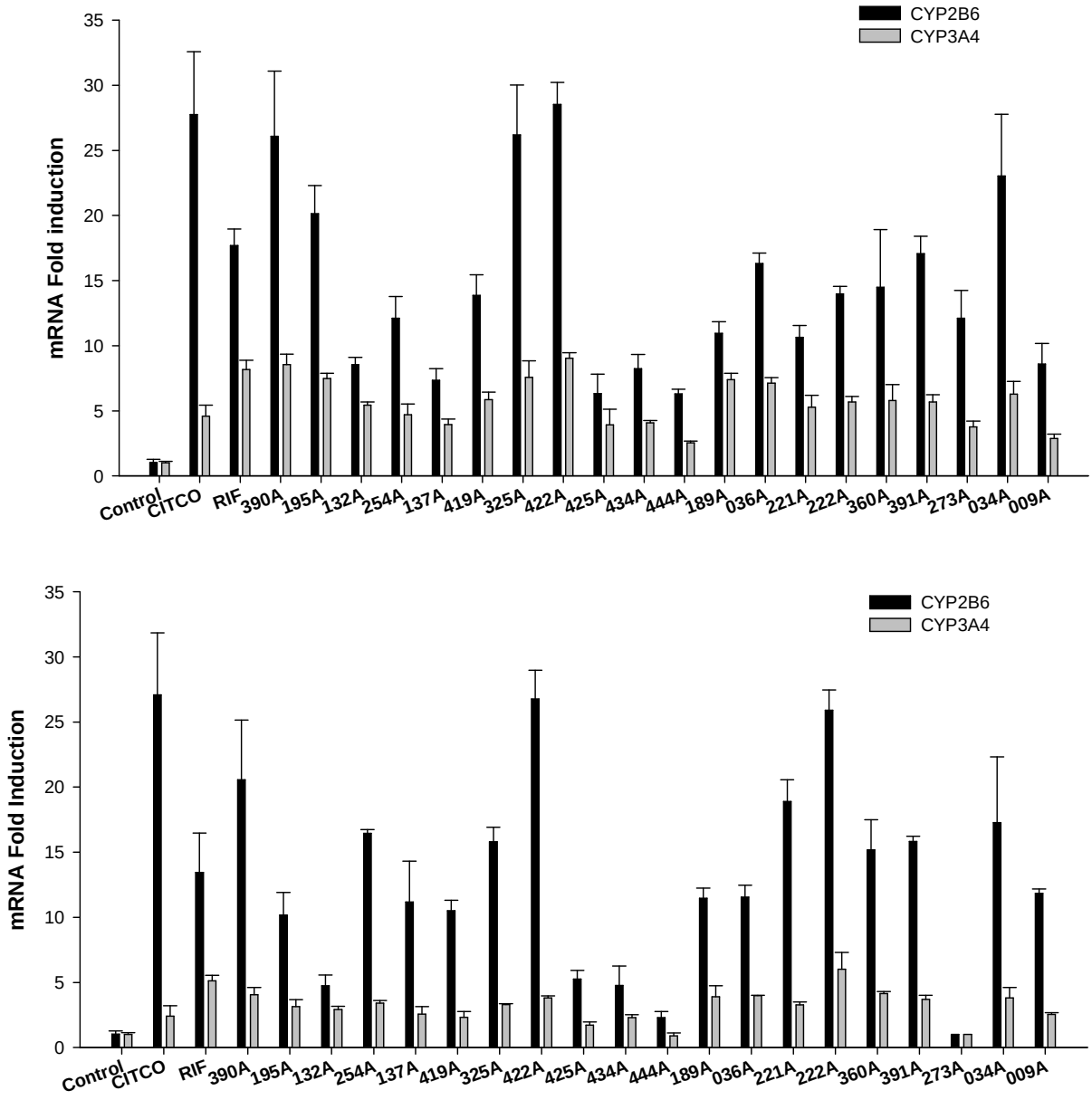


Figure 3.6 Induction of CYP2B6&CYP3A4 by the 20 candidates in human primary hepatocytes. Human hepatocytes from liver donors (HL#64 and HL#69) were treated with CITCO (1 μM), RIF (10 μM) or 100μg/mL samples for 24 h. The expression of mRNA for CYP2B6 and CYP3A4 was analyzed using RT-PCR.

Table 3.2 Comparison of induction profile of 20 candidates in reporter assays and mRNA assays.

Sample	English Name	F CITCO/F RIF 96 well	F CITCO/F RIF HPH-64	F CITCO/F RIF HPH-69
PBC-419A	Kalopanacis Cortex	1.36	0.69	0.87
PBC-425A	Elscholtziae Herba	1.23	0.48	0.56
PBC-434A	Nepetae Spica	0.98	0.60	0.40
PBC-422A	Cyperi Rhizoma	1.74	0.93	1.34
PBC-390A	Biotae Orientalis Folium	3.01	0.90	0.96
PBC-391A	Biotae Orientalis Folium	4.66	0.88	0.81
PBC-360A	Atractylodis Rhizoma	2.26	0.73	0.69
PBC-195A	Sophorae Subprostratae Radix	2.93	0.79	0.59
PBC-325A	Peucedani Radix	2.76	1.02	0.89
PBC-273A	Linderae Radix	3.32	0.95	0.00
PBC-034A	Ligustici sinensis Rhizoma et Radix	1.70	1.08	0.86
PBC-036A	sophorae Radix	3.92	0.68	0.54
PBC-009A	Nardostachys Rhizoma	4.52	0.88	0.88
PBC-444A	Cartami Semen	0.79	0.74	0.47
PBC-222A	Acori Graminei Rhizoma	2.21	0.72	0.82
PBC-221A	Acori Graminei Rhizoma	1.67	0.58	1.09
PBC-254A	Ponciri Fructus	2.00	0.76	0.92
PBC-137A	Peucedani Japonici Radix	1.55	0.52	0.55
PBC-132A	Potulacae Grandiflorae Herba	1.01	0.46	0.54
PBC-189A	Belamcandae Rhizoma	1.81	0.49	0.64

Green: weak/non PXR activators as defined by reporter assay. Yellow: Top 4 candidates ranked by reporter assays. Red: Top 4 candidates ranked by mRNA assays in two different batches of HPH.

3.3.7 Matrine enhanced CPA therapeutic effect through preferential induction of CYP2B6 over CYP3A4

Given the overall purpose of the current study being to find clinically relevant compounds that may enhance CPA-based chemotherapy against cancer, and the above 8 candidates exhibited strongest preferential induction of CYP2B6 over 3A4 with minor exceptions, it is reasonable to perform a literature research of the 8 candidates to identify specific ones that have already been used in cancer treatments. Interestingly, it is documented that a major component of both sample PBC-195A and PBC-036A is

matrine, a compound that exhibits anti-hepatitis, antipyretic, anti-inflammatory, anti-arrhythmic and anti-tumor properties[268; 269; 270; 271; 272]. Moreover, matrine has been applied as an adjuvant therapy to other medicines including CPA in cancer treatments in China[273]. Nonetheless, little mechanism-based study was accomplished thus far with respect to the P450 induction or nuclear receptor activation profile of the compound.

To answer these questions, a series of experiments were performed. Firstly, matrine was introduced to human primary hepatocytes at two concentrations for its P450 induction profile. At mRNA level, matrine exhibited preferential induction of CYP2B6 over CYP3A4, with the magnitude of induction of CYP2B6 and preference over CYP3A4 stronger than PCB-391A, the most favorable compound in reporter assays, but weaker than CITCO (Figure 3.7A). However, 300μM matrine did not deliver stronger induction of CYP2B6 than that by 100μM matrine, possibly due to reaching the plateau of induction. In contrast, matrine induced CYP2B6 protein expression in a dose dependent manner, with the magnitude of induction overpassed both PCB-391A and CITCO. Consistent with mRNA results, no induction of CYP3A4 was observed (Figure 3.7B). In summary, matrine exhibited superior preferential induction of CYP2B6 over CYP3A4 than its parent.

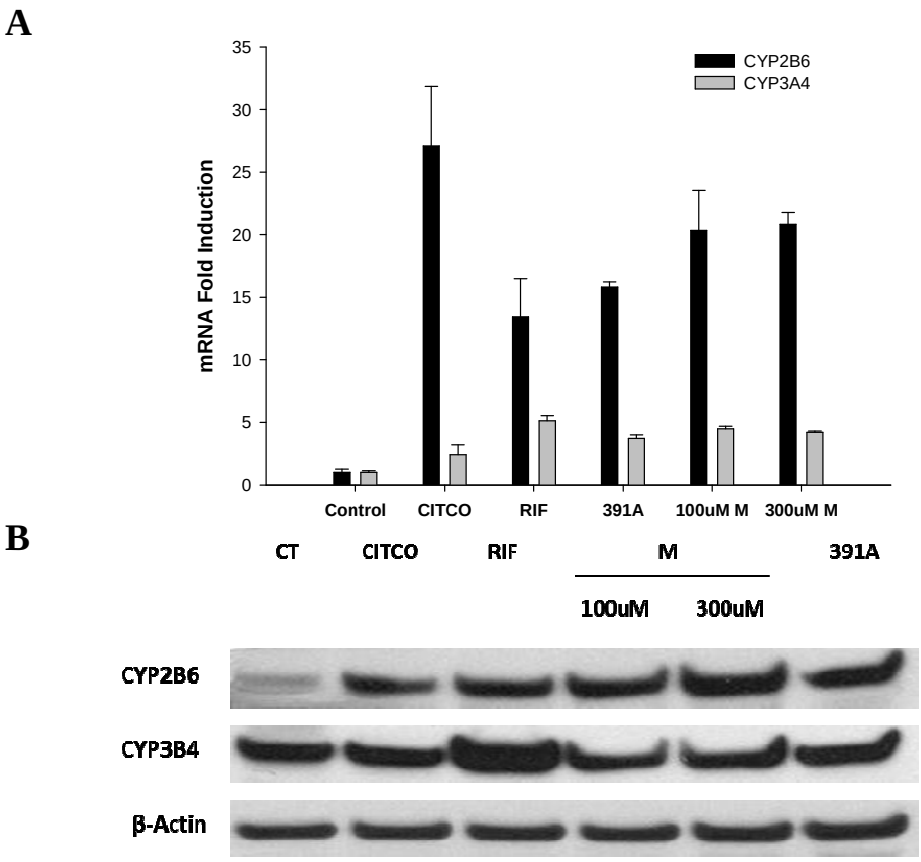


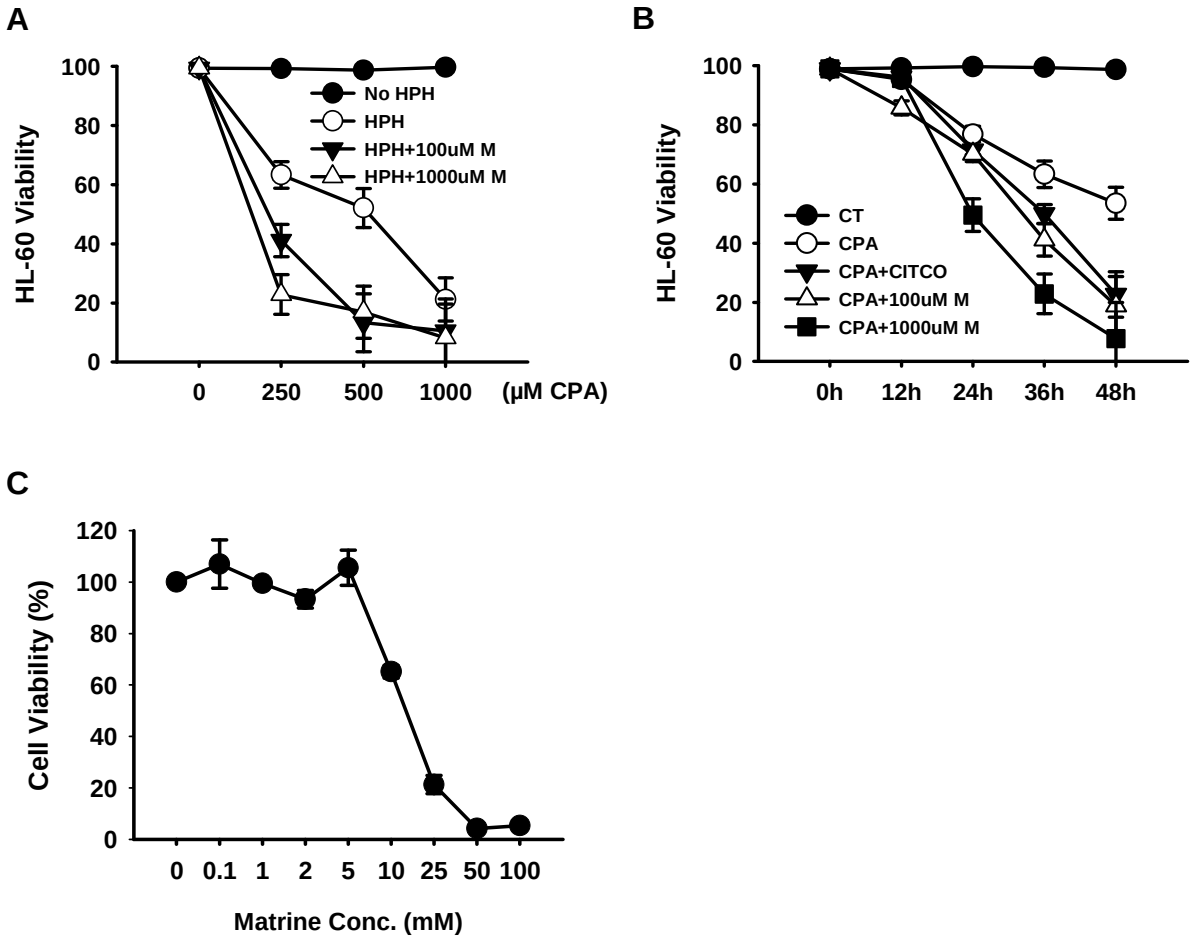
Figure 3.7 Induction of CYP2B6&CYP3A4 by matrine in human primary hepatocytes. Human hepatocytes from liver donor (HL#73) was treated with CITCO (1 μM), RIF (10 μM),391A (100 μg/mL) or matrine (100 μM or 300 μM) for 24 h (A) and 72 h (B), respectively. The expression of mRNA was analyzed using RT-PCR; related protein expression was detected using immunoblotting analysis as outlined in "Materials and Methods."

To investigate the effect of matrine in promoting CPA anti-cancer activity, the compound was applied to HPH-HL-60 co-culture system at aforementioned concentrations. At 36h, co-treatment of matrine and CPA markedly decreased HL-60 viability compared to CPA alone. Notably, 250 μM of CPA + 1000 μM matrine could achieve equal to greater magnitudes of anticancer activity than 1000 μM CPA. In 250 μM CPA treatment group, co-treatment of 1000 μM matrine resulted in even further decrease in HL-60 viability

than that by 100 μM matrine. The difference caused by different matrine concentrations was not statistically significant in higher CPA concentration groups due to the low viability of HL-60 (Figure 3.8A). Like in previous studies, the temporal effects of matrine in conjugation of CPA were also evaluated in parallel experiments. As shown in Figure 3.8B, CPA-induced cytotoxicity in HL-60 cells is time-dependent and the presence of CITCO or matrine can significantly enhance CPA-induced cytotoxicity as early as 24 h after co-treatment. It is worthy to note that 100 μM matrine exerted a similar therapeutic effect as CITCO, while 1000 μM matrine further enhanced CPA-induced HL-60 cytotoxicity. In addition, the cytotoxicity of matrine itself was tested in human primary hepatocytes. No obvious cytotoxicity was observed up to 5 mM of matrine based on MTT assay (Figure 3.8C). Matrine did exhibit significant cytotoxicity at higher concentrations that far beyond the range of clinical application.

Figure 3.8Matrine promotes CPA anticancer activity in the human primary hepatocyte-HL-60 cell co-culture system. The effect of matrine in promoting CPA anti-cancer activity was analyzed in co-culture model. (A) Effects of matrine on the concentration-dependent anticancer activity of CPA in HL-60 cells. As detailed in the "Methods", the HPH-HL-60 co-cultures were treated with vehicle control (0.1% DMSO) or CPA (250, 500, and 1000 μM) in the presence and absence of matrine (100,1000 μM). Cell viability was analyzed 36 h after the co-treatment. (B) Effects of matrine on the temporal changes of CPA-mediated anticancer activity in HL-60 cells. In the co-cultures, cells were treated with vehicle control (0.1% DMSO) or CPA (500 μM) with or without CITCO (1 μM) or matrine (100,1000 μM). Cell viability was measured at 0, 12, 24, 36, and 48 h after the co-treatment. All viability data represent mean ± SD from three independent experiments and are expressed as percent viability of vehicle control. **, $p < 0.01$. (C) Human

hepatocytes from liver donor (HL#74) in 96-well coated plate were treated with matrine at the wide range of concentrations. Prototypical MTT assays were performed 48 h after the treatments as described in the "Methods". Cell viability data represent mean ± SD from three independent measurements and are expressed as percent of vehicle control.



3.4 Discussion

CYP2B6 and CYP3A4 are the two most important P450 isozymes primarily responsible for the bioactivation and inactivation of CPA, respectively[8]. Previous findings suggest that preferential induction of CYP2B6 over CYP3A4 may benefit the anti-cancer activity of CPA[274]. In the last chapter, hCAR specific activator CITCO and mCAR specific

activator TCPOBOP have been demonstrated to enhance CPA therapeutic effect by activating CAR, which in turn induced CYP2B over CYP3A4 and promoted CPA bioactivation. Those observations may have potential clinical implication that enhanced CPA anti-cancer acitiviy may be achieved by the addition of a compound to the CPA chemotherapy regimen that will promote the bioactivation of the drug. However, the fact that neither CITCO nor TCPOBOP is a drug renders our findings compromised clinical importance. Thus, a search within clinically available compounds for a preferential induction profile of CYP2B6 over 3A4 will take our findings a huge step forward from a prove-of-concept experiment to a novel therapeutic strategy.

A panel of FDA-approved drug has been reported to activate hCAR with less potency and specificity than CITCO[192; 205; 261]. In agreement with previous publications, all these compounds exhibited significant induction of CYP2B6 mRNA with magnitudes lower than CITCO. At protein level, only EVF and ART slightly induced the expression of CYP2B6. It is reasonable to conclude that none of these weak hCAR activators may qualify a potent preferential inducer given their CYP2B6 induction profile and previous reports that CMZ, EFV, NVP and PHN are at the same time, CYP3A4 inducers[275]. Our CYP3A4 mRNA results partially confirmed these findings with the exception of PHN and NVP which induced CYP3A4 mRNA by less than 2 folds. The discrepancy may be explained by the different expression patterns between mRNA and protein, and lack of repeated experiments in different liver samples in the current study. CAR is the closest relative of PXR in the orphan nuclear receptor family and there is significant cross-talk between these receptors. As a result, many hCAR activators exhibit hPXR activation capacity and up-regulate CYP3A4 hand in hand with CYP2B6[171]. This is another reason

that could explain the lack of strong preference in the induction of the two isozymes by non-specific hCAR activators tested above.

To circumvent this problem, another approach was introduced. Ritonavir was identified as one of the most potent CYP3A4 specific inhibitors that exert their inhibitory effect through covalent, irreversible binding to the enzyme[263]. At the same time, the drug was proved to induce CYP2B6[262]. In fact, other members of the HIV protease inhibitor family, including indinavir,nelfinavir and saquinavir were also identified as CYP3A4 inhibitors with less potency. Another important class of CYP3A4 inhibitors is the anti-fungal azoles. However, since those compounds exhibit strong inhibition to CYP2B6 activity at the same time[276], they are pre-excluded from the current study. It is admitted that other identified CYP3A4 specific inhibitors might induce CYP2B6, but given the scarcity of human primary liver samples and time limit of our study, only ritonavir was selected to be tested in the co-culture model. Results from two independent experiments using hepatocytes from different donors confirmed that ritonavir did not enhance the CPA anti-cancer activity to SUDHL-6 cells or promote CPA cytotoxicityin hepatocytes *in vitro*. One of the possible explanations might be a mixed result of strong CYP3A4 inhibition and moderate CYP2B6 induction. Although CYP2B6 is primarily responsible for CPA bioactivation, CYP3A4 also contributes to this process to a less extend. It is possible that the potent inhibition of CYP3A4 by ritonavir caused a decreased bioactivation of CPA that was not completely compensated by the moderate induction of CYP2B6 and decreased inactivation of the drug, leading to a neutral overall result. Nonetheless, the co-culture system remains a useful tool to mimic the *in vivo* condition of drug metabolism and serves as a good measurement of overall therapeutic effects of the proposed strategy.

Chinese herbal medicine has been used in clinical application since ancient China and is more and more widely accepted with their pharmacology and toxicity explored in modern scientific settings. There are roughly 900~1000 Chinese herbal medicines in record, based on different sources and methods of preparation that dictate their characteristics and application[277]. The collection of 800 Chinese herbal medicines thus represents a great portion of all clinically available medicines and serves as a bank through which specific medicines can be easily identified and analyzed. Twenty candidates were selected through a 96-well plate based CAR1+A reporter assay. CITCO as a positive control generated an average of 4 fold signal versus negative control while in 24-well plate assay the ratio normally varies between 8 and 20[204]. The difference might due to different transfection conditions and different means of measurements. Nonetheless, the quality of data obtained was acceptable given the consistency in positive control signal strength and tight standard deviation of samples across different batches of experiments. The 20 candidates were further tested on a 96-well based PXR reporter assay and 8 candidates were identified as weak or non-PXR activators with their ration of preferential activation of CAR against PXR calculated. Notably, mRNA induction profile of the 20 candidates in large agree with the reporter assay results in that most of these samples exhibited decent induction of CYP2B6 mRNA while the samples most preferentially induced CYP2B6 over CYP3A4 falls into the 8 candidates list with minor exceptions. These results indicate that 96-well plate based reporter assay results can be used as a good precursor of mRNA expression profile in drug screening for preferential induction of CYP2B6 over CYP3A4.

An even stronger evidence of the success of our screening strategy was the identification

of matrine, which is the major component of PBC-036A and PBC-195A. The two samples ranked the 3rd and 6th in reporter based preferential activation ratio, respectively. Matrine delivered a stronger preferential induction profile than the two samples and its capacity of promoting CPA therapeutic effect was further confirmed by co-culture assay. In line with our observations, matrine has been reported to induce apoptosis in a variety of cancer cell lines such as hepatocacinoma cell lines[278], leukemia cell lines[279], pancreatic cancer cell lines[280] and ovary teratoma cell lines[281] both *in vitro* and in rodent models. Moreover, matrine has been used as an adjuvant therapy in cancer treatments to achieve better healing efficacy[282]. In particular, oxymatrine, the major metabolite of matrine was found to be an inducer of CYP2B in mice and promotes CPA bioactivation[283; 284]. Further exploration is warranted to elucidate the underlying mechanism of the preferential induction of matrine with respect to CAR and PXR and bridge the gap between rodent model and clinical research.

3.5 Conclusion

In summary, the current study explored several strategies to identify compounds that may preferentially induce CYP2B6 over CYP3A4. A panel of FDA-proved drugs which are reported to be hCAR activators exhibited marginal induction of CYP2B6 protein. Alternatively, a potent CYP3A4 inhibitor, ritonavir was tested in the co-culture system but failed to enhance CPA anti-cancer activity possibly due to the combined effects of strong CYP3A4 inhibition and moderate CYP2B6 induction. Last but not least, a collection of 800 Chinese herbal medical products were screened through 96-well based CAR/PXR activation assays. The effectiveness of this screening strategy was

demonstrated by preferential induction profiles of the 20 candidates selected in HPH mRNA assay. Most importantly, matrine was identified to be the major contributor of this profile in two of the top-ranked candidates. In agreement with previous publications, matrine promoted CPA therapeutic effect in our unique co-culture system. Further exploration will be focused on the underlying mechanism of matrine with respect to nuclear receptors and more compounds with preferential induction of CYP2B6 over CYP3A4 in the composition of selected candidates.

CHAPTER FOUR
OXAZAPHOSPHORINE-MEDIATED INDUCTION OF DRUG-METABOLIZING ENZYMES

4.1 Introduction

CPA, along with other members of the oxazaphosphorines, was developed by Norbert Brock and ASTA (now Baxter Oncology) in the 1950s[82].It has been the most widely used alkylating agent for the last 50 years in the treatment of hematological malignancies as well as solid tumors[160; 285; 286]. CPA has also been used at higher doses in the treatment of aplastic anemia leukemia prior to bone marrow transplantation and other malignancies. Moreover, it has been used as an immunosuppressor to treat several autoimmune disorders[287; 288]. Recently, lower dose of CPA has been used as either an antiangiogenic or an immunostimulatory agent in combination with immunotherapies as a cancer treatment[289; 290; 291]IFO is a close derivative of CPA and has been shown to be more effective in a wide range of malignant diseases[85]. In clinical application, CPA and IFO are frequently used in combination with other drugs and supportive therapies to produce synergistic or additive effect, which give rise to concerns of durg-drug interactions and the underlying pharmacological mechanisms.

Both CPA and IFO are metabolized by two alternative P450 pathways, drug activation via 4-hydroxylation and drug inactivation via N-dechloroethylation. CYP2B6 have been demonstrated to play a major role in the activation of CPA, while CYP3A4 counts for a large portion of its inactivation[116]. In the case of IFO, CYP2B6 and CYP3A4 are believed to be responsible for both its activation and inactivation[123]. Interestingly, the autoinduction effect of CPA has been observed in HPH [292]and in rats[293; 294], through

which the mRNA and protein levels of relevant P450s have been up-regulated and CPA metabolism has been promoted. Similar effect has been observed for IFO[295].During the past decade, significant progress has been made in our understanding of the mechanism involving drug-induced expression of CYP2B and CYP3A. Two xenobiotic nuclear receptors PXR and constitutive androstane receptor CAR have been documented as important xenobiotic sensor mediating the transcription of multiple DMEs and transporters[171]. PXR has been demonstrated to play a crucial role in the autoinduction effect of CPA, but it is not enough to fully account for the induction profile[177; 296; 297].CAR, which is the closest relative of PXR in the human nuclear receptor family tree, has also been proposed to play a role, but with very limited evidence. Given the close relationship between PXR/CAR and CYP3A4/CYP2B6 which dictate the metabolism of CPA and IFO, it is reasonable and necessary to explore the role of CAR in the autoinduction effect of these oxazazphospharines. Besides, a broad spectrum of Phase I and Phase II drug metabolizing enzymes are involved in the metabolism of CPA and IFO, but the induction profile of related genes by these drugs has never been studied systematically with respect to the role of CAR.

In this report, we studied the involvement of CAR in CPA and IFO induced drug metabolizing enzyme (DME) regulation by a variety of HPH-based assays and HepG2-based assays. Our results showed that CAR plays an important role in CPA induced DME regulations. The induction profile of IFO is very similar to that of CPA, but CAR is not activated upon IFO treatment. The difference in CAR activity under CPA and IFO treatments suggests different roles of CAR in oxazaphospharine- mediated DME regulation.

4.2 Material and Methods

4.2.1 Chemicals and biological reagents

CPA, IFO, RIF, PB and 3-methylcholanthrene (3MC) were purchased from Sigma-Aldrich (St. Louis, MO). CITCO was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). FuGENE® 6 transfection reagent was obtained from Roche (Basel, Switzerland). Matrigel, insulin and ITS^{+} were obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Calsbad, CA) or Sigma-Aldrich.

4.2.2 Plasmids and generation of adenovirus-EYFP tagged hCAR

The pSG5-hPXR expression vector was obtained from Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). The pCR3-hCAR1+A expression vector and the CYP2B6 reporter construct, containing both phenobarbital-responsive enhancer module and the distal XREM (CYP2B6–2.2kb), were generated as described previously [112; 204]. The CYP3A4-PXRE/XREM reporter vector was obtained from Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC). The pRL-TK Renilla reniformis luciferase plasmid used to normalize firefly luciferase activities was obtained from Promega. The pCR3-hCAR and the enhanced yellow fluorescent protein tagged hCAR (EYFP-hCAR) expression plasmids were kindly provided by Dr. Masahiko Negishi (National Institute of Environmental and Health Sciences, National Institutes of Health, Research Triangle Park, NC). As reported previously, the CYP2B6-2.2kb firefly

luciferase construct is composed of the PBREM containing 1.8 kb of the native promoter, and the 400 bp distal XREM region [112]. The pRL-TK Renilla luciferase plasmids used to normalize firefly luciferase activities were from Promega. The EYFP tagged hCAR was sub-cloned into pShuttle-CMV expression vector at SaI I and Xba I sites. The linearized shuttle vector and AdEasy vector were then cotransformed into BJ5183 cells. Positive recombinant Ad/EYFP-hCAR plasmids were selected, and transduced into HEK293 cell cultures for virus packaging and amplification. Viruses were purified by using ViraBindTM Adenovirus Purification kit (Cell Biolabs, San Diego, CA). hCAR1+A was generated by Tao Chen in our lab by introducing nucleotides corresponding to the alanine residue at site 270 of hCAR3 into the pCR3-hCAR1 expression vector by use of the QuikChange Site-directed Mutagenesis System (Stratagene, La Jolla, CA).

4.2.3 Human primary hepatocytes culture and treatments

Human liver tissues were obtained following surgical resection by qualified pathology staff after diagnostic criteria were met and prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously[298]. Hepatocytes were seeded at 3.75 × 105 cells/well in 24-well biocoat plates in DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 μg/mL insulin, and 1 μM dexa methasone, and cultured as described previously[112]. Hepatocyte cultures were infected with 2 μl Ad/EYFP-hCAR for 12 hrs before the treatment with vehicle control (0.1% DMSO) or test compounds. After 24 hrs treatment, cells were washed twice with phosphate-buffered saline and fixed for 30 min in 4% buffered paraformaldehyde. The cells were then stained with 4,6-diamidine-2-

phenylindole dihydrochloride (DAPI) for 30 min. PB, CPA and IFO were tested in Ad/EYFP-hCAR infected HPH. In separate experiments, HPH seeded in 6-well biocoat plates were treated with RIF(10 μM), CITCO (1 μM), SFN(25 μM), 3-MC(5 μM),CPA(various concentrations),IFO(various concentrations), or 0.1% DMSO as vehicle control for real-time PCR and Western blotting analysis.

4.2.4 Confocal laser scanning microscopy imaging

Confocal laser scanning microscopy was performed with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE000 microscope. The confocal system was equipped with three fluorescence detection channels (photomultipliers) and a nonconfocal transmitted light detector. One of the photomultipliers was used to collect fluorescence signals from the green and yellow region of the fluorescence emission, and the nonconfocal transmitted light detector was used to collect bright-field images

4.2.5 Real-time PCR analysis

Total RNA was isolated from treated hepatocytes using the RNeasy Mini Kit (Qiagen) and reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instructions. CYP2B6 and CYP3A4 mRNA expression was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Primers for CYP2B6, CYP3A4, and GAPDH mRNA detection are detailed in Supplemental Table 1.Fold induction values were calculated according to the equation:fold over control = $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle

threshold numbers between the target gene and GAPDH, and ΔΔCt represents the relative change in these differences between control and treatment groups.

4.2.6 Western Blot Analyses

Homogenate proteins (20 µg) from treated HPH were resolved on SDS -polyacrylamide gels, and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes. Subsequently, membranes were incubated with specific antibodies against CYP2B6 or CYP3A4 (Millipore-Chemicon, CA) diluted 1:4000 and 1:5000, respectively. β-actin (Sigma-Aldrich. St. Louis, MO) was used as internal control. Blots were washed and incubated with horseradish peroxidase goat anti-rabbit IgG antibody diluted 1:4000. Blots were developed using ECL Western blotting detection reagent (GE Healthcare).

4.2.7 Transfection of cell lines

HepG2 cells in 24-well plates were transfected with hPXR, hCAR1 or hCAR1+A expression vector, and CYP2B6-2.2kb or CYP3A4-PXRE/XREM reporter construct using Fugene 6 Transfection Kit following the manufacturer's instruction. 24 hours after transfection, cells were treated with solvent (0.1% DMSO) or test compounds at indicated concentrations .24 hours later, cell lysates were assayed for firefly activities normalized against the activities of cotransfected Renilla luciferase using Dual-Luciferase Kit (Promega, WI). Data were represented as mean±S.D. of three individual transfections.

4.2.8 Statistical analysis

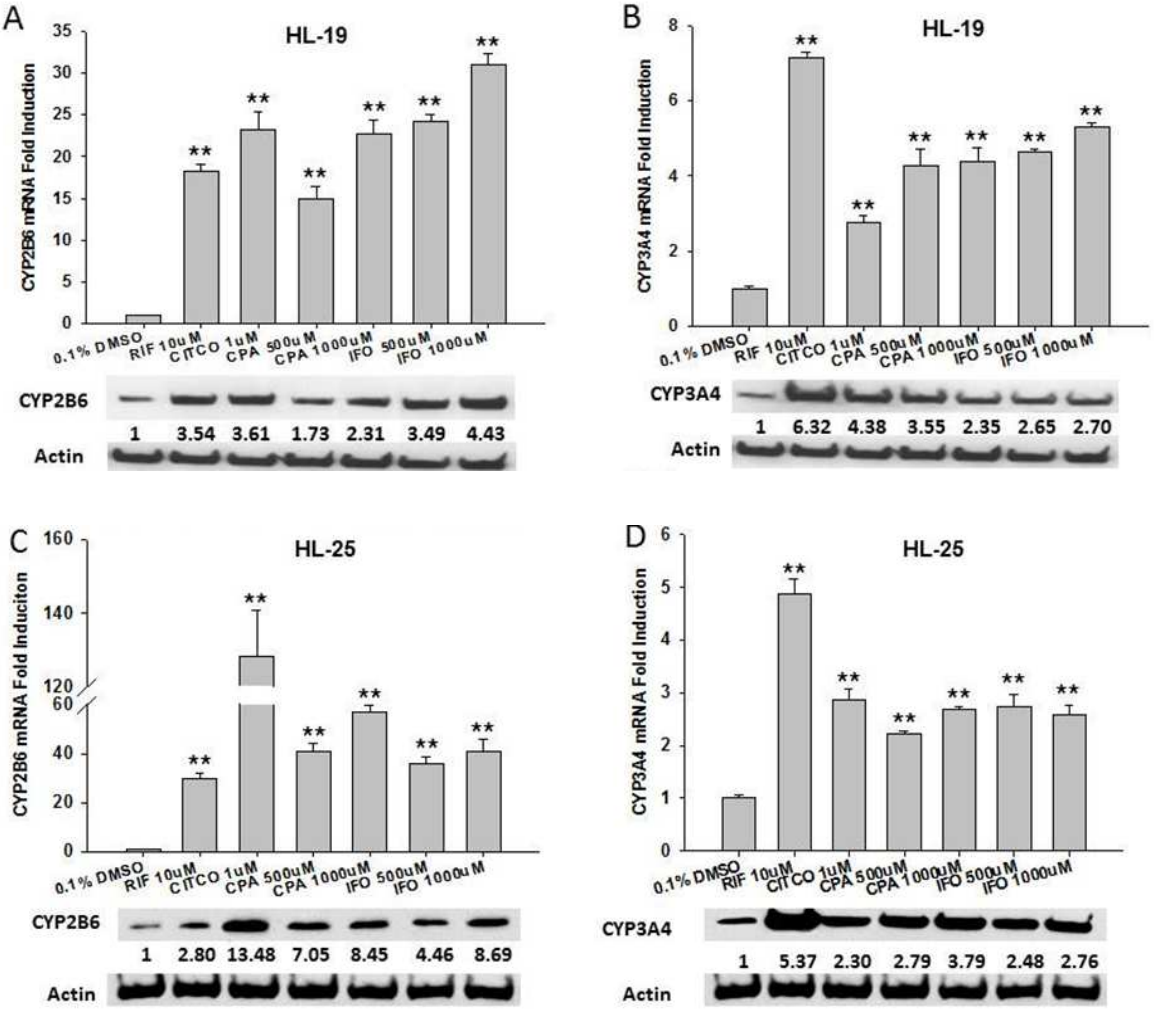
Experimental data are presented as a mean of triplicate determinations ± S.D. unless otherwise noted. Statistical comparisons were made using one-way analysis of variance (ANOVA). The statistical significance was set at p values <0.05 (*), or <0.01 (**).

4.3 Results

4.3.1 Effects of CPA and IFO on the expression of DMEs in HPHs

HPHs are well accepted as an appropriate *in vitro* model mimicking human liver for drug metabolism and preclinical induction studies[299]. In the current investigation,we evaluated the effects of CPA and IFO on the expression of several DMEs in HPH cultures using RTPCR and Western Blotting assays. As demonstrated in Figure 4.1, the mRNA and protein expressions of CYP2B6 and CYP3A4 were significantly induced by CPA and IFO at concentrations bracketing their pharmacologically relevant levels[177; 295], and there was no identifiable cytotoxicity observed throughout the experiments. Notably, CYP2B6 expression was induced to a greater extent by both CPA and IFO treatment than that of CYP3A4, in particular, at the mRNA level. In HPH preparation from donor HL#19, the maximal induction of CYP2B6 mRNA by CPA and IFO challenges the induction by positive controls (RIF and CITCO).

Figure 4.1 Induction of CYP2B6&CYP3A4 by CPA and IFO in human primary hepatocytes. Human hepatocytes from liver donors (HL#19 and HL#25) were treated with CITCO (1 μM), RIF (10 μM) or CPA and IFO at indicated concentrations for 24 h (mRNA) and 72 h (protein), respectively. The expression of mRNA for CYP2B6 (a, c) and CYP3A4 (b, d) was analyzed using RT-PCR; related protein expression was detected using immunoblotting analysis as outlined in "Materials and Methods." RT-PCR data are expressed as mean ± SD (n=3). **: $p<0.01$. Densitometry of CYP2B6 and CYP3A4 proteins was normalized against β-actin expression.



In parallel experiments, we further investigated the role of CPA and IFO in the induction of UGT1A1 and CYP1A2, which are also involved in the metabolism of a broad spectrum of drugs and environmental compounds. Results showed that CPA and IFO treatment of HPHs resulted in a clear induction of UGT1A1 mRNA expression, while exhibiting no effects on the regulation of CYP1A2 expression (Figure 4.2A, B). These results indicate that CPA and IFO may enhance the expression of CYP2B6, CYP3A4, and UGT1A1 through shared transcriptional regulatory machineries that are distinct from the induction of CYP1A2.

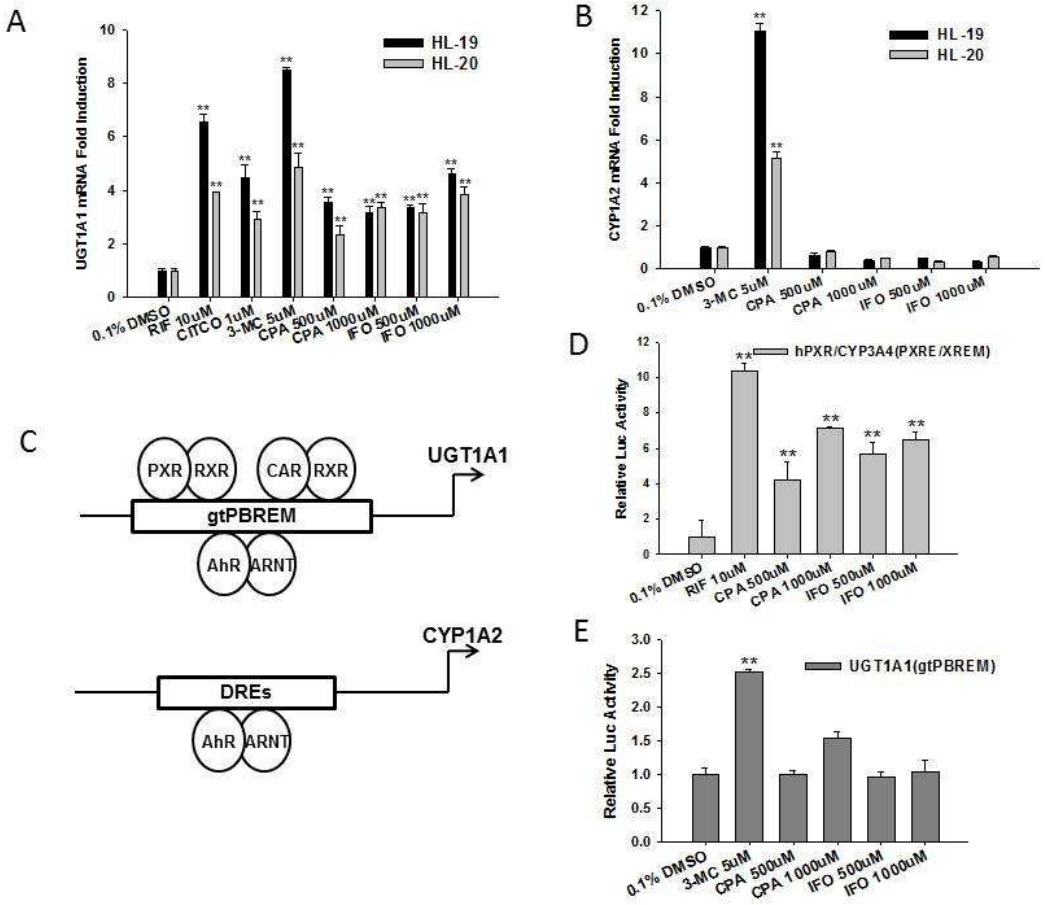


Figure 4.2 CPA and IFO induce the expression of UGT1A1 but not CYP1A2. Human primary hepatocytes from liver donors (HL#19, and HL#20) were treated with CITCO (1 μM), RIF (10 μM), 3MC (5 μM) or CPA and IFO at indicated concentrations for 24 h. The mRNA expression of UGT1A1 (A) and CYP1A2 (B) was analyzed using real-time RT-PCR. In parallel experiments, CPA- and IFO-mediated activation of PXR (D) and AhR (E) was assessed in HepG2 cell-based reporter assays as described in "Materials and Methods." All data are presented as mean ± SD (n=3), **: $p<0.01$. Panel (C) represents a schematic diagram depicting the CAR, PXR, and AhR response elements in the UGT1A1 and CYP1A2 promoters.

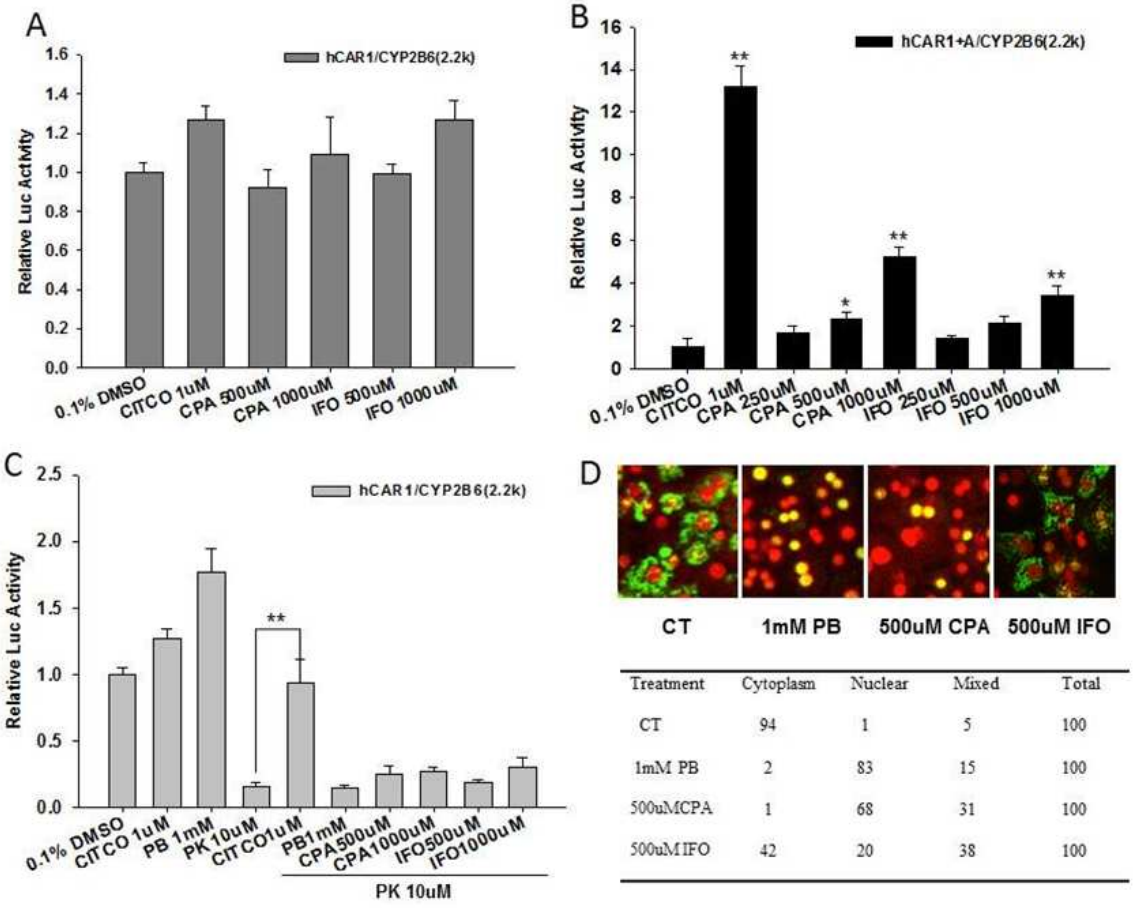
4.3.2 CPA and IFO activate human PXR but not AhR

It has been well established that drug-induced expression of UGT1A1 is primarily regulated at the transcriptional level by three xenobiotic receptors, PXR, CAR and AhR, while CYP1A2 is predominantly transactivated by AhR (Figure 4.2C)[213; 300; 301]. To illustrate mechanisms pertaining to the differential induction of UGT1A1 and CYP1A2 by CPA and IFO, we tested the role of these oxazaphosphorines in the activation of PXR and AhR in HepG2 cell-based reporter assays. In agreement with previous reports, CPA and IFO significantly enhanced PXR-mediated transactivation of CYP3A4 luciferase activity (Figure 4.2D)[177; 181]. In determining the contribution of AhR in CPA- and IFO-mediated UGT1A1 induction, HepG2 cells, which express endogenous AhR abundantly[302], were transfected with UGT1A1-gtPBREM reporter construct alone. As expected, 3MC, a prototypical AhR activator, remarkably transactivated UGT1A1 luciferase activity, while neither CPA nor IFO activated AhR (Figure 4.2E), indicating the CPA- and IFO-mediated induction of UGT1A1 is AhR-independent.

4.3.3 Differential Activation of human CAR by CPA and IFO

Since CYP2B6 and CYP3A4 are typical target genes for both CAR and PXR, and CPA- and IFO-mediated induction of UGT1A1 is AhR-independent, we speculated that PXR and CAR play pivotal roles in the overall metabolic elevation by these oxazaphosphorines. In particular, the role of CAR in the metabolic inducibility of CPA and IFO remains unexplored, due partly to its high basal activity and lack of response to chemical stimulation in immortalized cell lines (Figure 4.3A)[171]. Utilizing a recently established chemical-responsive hCAR variant with an alanine insertion at 271 (hCAR1 + A)[204], our reporter assays revealed that CPA and IFO can enhance CYP2B6 luciferase

activity through activation of CAR in a concentration-related manner, and CPA appears to be a stronger CAR activator than IFO (Figure 4.3B). Additional luciferase reporter experiments demonstrated that PK11195 (a potent hCAR antagonist-deactivated CAR activity (80% of the constitutive control) could be efficiently reactivated by the direct hCAR activator CITCO but not by the indirect activator PB (Figure 4.3C). CPA and IFO failed to reactivate PK11195-repressed hCAR activity in this assay, suggesting these oxazaphosphorines may activate hCAR through PB-type indirect mechanism.



Treatment	Cytoplasm	Nuclear	Mixed	Total
CT	94	1	5	100
1mM PB	2	83	15	100
500uMCPA	1	68	31	100
500uMIFO	42	20	38	100

Figure 4.3 Differential activation of hCAR by CPA and IFO. HepG2 cells were transfected with hCAR1 (A,C) or hCAR1 + A (B) expression vector in the presence of CYP2B6-2.2 kb reporter construct and the pRL-TK control vector. Transfected cells were treated for 24 h with

CITCO (1 μM), PB (1 mM), PK11195 (10 μM) or CPA and IFO at indicated concentrations alone or cotreated with PK11195 as depicted in 3 C. Luciferase activities were determined and expressed as fold relative to vehicle control. All data are expressed as mean ± SD (n=3). **: $p<0.01$. In separate experiment, human primary hepatocytes (HL#20) were infected with Ad/EYFP-hCAR and treated with negative control (0.1% DMSO), positive control (PB, 1 mM), or CPA and IFO (500 μM) for 24 h. After fixation and DAPI staining, hepatocytes were subjected to confocal microscopy analysis as described in "Materials and Methods." Representative Ad/EYFP-hCAR localization was demonstrated in (D). The table shows the percentage of hepatocytes exhibiting the different types of hCAR localization from each treatment group.

Different from the constitutive activation in immortalized cell lines, CAR is primarily localized in the cytoplasm of HPHs prior to activation, and nuclear accumulation occurs only after treatment with CAR modulators[191]. To further examine the capability of CPA and IFO in hCAR activation, a hCAR nuclear translocation experiment was carried out in cultured HPHs infected with the Ad/EYFPhCAR, which has demonstrated exceptional efficiency in infecting HPH[193]. As expected, in the virus-infected HPHs, the EYFP-hCAR was primarily expressed in the cytoplasm (94%) without activation (vehicle control) and accumulated in nucleus (83%) upon PB treatment (Figure 4.3D). Intriguingly, treatment with CPA at 500 μM resulted in remarkable EYFP-hCAR nuclear translocation (63%), while IFO at the same concentration only led a moderate nuclear accumulation of CAR (20%) (Figure 4.3D). In agreement with aforementioned reporter assays, these results suggest that CPA is a stronger hCAR activator in comparison to IFO, and IFO may exert its inductive activity primarily through the activation of PXR.

4.3.4 Effects of selective inhibition of PXR on the Induction of CYP2B6 by CPA and IFO

Through mechanistic cross-talking, PXR and CAR share a large number of target genes as well as many xenobiotic activators[171]. To further explore the contribution of CAR in CPA and IFO induction of DME, a co-treatment experiment was carried out in HPHs using sulforaphane (SFN) as a selective inhibitor of hPXR[303]. As expected, concomitant treatment of SFN and the selective hPXR activator RIF completely abolished RIF-mediated induction of CYP2B6, while CITCO-mediated CYP2B6 induction was only moderately repressed by SFN (Figure 4.4)[248; 303] .Notably, although CPA-mediated induction of CYP2B6 in HPHs was suppressed moderately by the co-treatment of SFN, the induction by IFO was dramatically decreased (Figure 4.4). Taken together, these findings corroborate the differential contribution of CAR in CPA- and IFO mediated induction of CYP2B6.

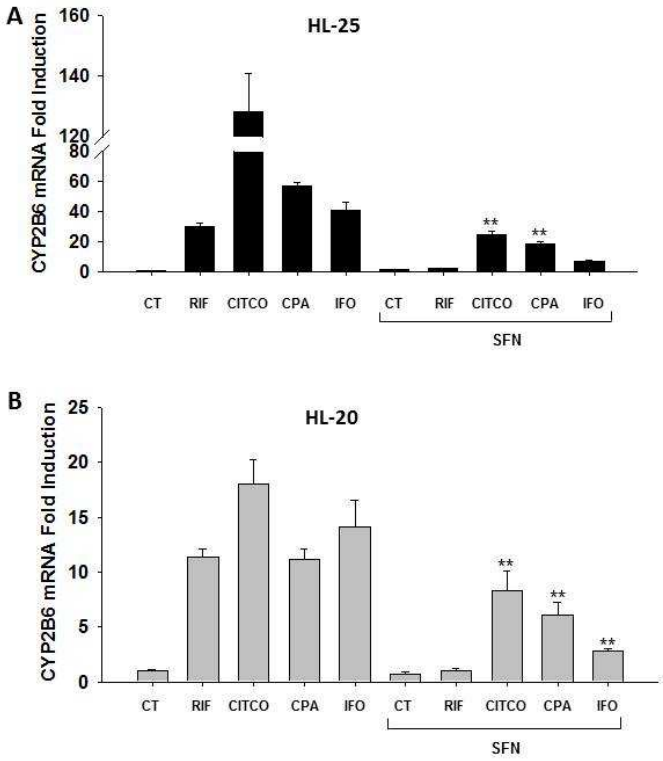


Figure 4.4 Effects of PXR inhibition on the induction of CYP2B6 by CPA and IFO. Human primary hepatocytes from donors (HL#20 and HL#25) were treated for 24 h with CITCO (1 μM), RIF (10 μM), SFN (25 μM), CPA (1 mM), and IFO (1 mM), and cotreatment of SFN (25 μM) with CITCO, RIF, CPA, and IFO, respectively. The expression of CYP2B6 mRNA was assessed in real-time RT-PCR assays (Materials and Methods). All data are expressed as mean ± SD (n=3). **: $p<0.01$.

4.4 Discussion

Although CPA and IFO have long been used as anti-cancer alkylating agents in clinical practice with their pharmacology, metabolism and pharmacokinetic/pharmacodynamics profiles well elucidated, there have been rising concerns regarding drug-drug interactions, and autoinduction along with the increase of combinatory therapeutic strategies involving

these drugs[229]. Accumulating evidence thus far, revealed that hepatic metabolism of CPA and IFO is autoinducible and repeated administration of CPA and IFO was associated with elevated 4-hydroxylation of the oxazaphosphorines and expression of several CYP enzymes[292; 295]. However, the molecular mechanisms behind the induction are not fully understood. In this report, we demonstrated that in addition to the promiscuous xenobiotic receptor PXR, CAR plays an important role in the enzymatic autoinduction of these oxazaphosphorines. More importantly, although PXR appears to be equally involved in the inductive activity of CPA and IFO, CAR functions as a preferential mediator of CPA—rather than IFO-mediated induction of hepatic DMEs. This evidence suggests that co-administration of drugs that selectively disturb the expression and function of CAR may differentially affect the autoinduction and drug-drug interactions of CPA rather than IFO.

In order to accommodate their own metabolism and clearance, xenobiotics including drugs and environmental chemicals can alter the expression of DMEs and transporters through the transactivation of a group of xenobiotic receptors. Previously, PXR was reported as the mediator of CPA and IFO autoinduction by transcriptional up-regulation of CYP2B6 and CYP3A4[181177]. Both CPA and IFO increased PXR activity in luciferase reporter assays at the concentrations that significantly induced CYP2B6 and CYP3A4 expression in HPH cultures[177; 181]. Interestingly, we showed that CPA and IFO treatment resulted in a greater increase in CYP2B6 mRNA than that of CYP3A4, which is in agreement with data from a previous report[177]. Given that CYP2B6 is a favorable target gene of CAR over PXR and potent induction of CYP2B6 by CPA and IFO was observed, activation of PXR alone by these oxazaphosphorines may not be able to sufficiently

accommodate the extent of DME induction in hepatocytes. Accordingly, it is conceivable that CAR, the closest relative of PXR, may mediate a compensatory role in CPA- and IFO-associated induction. However, unlike that of PXR, investigation of CAR activation *in vitro* has been hindered by several specific features of this receptor, including that 1) CAR is constitutively activated and spontaneously nuclear localized in immortalized cell lines and 2) it can be activated by both direct ligand binding and indirect ligand independent mechanisms[191; 192]. Recently, we have generated a chimerical construct, namely hCAR1 + A, with an alanine insertion at 271, which converts the constitutive wild type hCAR into a chemically responsive xenobiotic sensor[204]. In cell-based reporter assays utilizing this chimerical vector, our results revealed that CPA and IFO dose dependently enhanced CYP2B6 luciferase expression through the activation of hCAR1 + A. Given that over 90% of known hCAR activators positively respond to this chimerical construct[204], this initial experiment supports the involvement of CAR in the inductive activity of CPA and IFO.

Alternatively, CAR exhibits significant nuclear translocation from cytoplasm of primary cultured hepatocytes in the presence of CAR activators such as PB and CITCO[193]. This initial step of CAR activation in primary hepatocytes has been successfully established as an efficient approach to identify hCAR activators *in vitro*[193]. Remarkably, both direct activators (e. g. CITCO, and artemicinin) and indirect activators (e. g. PB, and phenytoin) are effective in the nuclear accumulation of CAR in hepatocyte cultures[191; 192; 193]. Intriguingly, our results showed that CPA but not IFO displayed marked effects on nuclear accumulation of CAR in HPHs. Although activation of CAR is a multistep process, the lack of initial nuclear translocation of CAR by IFO suggests that IFO is less

likely to be an effective activator of hCAR.

In contrast to other nuclear receptors, activation of CAR doesn't require ligand binding (indirect activation), and as a matter of fact the majority of known human CAR activators identified thus far activate CAR through PB-like indirect machinery rather than CITCO-like ligand binding[191; 193]. Recent reports from this laboratory demonstrated that in HepG2 cells, the PK11195 (a prototypical peripheral benzodiazepine receptor and potent hCAR deactivator)-repressed hCAR activity can be efficiently reactivated by direct activator CITCO but not the prototypical indirect activator PB[248] . Akin to PB, CPA was unable to reactive PK11195-inhibited CAR activity, suggesting CPA may function as a PB-type indirect CAR activator.

To date, mounting evidence suggests that CAR and PXR share their target genes through recognizing of, and binding to, xenobiotic responsive elements located in the upstream of these genes[231; 304]. An asymmetrical cross-regulation of CYP2B6 and CYP3A4 by hCAR but not hPXR has been established in that hCAR exhibits preferential induction of CYP2B6 over CYP3A4, while hPXR exerts less-selective induction of both genes[207]. In an effort to determine the relative involvement of CAR in CPA- and IFO-mediated CYP2B6 induction, experiments in HPHs revealed that SFN, a selective inhibitor of hPXR, dramatically repressed IFO-induced expression of CYP2B6 mRNA, while to a lesser extent in response to the CPA-mediated induction. In concert with aforementioned luciferase reporter and nuclear translocation data, these results strongly suggest that CPA is a stronger activator of hCAR compared to IFO.

4.5 Conclusion

In summary, our results from the current study indicate that although PXR indiscriminately mediates the inductive activity of both CPA and IFO, CAR exhibits marked differential roles in CPA- and IFO-associated induction of DMEs. CPA can activate both PXR and CAR; IFO appears primarily to transactivate PXR. Furthermore, CPA functions most likely through PB-type indirect mechanisms, by which it predominantly influences the nuclear translocation of CAR with no obvious effects on the nuclear localized CAR activity. Due to the insufficient understanding of the mechanism(s) underlying CAR indirect activation, particularly the lack of knowledge regarding which molecule these activators initially interact with, it is difficult to explain the differential roles on CAR activation between CPA and IFO. Moreover, extra caution is required in the interpretation of these *in vitro* discoveries due to the range of drug concentrations where a response was observed. Nevertheless, these findings may be potentially of clinical importance where drug-mediated manipulation of hCAR activity could selectively alter the autoinduction and pharmacokinetic profile of oxazaphosphorines, as well as co-administered drugs in the regimen.

CHAPTER FIVE
CONCLUSIONS

Drug metabolism and its regulation is a dynamic research field in which various enzymes, co-factors and regulation mechanism have recognized and studied with respect to their clinical importance and drug research & development potential[8; 171]. In this dissertation, the role of constitutive androstane receptor in the metabolism of oxazaphosphorines was investigated.

Prodrugs are pharmaceutical substances that administered in an inactive or partially active form and subsequently converted to the active therapeutic moiety through bioactivation. In most cases prodrugs are designed to achieve better bioavailability , or to overcome unwanted toxicities and side-effects, which has been a major concern in chemotherapies[8]. Among them, oxazaphosphorines is a class of alkylating agents designed to deliver anti-cancer and immune-suppressing effects through binding to DNA in active-proliferating cells. CPA and IFO, as the first two members of this family, are among the most widely used anti-cancer prodrugs in the last 50 years. The metabolism, transportation, and elimination of these compounds have been well characterized and thoroughly studied[8]. However, because of the increased polypharmacy in oxazaphosphorine-based chemotherapy, drug-drug interactions associated with CPA and IFO multidrug regimens have been raising concerns in clinical application. It has been well established that hepatic CYP2B6 and CYP3A4 differentially contribute to the 4-hydroxylation and *N*-dechloroethylation of CPA and IFO and many clinical used drugs and environmental compounds can stimulate the inductive expression of these two CYP

enzymes. In the past ten years, progressive research has unveiled the importance of CAR and PXR in the regulation of CYP2B6 and CYP3A4 expression. Notably, activation of PXR induces both isozymes, while selective activation of CAR leads to preferential induction of CYP2B6 over CYP3A4 in human liver[207]. Given the fact that CPA is predominantly bioactivated by CYP2B6 while deactivated through CYP3A4, this important discovery leads to our hypothesis that a combination of CPA and CAR activators may enhance the therapeutic effect of the anti-cancer drug by preferential induction of CYP2B6 over 3A4. This center hypothesis was tested and developed in different approaches in following chapters.

In chapter 2, an innovative hepatocyte-hematopoietic cell co-culture system was established and demonstrated it to be a useful *in vitro* model for studying the biotransformation and therapeutic effects of CPA and potentially other prodrugs in an environment that closely mimics *in vivo* conditions. Based on this system, CITCO was proved to preferentially induce CYP2B6 over CYP3A4 and subsequently enhance CPA anti-cancer activity. Similar trends were observed using rodent primary hepatocytes and their corresponding CAR activators. These findings support the aforementioned center hypothesis that inclusion of a selective hCAR activator in CPA-based regimen may benefit cancer patients, at least patients with hematological malignancies. It is important to note that more studies have to be conducted to bridge the current concept-proving research with actual clinical needs, especially when no FDA-approved drug has been demonstrated thus far to activate hCAR selectively. The comprised clinically relevance of the current study presents an urgent need for the identification of more candidates that exhibit preferential induction of CYP2B6 over CYP3A4 from the universe of drugs

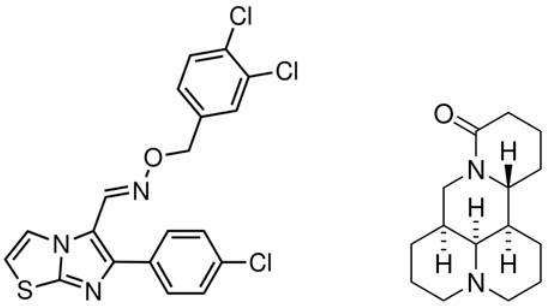
already in clinical application. Different approaches was proposed and tested in chapter 3. In another attempt to move the proposed therapeutic strategy towards clinical application, an *in vivo* study was conducted to test the combined effect of CPA and mCAR specific activator TCPOBOP in a SMID mouse model with xenografted lymphoma. Though *in vitro* studies in co-culture model demonstrated significantly enhanced therapeutic effect of CPA when co-treated with TCPOBOP, the effect of TCPOBOP was negligible in promoting anti-cancer effect of CPA *in vivo*. The fact that this mCAR activator is a potent tumor promoter may contribute to the discrepancy between *in vitro* and *in vivo* results[257; 258]. Nonetheless, further studies are warranted to improve CPA-based chemotherapy given the clinical importance of this anti-cancer drug and promising future of the proposed strategy.

In chapter 3, compounds that may potentially induce CYP2B6 over CYP3A4 were screened and tested in three different approaches. Firstly, a panel of FDA-approved drugs that are reported to be hCAR activators was evaluated with respect to their induction profile. However, none of these drugs exhibited significant preferential induction of CYP2B6 over CYP3A4 possibly due to their limited specificity for hCAR. Secondly, ritonavir was evaluated in the co-culture model as a representative of potent CYP3A4 activity inhibitors. Ritonavir did not enhance the CPA anti-cancer activity *in vitro* with a possible explanation that the induction of CYP2B6 by ritonavir was not enough to compensate the loss of CYP3A4 activity, leaving a neutral overall effect. The third approach was to screen qualified candidates from 800 Chinese herbal medicines, which have a long history of clinical application in China. Through 96-well based hCAR and PXR activation assays, 20 candidates were selected and ranked by their capacity in

preferentially activating hCAR versus PXR, which can presumably be translated to a preferential induction of CYP2B6 over CYP3A4. HPH-based mRNA induction results were generally in line with the reporter screening results and thus proved its validity. Notably, a single compound, matrine was identified through these assays as a promising candidate in combined CPA chemotherapy that it enhanced CPA therapeutic effect in co-culture assay. Although matrine has already been used an adjuvant therapy in cancer treatments to achieve better healing efficacy[282], this is the first time that its capability is address with a P450 and nuclear receptor related mechanism. In light of their potential clinical value in the international community, identification and further study of more individual compounds like matrine in those 20 Chinese herbal medical products are warranted.

Chapter 4 mainly focuses on the autoinduction profile and the underlying mechanism of CPA and IFO. Both compounds induced CYP2B6 and CYP3A4 at mRNA and protein levels in HPH. While both drugs were proved to be PXR activators which indiscriminately induces CYP2B6 and CYP3A4, CPA but not IFO was demonstrated to be a PB-like, indirect hCAR activator by translocation assay and reporter assays. These findings may be of potential clinical importance where drug-mediated alteration of hCAR activity could selectively influence the autoinduction and pharmacokinetic profile of oxazaphosphorines.

APPENDIX:
SUPPLEMENTAL DATA



CITCO Matrine

Figure S1 Chemical structures of CITCO and Matrine

Table S1 List of primers

Gene	Primer Sequence	Amplicon Size (bp)	Reference
CYP2B6	F: 5′-AGACGCCTTCAATCCTGACC-3′ R: 5′-CCTTCACCAAGACAAATC-CGC-3′	105	Reference #207
CYP3A4	F: 5′-GTGGGGCTTTTATGATGGTCA-3′ R: 5′-GCCTCAGATTTCTCACCAACACA-3′	272	Reference #207
hCAR	F: 5′-GAGCTGAGGAACTGTGTGGTA-3′ R: 5′-CTTTTGCTGACTGTTCTCCTGAA-3′	92	Reference #207
ADH1B	F: 5′-CCCGGAGAGCAACTACTGC-3′ R: 5′-AACCAGTCGAGAATCCACAGC-3′	224	PrimerBank ID 4501931a1
ADH1C	F: 5′-CTCGCCCCTGGAGAAAGTC-3′ R: 5′- GGCCCCCAACTCTTTAGCC-3′	223	PrimerBank ID 71565153c3
ALDH1A1	F: 5′-CCGTGGCGTACTATGGATGC-3′ R: 5′-GCAGCAGACGATCTCTTTCGAT-3′	82	PrimerBank ID 327365354c2
ALDH3A1	F: 5′-CTCTGTGACCCCTCGATCCA-3′ R: 5′-TCTTCCCCGTAGAACTCTTTCA-3′	74	PrimerBank ID 206597438c2
ALDH5A1	F: 5′-AGTCATCACCCCGTGGAATTT-3′ R: 5′-GAGAAGGGCGTGTCTTCGG-3′	111	PrimerBank ID 25777720c2
GAPDH	F: 5′-CCCATCACCATCTTCCAGGAG-3′ R: 5′-GTTGTCATGGATGA- CCTTGGC-3′	285	Reference #207

Table S2 Liver donor information

Liver sample	Age	Sex	Race	Cause of surgery
HL#7	45	Female	n/a	n/a
HL#10	n/a	n/a	Caucasian	n/a
HL#18	65	Female	n/a	Metastatic colon cancer
HL#19	45	n/a	n/a	Metastatic breast cancer
HL#20	75	Female	n/a	Metastatic colon cancer
HL#22	49	Male	n/a	Metastatic colon cancer
HL#23	53	Male	Caucasian	n/a
HL#24	69	Male	African American	n/a
HL#25	77	Male	Caucasian	n/a
HL#35	45	n/a	n/a	Metastatic breast cancer
HL#37	38	Male	Caucasian	n/a
HL#38	46	Female	Caucasian	n/a
HL#42	64	Male	Caucasian	Metastatic colon cancer
HL#45	50	Female	Caucasian	Metastatic colon cancer
HL#54	43	Female	Caucasian	n/a
HL#64	n/a	n/a	n/a	n/a
HL#69	78	Female	Caucasian	n/a
HL#73	53	Male	Caucasian	n/a
HL#74	n/a	n/a	n/a	n/a

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