RITONAVIR - A NOVEL MULTIDRUG RESISTANCE MODULATOR IN CANCER CHEMOTHERAPY AND OCULAR NEOVASCULAR DISEASES

A DISSERTATION IN

Pharmaceutical Sciences
and
Chemistry

Presented to the Faculty of the University
of Missouri-Kansas City in partial fulfillment of
the requirements for the degree

DOCTOR OF PHILOSOPHY

By

RAMYA KRISHNA VADLAPATLA

B. Pharm., Andhra University, 2008

Kansas City, Missouri

2014

RITONAVIR - A NOVEL MULTIDRUG RESISTANCE MODULATOR IN CANCER CHEMOTHERAPY AND OCULAR NEOVASCULAR DISEASES

Ramya Krishna Vadlapatla, Candidate for the Doctor of Philosophy degree,

University of Missouri-Kansas City, 2014

ABSTRACT

Multidrug resistance (MDR), a clinical outcome characterized by subtherapeutic intracellular drug concentration, is one of the predominant factors limiting effective cancer chemotherapy. Several possible mechanisms and molecular alterations have been implicated in the development of MDR, including activation of efflux transporters and metabolizing enzymes in response to therapeutic agents. Therefore, the primary objective of my dissertation project is to develop strategies for overcoming drug resistance in cancer chemotherapy. Human adenocarcinoma cells (LS-180) were treated for 72 hours with vinblastine alone and in the presence of ritonavir. The expression of efflux transporters (MDR1 and MRP2), metabolizing enzyme (CYP3A4) and nuclear hormone receptor (PXR) was induced in response to vinblastine. This overexpression was completely neutralized when cells were cotreated with ritonavir. Uptake of [^{3}H] lopinavir and Vivid™ assay further confirmed the functional activity of transcribed genes upon cotreatment. Reduced cell proliferation, migration and increased apoptosis of cancer cells were further indicative of enhanced activity of chemotherapeutics (doxorubicin, paclitaxel, tamoxifen and vinblastine) in the presence of ritonavir. Combination therapy of anticancer drug with ritonavir may overcome drug resistance by neutralizing overexpression of efflux transporters and metabolizing enzymes.

Hypoxia leading to neovascularization has also been implicated in the development of MDR and ocular neovascular diseases. Despite introduction of novel therapeutics, treatment of

retinal disorders remains challenging, possibly due to complex nature of hypoxia signaling. This study demonstrates for the first time that hypoxic conditions may alter expression of efflux and influx transporters in retinal pigment epithelial (RPE) cells. These findings suggest that hypoxia may further alter disposition of ophthalmic drugs. Inhibiting this signaling mechanism with an already approved therapeutic molecule may have promising antiangiogenic role with fewer side effects. Our studies (quantitative PCR, immunoblot analysis, ELISA and angiogenic assay) have demonstrated that ritonavir inhibits the expression of hypoxia-inducible factor-1α (HIF-1α) mediated vascular endothelial growth factor (VEGF) expression in RPE cells probably *via* inhibition of PI3K/AKT pathway. This inhibition may reduce retinal neovascularization. These findings shed new light on the possibility of incorporating ritonavir in the treatment regimen of ocular angiogenic diseases. Although many inhibitors of HIF-1α are in clinical trials, additional benefit of using ritonavir is that it has been given to HIV patients with relatively low toxicity. The process of traditional drug development could be fast tracked since ritonavir is clinically approved for human use. However, further preclinical and clinical experiments are necessary to determine the repositioning of ritonavir in the treatment of ocular neovascular diseases.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies have examined a dissertation titled “Ritonavir - A Novel Multidrug Resistance Modulator in Cancer Chemotherapy and Ocular Neovascular Diseases” presented by Ramya Krishna Vadlapatla, candidate for the Doctor of Philosophy degree and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

Ashim K. Mitra, Ph.D., Committee Chair

Department of Pharmaceutical Sciences

Kun Cheng, Ph.D.

Department of Pharmaceutical Sciences

Santosh Kumar, Ph.D.

Department of Pharmacology and Toxicology

Nathan A. Oyler, Ph.D.

Department of Chemistry

Jacob Marszalek, Ph.D.

Department of Educational Research and Psychology

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to express my sincere acknowledgment and deepest gratitude to my advisor Dr. Ashim K. Mitra. Dr. Mitra has shown continuous confidence in my candidature and has been very influential in ensuring my professional well-being. He is a great mentor, whose constructive feedback and support always motivated me to focus and excel in my research. I am thankful to Drs. Cheng, Oyler, Kumar and Marszalek for serving on my PhD supervisory committee and for their consistent support and encouragement. It is a great honor to have such outstanding researchers on my PhD supervisory committee. Thanks are due to Dr. Russell Melchert for his constant encouragement and timely support. Also, I would like to thank Dr. Mridul Mukherji for his helpful discussions.

I am extremely grateful to Dr. Dhananjay Pal for his professional and personal support all through my graduate career. I am also very thankful to Mrs. Ranjana Mitra for her cheerful encouragement and help. A special thanks to Drs. Kwatra and Vadlapudi for their constant support, time, fruitful discussions, technical assistance and scientific input into my research. I sincerely thank Anusha Ande, Varun Khurana, Sujay Shah, Ananth Nookala, Dr. Nanda Mandava and Sandeep Putty for making my stay at UMKC a memorable one. My sincere thanks to Joyce Johnson and Sharon Self at UMKC School of Pharmacy; and Connie Mahone, Jennifer Friend, Nancy Hoover, Michelle Heiman and Alphia Curry at School of Graduate Studies for their help at various stages during my graduate career. My sincere gratitude to my close friends - Syamala Tipirneni, Vijetha Kode, Lakshmi Kaza and Vennela Mullangi who has been my tower of strength so many times. Finally, I am very thankful to my parents, my sisters, other family members and friends for their invaluable sacrifices, constant encouragement, moral support and love.

DEDICATED TO MY FAMILY

CHAPTER 1

MECHANISMS OF DRUG RESISTANCE IN CANCER CHEMOTHERAPY: COORDINATED ROLE AND REGULATION OF EFFLUX TRANSPORTERS AND METABOLIZING ENZYMES

Introduction

Cancer remains a major health problem accounting for one in every four deaths in the United States. In 2013, a total of 1.6 million new cases and 0.6 million cancer deaths are predicted to occur [1]. Chemotherapy is one of the major therapeutic interventions in the field of oncology. Chemotherapy can either be administered alone or as an adjuvant therapy depending on the stage of cancer progression. Despite numerous advances and intensive research, a large number of patients acquire multidrug resistance (MDR) and no longer respond to chemotherapy. MDR is a phenomenon where resistance develops to functionally and structurally unrelated drugs. This represents a major obstacle to successful chemotherapy. Resistance, either *acquired* or *inherent*, can be developed *via* multiple pathways. These mechanisms may function independently or synergistically, leading to treatment failure and rise in cancer deaths [2]. Therefore, the focus of this chapter is to discuss the integrated role of efflux transporters (P-glycoprotein, multidrug resistant proteins and breast cancer resistance protein), metabolizing enzymes (cytochrome P450) and nuclear receptors (pregnane X receptor) in development of MDR.

Mechanisms of Cancer Drug Resistance

Several mechanisms of MDR have been suggested, which can be categorized into pharmacokinetic and pharmacodynamic resistance pathways [3]. These mechanisms include i) decreased influx, ii) increased efflux, iii) activation of detoxifying systems, iv) drug

sequestration, v) alteration of specific drug targets, vi) activation of DNA repair and vii) modulation of cellular death pathways and viii) impact of microenvironment (**Figure 1**). The first four mechanisms leading to reduced intratumor drug levels account for pharmacokinetic resistance while changes in microenvironment or biology of the cancer cells (the last four mechanisms) lead to pharmacodynamic resistance.

Decreased Influx

MDR can be mediated by a decrease in drug uptake into target cells. Many drug molecules find their way into the cell by means of passive diffusion, endocytosis or facilitated transport [4, 5]. Notably, the solute carrier family (SLC) comprising of 43 families and 298 transporters is involved in the uptake of several anticancer drugs [6]. Therefore, a decrease in the expression or mutation of these transporters results in drug resistance. For example, methotrexate (antifolate chemotherapeutic) utilizes reduced folate carrier (RFC/SLC19A1) for cellular accumulation. Downregulation and/or mutation of this carrier develops antifolate resistance [7]. Similarly, mutations in human equilibrative nucleoside transporter 1 (ENT1/SLC29A1) and organic cation/carnitine transporter 1 (OCTN1/ SLC22A4) confer sensitivity to gemcitabine and doxorubicin, respectively by reducing cellular uptake [8, 9].

Increased Efflux

A family of ATP-binding cassette (ABC) efflux transporters expels drug molecules out of the cell thereby lowering intracellular drug concentration. This is considered to be one of the most predominant mechanisms for development of MDR. The human genome encodes 48 ABC transporters, organized into seven distinct subfamilies (ABCA-ABCG) based on phylogenetic analysis [10]. The structure, biology and mechanism of action of ABC transporters are thoroughly described in the later sections of this chapter.

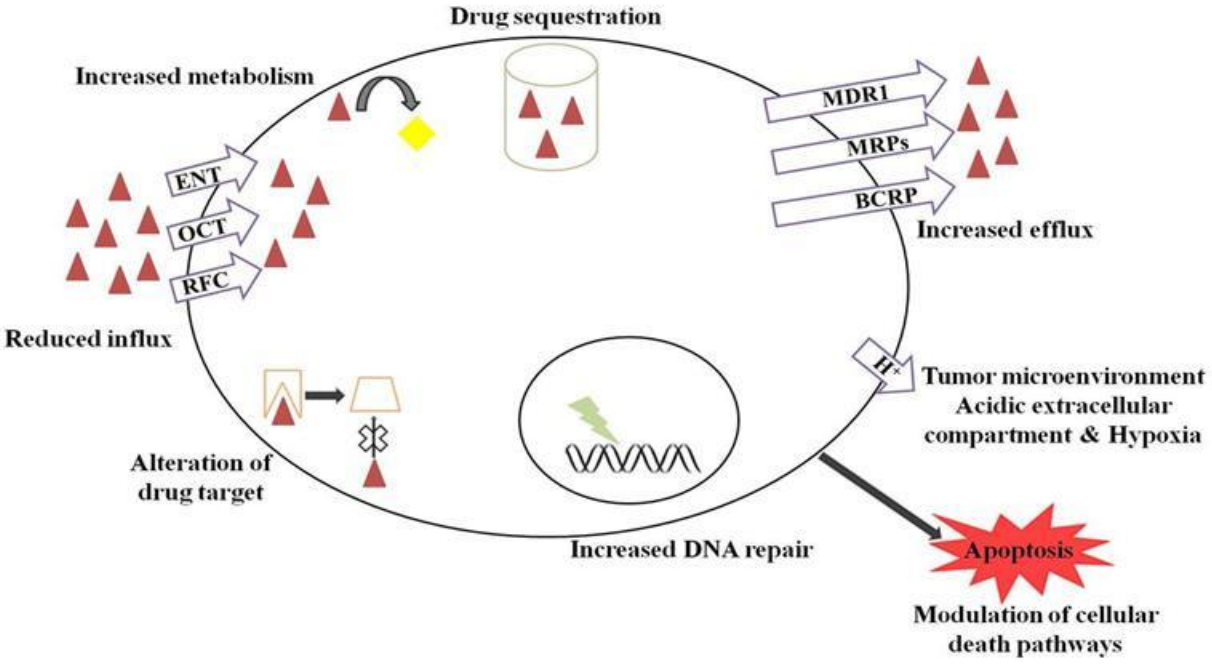

Figure 1. Mechanisms of cancer drug resistance.

Activation of Detoxification

Drug detoxification is considered as the second line for development of drug resistance. Metabolism involves two distinctive pathways - phase I and phase II. Phase I reactions include oxidation, reduction or hydrolysis reactions. These functionalization reactions either introduce a new polar functional group or modify an existing functional group [11, 12]. The key players involved in phase I metabolism are cytochrome P450 enzymes (CYP450). Since CYP450 plays an important role in mediating MDR, more details are described in subsequent sections. Phase II reactions are termed as conjugation reactions and include acetylation, glucuronidation, glutathionylation, methylation and sulfonation reactions [13-15]. These reactions transform the parent drug or phase I metabolite into hydrophilic conjugate and favors excretion. Also, phase II conjugates are actively effluxed *via* ABCC transporters [16]. One such example includes 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan. The glucuronide conjugate of SN-38 is actively effluxed by ABC transporters, enhancing drug clearance in liver [17]. Thus, the synergistic interplay between phase II enzymes and efflux transporters can dramatically decrease chemotherapeutic efficacy [18].

Drug Sequestration

Recent investigations have demonstrated that altered intracellular distribution of chemotherapeutic agents is another important factor involved in MDR. Intracellular drug sequestration as a result of defects in lysosomal and protein trafficking leads to subtherapeutic concentration in the target compartment, leading to treatment failure [19]. Cisplatin is commonly employed in treatment of many malignant tumors. However, development of resistance represents a major obstacle in its therapeutic utility. Cisplatin is extensively sequestered in the vesicular structures of lysosome, golgi and secretory compartments and then effluxed from the

cell [20-23]. Recent investigations have analyzed the expression of an ABC efflux transporter, ABCA3, on limiting membranes of lysosomes and other vesicular bodies. The expression of this transport protein correlated with poor survival rate in patients with acute myeloid leukemia. Intracellular drug sequestration mediated by ABCA3 transporter was reported to develop resistance to chemotherapeutics such as daunorubicin, etoposide, imatinib, mitoxantrone and vincristine [24-27].

Alteration of Drug Targets

Resistance can also be associated with changes in expression of target protein. A classic example of this mechanism is the decrease of DNA topoisomerase (Top) activity [28]. Top I and II play a key role in DNA replication, facilitating relaxation (untangling) and subsequent relegation of intertwined DNA strands. Top I induces single strand breaks while Top II cleaves both strands. Chemotherapeutics stabilize Top-DNA complex interaction, prevent religation, and promote cellular death. Camptothecins act on Top I while anthracyclines and epipodophyllotoxins act on Top II. Use of such drug molecules is limited due to acquisition of resistance *via* reduced expression of the enzyme or mutation thus preventing drug binding [29, 30]. Similarly, resistance to methotrexate acquires *via* elevated expression or mutation of intracellular target, dihydrofolate reductase (DHFR). L22R mutation of DHFR reduced the binding of methotrexate by approximately 270 fold, enabling acquisition of drug resistance [3, 31, 32].

Activation of DNA Repair

Exposure to radiation or use of classical anticancer drugs (including alkylating agents, anthracyclines and platinum based compounds) causes extensive DNA damage leading to cell death. However, activation of DNA repair significantly reduces therapeutic efficacy. These

repair pathways include i) direct reversal, (ii) mismatch repair (MMR), (iii) nucleotide excision repair (NER), (iv) base excision repair (BER), (v) homologous recombination (HR) and (vi) nonhomologous end joining (NHEJ) pathways [33-35]. DNA repair mechanism depends on the type of DNA-drug adduct, tissue location and the participating proteins. The important rate limiting protein of NER pathway is ERCC1. Various *in vitro* studies have demonstrated elevated expression levels of ERCC1 in drug-resistant cell lines [36]. Also, the survival rate of patients receiving cisplatin-based chemotherapy is reported to be higher in ERCC1-negative non-small cell lung tumors compared to ERCC1-positive tumors [37]. Similarly, resistance to alkylating agents was reported to occur by ubiquitously expressed, O^6-methylguanine DNA methyltransferase (MGMT) repair protein. The expression levels of this protein were correlated with resistance to carmustine and temozolamide in glioblastoma. Patients with higher expression of MGMT exhibited treatment failure and increased death rate compared to patients with lower expression levels [38, 39].

Modulation of Cellular Death Pathways

Disruption of cellular death pathways is an important hallmark of cancer. Hence, the principal pharmacological purpose of chemotherapy is to trigger cell cycle arrest or cell death. Any alterations in these pathways appear to have critical impact in mediating drug resistance. Cellular death is mediated by both apoptotic and non-apoptotic mechanisms (autophagy, mitotic catastrophe, necrosis and senescence) [40, 41]. The programmed cell death, apoptosis, is characterized by morphological changes including chromatin condensation, nuclear fragmentation and DNA laddering in the nucleus; formation of apoptotic bodies in the cytoplasm and blebbing of cell membrane. Apoptosis occurs *via* two pathways - *extrinsic* (involving activation of tumor necrosis factor receptor family) and *intrinsic* (involving mitochondria) [42,

43]. A balance between pro- and antiapoptotic proteins governs chemotherapy induced resistance. A relation between Bcl-2 (antiapoptotic protein) expression and response to chemotherapy has been investigated in breast cancer treatment. The study revealed that a complete pathological response was noted in Bcl-2 lacking tumors while tumors with stable levels of Bcl-2 exhibited no response to chemotherapy [44]. Similar chemotherapeutic resistance in acute myeloid leukemia, non-Hodgkins lymphoma and advanced breast cancer was positively correlated with expression levels of Bcl-2 [45, 46].

Impact of Microenvironment

Tumors are characterized by impaired blood supply, abnormal vasculature and decreased lymphatic clearance. Consequently, the availability of nutrients and oxygen is reduced and the clearance of metabolic byproducts is impaired. This leads to formation of hypoxic and acidic regions in tumors [47, 48]. Such heterogeneous tumor microenvironment can trigger a cascade or reactions that influence treatment outcome [49, 50].

Acidic pH: Tumor cells meet their energy requirements through glycolytic pathway rather than oxidative phosphorylation pathway due to lack of oxygen. Lactic acid produced during glycolysis is not cleared due to high interstitial pressure, leading to intracellular acidification. In a process to maintain pH homeostasis, the expression of proton pumps is increased, rendering the extracellular milieu acidic. This extracellular pH influences chemotherapy. Weakly basic chemotherapeutics such as doxorubicin, mitoxantrone, vinblastine and vincristine are ionized at acidic pH, hindering passive cellular uptake [51]. Also, the active uptake of methotrexate is lowered at acidic extracellular conditions [52]. Conversely, the cellular uptake of weakly acidic drugs such as camptothecins, chlorambucil and cyclophosphamide is enhanced at acidic extracellular pH [51, 53].

Hypoxia: Hypoxia, reduced tissue oxygen levels, represents a major hallmark of tumor microenvironment. Apart from efflux transporters and metabolizing enzymes, hypoxia is the other major factor contributing not only to developing MDR but also to intensifying or amplifying resistance acquisition. Tissue response to hypoxia is orchestrated by the transcription factor, hypoxia-inducible factor 1 (HIF-1), which regulates the expression of more than hundred target genes [54, 55]. The target genes play a key role in regulating angiogenesis, cell survival, chemotherapy, genetic instability, immortalization, immune evasion, invasion, metabolism, metastasis, proliferation, radiation resistance and stem cell maintenance [56-63]. For instance, hypoxia induces the expression of Bcl-2 genes rendering resistance to many chemotherapeutic agents. Furthermore, the expression of nucleophiles such as glutathione and metallothionein is upregulated in hypoxic cells. This significantly reduces the treatment outcome of alkylating agents and platinum based compounds [64, 65]. HIF-1 transcription factor also regulates the basal expression of important ABC efflux transporters [66, 67]. Taken together, independent of the mechanism involved, hypoxia is associated with increased patient mortality in many different cancers including bladder, brain, breast, colon, endometrium, head and neck, liver, lung, ovary, pancreas and skin [68-71].

In a nut shell, it appears that several resistance pathways pose a significant barrier to chemotherapy. Although these pathways can act independently, they are more often interconnected. Of the various mechanisms involved, upregulation of efflux transporters and metabolizing enzymes constitute a major resistance phenotype.

ABC Efflux Transporters

Overview

Significant research from the past 30 years has demonstrated the importance of ABC transporters in development of resistance [72, 73]. Efflux transporters are ubiquitous and identified in both prokaryotes and eukaryotes. Phylogenetic analysis categorized ABC proteins into seven subfamilies, ABCA-ABCG, and fifteen of these members have been associated with MDR [74]. ABC efflux transporters provide body's first line of defense against xenobiotics and endogenous metabolites. They constitute a large family of membrane proteins involved in translocation of diverse compounds including amino acids, bile salts, drug molecules, metal ions, metabolites, nucleotides, peptides, sterols and sugars. ABC transporters utilize the energy harnessed from ATP hydrolysis and thus considered an active transport process [75].

A typical ABC transporter consists of two membrane spanning domains (MSDs) and two nucleotide binding domains (NBDs). The structural requirement is met by single polypeptide chain or dimerization of two polypeptide chains. MSDs and NBDs form the backbone of ABC transporter and are required for transporter activity. MSDs exhibit glycosylation sites at specific extracellular domains. Mutagenic studies revealed that these sites are not required for transporter function. However, they are needed for proper expression and membrane insertion [76-78]. The sequence of MSDs is not conserved across different members, the length and framework of each MSD determines ligand binding and substrate specificity. Unlike MSD, the sequence of NBD is conserved [79]. Each NBD contains i) Walker A motif (or P-loop) ii) Walker B motif iii) signature C motif iv) Q-loop v) D-loop vi) A-loop and vii) H-loop. The highly conserved Walker A motif binds ATP and the Walker B motif initiates nucleophilic attack and ATP hydrolysis. Signature C motif (LSGGQ motif) is considered as the hallmark of ABC transporters and helps in communicating MSDs. Q-loop mediates interactions between MSD and NBD while D-loop communicates the two NBDs. A-loop and H-loop contribute to the ATP binding and catalytic

cycle [80-83]. The structure of efflux transporters is depicted in **Figure 2** and important genomic characteristics are summarized in **Table 1**. Efflux transporters expel drug molecules out of the cell thereby reducing intracellular drug concentration leading to acquisition of drug resistance. A partial list of chemotherapeutics effluxed by ABC transporters is given in **Table 2**.

P-Glycoprotein (P-gp/MDR1/ABCB1)

Structure: P-gp was the first member of ABC superfamily to be studied. This surface glycoprotein was initially identified in chinese hamster ovary cells and found to modulate drug permeability of a wide range of amphiphilic drugs [84]. P-gp shares high sequence identity (78%) with another isoform of ABCB family (MDR2/3/ABCB4) involved in exporting phosphatidylcholine into bile. Structurally, P-gp comprises two MSDs and two NBDs. Each MSD comprises six hydrophobic transmembrane domains (TMDs). P-gp is a single polypeptide chain with two 2 homologous halves (each of 610 amino acids) linked by 60 amino acid region.

Tissue localization: P-gp is expressed in almost all the tissues. However, abundant expression is found at apical surface of small intestine, pancreatic ducts, bile ducts, kidney proximal tubules, and adrenal glands. Moderate expression is found in hepatocytes, spleen and lung while low expression is observed in prostate, ovary and skin. This localization suggests that P-gp plays a physiological role in protection of many tissues. Further, the protective role is also evident from its expression at blood-brain, blood-testis and blood-mammary tissue barriers [85-87]. Overexpression of P-gp leading to drug resistance has been reported in many tumor cells including breast, colon, gastric, kidney, leukemic, liver and pancreatic cells [88].

Structure-activity relationship (SAR): P-gp interacts with structurally diverse substrates. Although majority of them are amphipathic and relatively hydrophobic, few of them are water soluble. Extensive research has been conducted to predict SAR for P-gp substrates or inhibitors.

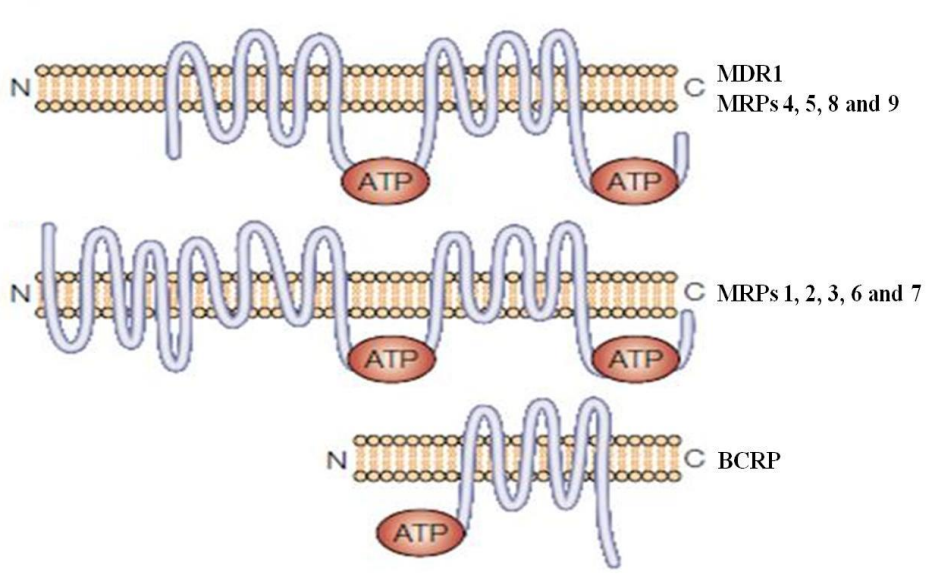

Figure 2. Structure of ABC transporters. Reproduced with permission from reference [73].

Table 1. Summary of genomic properties and tissue localization of ABC efflux transporters.

Member	Chromosomal location	Transcript length (bp)	Amino acids	Mol. weight (Da)	Exons	Tissue localization
P-gp/ MDR1/ ABCB1	7q21.12	4872	1280	141,463	28	Adrenal, blood-brain barrier, intestine, kidney, liver, placenta *Apical*
MRP1/ ABCC1	16p13.12	5927	1531	171,561	31	Ubiquitous *Basolateral*
MRP2/ cMOAT/ ABCC2	10q24.2	4930	1545	174,191	32	Colon, gall bladder, intestine, kidney, liver, lung, placenta *Apical*
MRP3/ MOAT-D/ ABCC3	17q21.33	5176	1527	169,343	31	Adrenal gland, gall bladder, gut, intestine, kidney, liver, pancreas, placenta, prostate *Basolateral*
MRP4/ MOAT-B/ ABCC4	13q32.1	5871	1325	149,541	31	Adrenal glands, lung, intestine, ovary, pancreas, prostate, testis *Apical and basolateral*
MRP5/ MOAT-C/ ABCC5	3q27.1	5851	1437	160,660	30	Ubiquitous *Apical and basolateral*
MRP6/ MOAT-E/ ABCC6	16p13.12	5111	1503	164,904	31	Kidney, liver, tracheal epithelium *Basolateral*
MRP7/ ABCC10	6p21.1	5118	1464	161,629	22	Low in all tissues *Basolateral*
MRP8/ ABCC11	16q12.1	4576	1382	154,301	29	Low in all tissues *Apical and basolateral*
MRP9/ ABCC12	16q12.1	5168	1359	152,244	29	Low in all tissues *?*
BCRP/ MXR/ ABCG2	4q22	4445	655	72,314	16	Breast, intestine, liver, placenta *Apical*

Table 2. Chemotherapeutic substrates of ABC efflux transporters.

Efflux transporter	Chemotherapeutic substrates
MDR1	Bisantrene, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, idarubicin, methotrexate, mitoxantrone, paclitaxel, teniposide, vinblastine, vincristine
MRP1	Daunorubicin, doxorubicin, epirubicin, etoposide, imatinib, melphalan, methotrexate, mitoxantrone, paclitaxel, vinblastine, vincristine
MRP2	Cisplatin, doxorubicin, etoposide, irinotecan, methotrexate, SN-38, vinblastine, vincristine
MRP3	Carboplatin, cisplatin, etoposide, methotrexate, teniposide
MRP4	Methotrexate, topotecan
MRP5	5-Fluorouracil, methotrexate
MRP6	Cisplatin, etoposide, teniposide
MRP7	Docetaxel, paclitaxel, vinblastine, vincristine
MRP8	5-Fluorouracil
BCRP	Bisantrene, daunorubicin, doxorubicin, epirubicin, etoposide, gefitinib, imatinib, irinotecan, methotrexate, mitoxantrone, SN-38, teniposide, topotecan

Seelig reported a general pattern for substrate recognition by P-gp by comparing more than hundred known substrates. Two types of recognition elements have been described. Type I group exhibits two electron donor groups with a spatial separation of 2.5 ± 0.3 Å while type II group exhibits two or three electron donor groups with a spatial separation of 4.6 ± 0.6 Å. Based on the type and number of groups, various molecules can be predicted as strong substrate, weak substrate or non-substrate [89]. A more recent pharmacophore hypothesis for P-gp substrate recognition was proposed by studying 129 diverse compounds. This approach suggested that the interaction of substrate with one or more binding sites plays a key role in transport. According to this three dimensional model, the minimal recognition elements for transport by P-gp are dimensions of the molecule, two hydrophobic groups 16.5 Å apart and two hydrogen bond acceptor groups 11.5 Å apart [90]. Another pharmacophore model proposes a pattern consisting of two hydrophobic units, three hydrogen bond acceptor units and one hydrogen bond donor [91]. These SAR methods can be a promising approach to study P-gp substrates and modulators [92].

Transport cycle: P-gp is considered as the archetype of ABC efflux transporters; hence the mechanism of substrate translocation has been highly investigated. The transport mechanism involves two interconnected cycles i) catalytic cycle involving ATP hydrolysis and ii) transport cycle involving translocation of the substrate. However, the molecular details still remain enigmatic [93, 94]. Two alternative models have been proposed describing the transport cycle of P-gp. The key difference in both these models is the nature of energy that expels the substrate. The first model proposed by Higgins and Linton (ATP switch model) hypothesizes ATP binding as the power stroke for translocation [95]. This model postulates the following steps. The transport cycle begins with binding of substrate to high-affinity site on TMD. This binding

increases affinity for ATP, reduces activation energy and results in dimerization of two NBDs. Subsequent conformational changes releases the bound substrate. Two sequential ATP hydrolysis events reset P-gp to the ground state. The second model described by Sauna and Ambudkar proposes ATP hydrolysis as the key step for translocation [96]. The second model proposes the flowing steps. The transport cycle begins with binding of both substrate and ATP simultaneously to the transporter. The ATP molecule is hydrolyzed resulting in conformational change of the transporter and release of bound substrate. A second ATP hydrolysis then resets the ground state of the molecule. The essential steps of two models are illustrated in **Figure 3**.

Multidrug Resistant Proteins (MRPs/ABCC)

Structure: ABCC represents the largest branch of ABC transporters. This family includes nine efflux transporters (MRP1-6/ABCC1-6 and MRP7-9/ABCC10-13), one gated chloride channel (cystic fibrosis transmembrane conductance regulator/CFTR/ABCC7) and two potassium channel regulators (sulfonylurea receptor1-2/SUR1-2/ABCC8-9) [97-100]. The founding member of this family, MRP1, was cloned by Cole et al., in doxorubicin-resistant lung cancer cells [101]. MRP2 was isolated from cisplatin resistant head and neck cancer cells [102]. Subsequent studies have identified additional members of this family. Although the molecular and functional mechanisms may vary, all members are structurally similar and ATP-dependent. Computational analyses, hydropathy profiles and sequence alignments have predicted the structure of MRPs. MRPs 4, 5, 8 and 9 are described as short MRPs with typical ABC structure comprising two MSDs and two NBDs. MRPs 1, 2, 3, 6 and 7 are described as long MRPs with an additional hydrophobic amino terminal domain (MSD0) of approximately 200 amino acids [103, 104]. In contrast to the intracellular amino terminus in P-gp and short MRPs; the long MRPs exhibit extracellular amino terminus [105, 106].

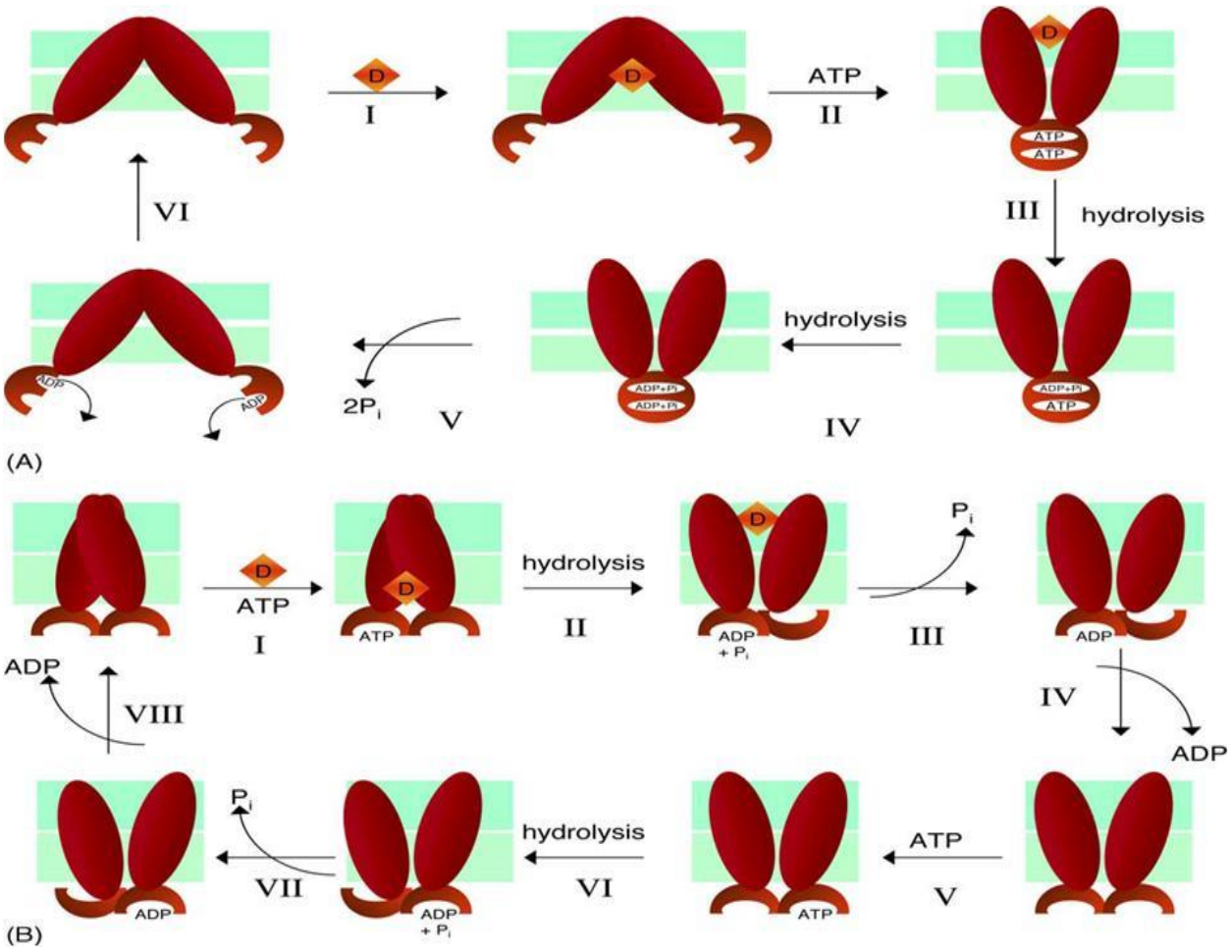

Figure 3. The transport cycle of P-glycoprotein. (A) ATP switch model proposed by Higgins and Linton (B) Alternate model proposed by Sauna and Ambudkar. Reproduced with permission from reference [94].

Tissue localization: MRP1 is ubiquitously found in a number of human cells. Higher expression is noticed in lung, testis, kidney and macrophages. MRP1 plays a protective role and is localized in the basolateral membrane of blood-testis barrier, blood-cerebrospinal fluid barrier and bronchial epithelium [107-109]. MRP2 is predominantly present in apical membrane of polarized cells including colon, gall bladder, intestine, kidney, liver, lung and placenta [110-114]. MRP1 and MRP2 are the most studied ABCC transporters believed to mediate MDR in cancer. Upregulation of MRP1 is correlated with poor patient outcome in retinoblastoma and neuroblastoma [115, 116]. Elevated levels of MRP1 have also been reported in breast, lung, and acute myeloid leukemic and prostate tumor cells [117, 118]. Similarly, overexpression of MRP2 is observed in breast, colorectal, hepatic, leukemic, lung and ovarian cancers [119-123].

Breast Cancer Resistant Protein (BCRP/MXR/ABCP/ABCG2)

Structure: The ABCG family includes six half-transporters and the second member of this family (ABCG2) is involved in mediating MDR [10]. Nakagawa et al., identified a distinct pathway mediating resistance in mitoxantrone resistant (MXR) human breast cancer cells [124]. However, the protein was first cloned in drug resistant human breast cancer cells, hence referred as BCRP [125]. Shortly after this cloning, a new human ABC transporter highly expressed in the placenta (termed as ABCP) was reported [126]. All these designations mapped the same transporter. BCRP is organized into two structural units comprising one NBD and one MSD. Since a functional ABC transporter requires a minimum of two NBDs and two MSDs, BCRP homodimerizes in a reverse orientation for transport activity [127, 128]. The dimerization of BCRP has been proposed to occur *via* disulfide bridge formed by cysteine moieties (Cys_{603}) located in the extracellular loop between TMDs 5 and 6 [129].

Tissue localization: BCRP is highly expressed on apical membranes of biliary canaliculi, brain capillary endothelial cells, colon, placenta, prostate, small intestine, testis and uterus [130, 131]. The pharmacological importance of BCRP in mediating MDR is evident by its expression in melanoma and tumors of digestive tract, endometrium, esophageal and lung cancers [132-134].

CYP450 Metabolizing Enzymes

Overview

Among the various enzyme systems involved in drug metabolism, CYP450 includes the most important constitutive and inducible superfamily (**Figure 4**). CYPs are heme containing monooxygenases and the number 450 indicates absorption maximum of the enzyme in reduced carbon monoxide bound form. To date, more than 57 active human P450 genes belonging to 17 distinct families have been described [135]. Each member consists of atleast two protein components (heme-protein and flavoprotein) and a lipid component (phophatidylcholine). The catalytic cycle of CYP450 utilizes molecular oxygen to oxidize a substrate with the release of a water molecule and utilizing electrons from NADPH. The cycle includes the following steps i) substrate binds to the resting ferric state of the enzyme, ii) an electron is transferred from NADPH-CYP reductase to the ferric enzyme resulting in formation of ferrous enzyme-substrate complex, iii) oxygen binding leads to an oxy-P450 complex, iv) a second electron is added resulting in formation of iron peroxo species, v) oxygen-oxygen bond is cleaved to form an iron oxo intermediate with release of water and finally vi) oxygen from iron oxo intermediate is transferred to the bound substrate, with release of product and regeneration of ground state of CYP [136, 137]. While the CYP450s are mainly localized in the liver, extrahepatic localization is also noted in brain, colon, esophagus, kidneys, lungs, nasal, respiratory and olfactory mucosa,

small intestine, stomach, and trachea [138-141]. P450s can be categorized into i) drug metabolizing enzymes (DMEs) mostly involving CYP families 1-4 (**Table 3**) and ii) enzymes involved in metabolism and synthesis of endogenous substrates (fatty acids, prostaglandins, steroids, vitamins) involving CYP11, 17 and 19 families [142-144].

CYP3A4

Among the different xenobiotic metabolizing enzymes, CYP3A 4 (family 3, subfamily A, member 4) accounts for metabolism of more than 50% of the marketed drugs [145]. Although CYP3A4 plays an important role in detoxification of many chemotherapeutic agents, certain prodrugs require bioactivation by this enzyme for therapeutic activity [146]. CYP3A4 is expressed in many tumors including breast, colon, esophagus, lung, ovarian and soft tissue sarcomas [143]. A higher CYP3A4 activity would generally result in increased detoxification of the drug and subsequent development of MDR. For example, patients with lower expression of CYP3A4 showed a higher response rate to docetaxel in treatment of breast tumors [147]. Similarly, higher expression of CYP3A4 is associated with metastasis and poor prognosis in osteosarcoma patients [148].

Combined Action and Regulation of Efflux and Metabolism

Efflux transporters and phase I DMEs orchestrate a defensive system against various chemotherapeutics. Synergistic action leading to MDR could possibly arise due to two different factors - overlapping substrate specificity and coordinated regulation of their expression. Oncolytic drugs such as docetaxel, doxorubicin, etoposide, imatinib, paclitaxel, teniposide, vinblastine and vincristine are substrates for both P-gp and CYP3A4. This close overlap of substrates and tissue distribution may lead to development of MDR [149, 150]. Also, their inducible expression is governed by molecular mechanisms involving nuclear receptors (NRs).

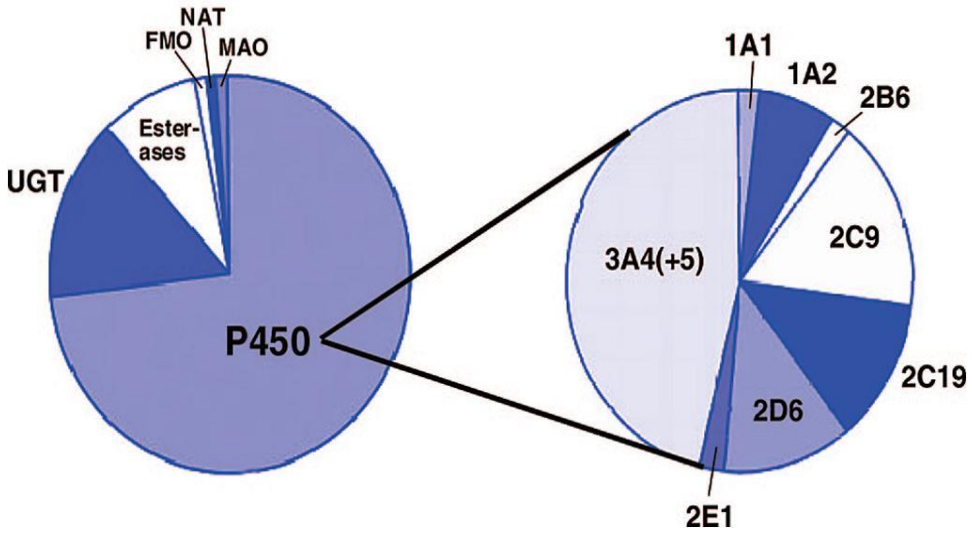

Figure 4. Contribution of enzyme systems and individual P450s in metabolism of marketed drugs. Reproduced with permission from reference [11].

Table 3. Chemotherapeutic substrates of CYP450 enzymes.

CYP450 enzyme	Chemotherapeutic substrates
1A1	Docetaxel, erlotinib, tamoxifen
1A2	Erlotinib, etoposide, flutamide, imatinib, tamoxifen
2B6	Cyclophosphamide, ifosfamide, tamoxifen
2C9	Cyclophosphamide, ifosfamide, imatinib, tamoxifen
2C19	Cyclophosphamide, ifosfamide, imatinib, tamoxifen, teniposide
2D6	Imatinib, tamoxifen, vinorelbine
2E1	Cisplatin, etoposide, tamoxifen, vinorelbine
3A4/5	Cisplatin, cyclophosphamide, cytarabine, dexamethasone, docetaxel, doxorubicin, erlotinib, etoposide, exemestane, flutamide, fulvestrant, gefitinib, ifosfamide, imatinib, irinotecan, letrozole, medroxyprogresterone acetate, mitoxantrone, paclitaxel, tamoxifen, targretin, teniposide, topotecan, vinblastine, vincristine

Certain chemotherapeutics can activate NRs, leading to increased expression of both efflux transporters and metabolizing enzymes [18, 151]. This untoward activation can lead to decreased oral uptake in the intestine, increased excretion in the liver and decreased concentrations at the tumor site **Figure 5**. These factors substantially increase the severity of drug resistance. NRs include a superfamily of transcription factors with more than 70 distinct members [152]. The characteristic structural features are shared in most of the NRs, exhibiting a highly conserved central DNA binding domain (DBD) and a ligand binding domain (LBD) at the C-terminal. Two transactivating domains, activation function 1 and 2 (AF-1 and 2) are located at the N- and C- terminus, respectively [153]. These domains help in recruiting various transcriptional coactivators. While AF-1 allows ligand-independent regulation, AF-2 acts in a ligand-dependent manner [153]. NRs are categorized into two different subclasses, ligand-dependent and orphan NRs, depending on their ligand-binding requirement. The regulatory ligands have not been identified for orphan NRs. Pregnane X receptor (PXR) and constitutive androstane/activated receptor (CAR) are the master orphan NRs involved in xenobiotic metabolism and elimination [154, 155]. The pharmacological action of PXR and its potential role in mediating MDR to cancer chemotherapy has been extensively investigated.

Pregnane X Receptor (PXR)

PXR (the gene product of NR1I2) was cloned in 1998 by three independent groups [156-158]. This NR was initially found to be activated by endogenous pregnanes, which gave rise to its name. PXR is considered as a ‘molecular sentinel’ localized to both cytoplasm and nucleus. In cytoplasm, PXR is sequestered by heat shock protein 90 (Hsp90) or cytoplasmic CAR retention protein (CCRP) [159]. Following ligand binding, PXR dissociates and translocates to nucleus *via* nuclear localization signal (NLS) located with residues 66 to 92 of DBD [160].

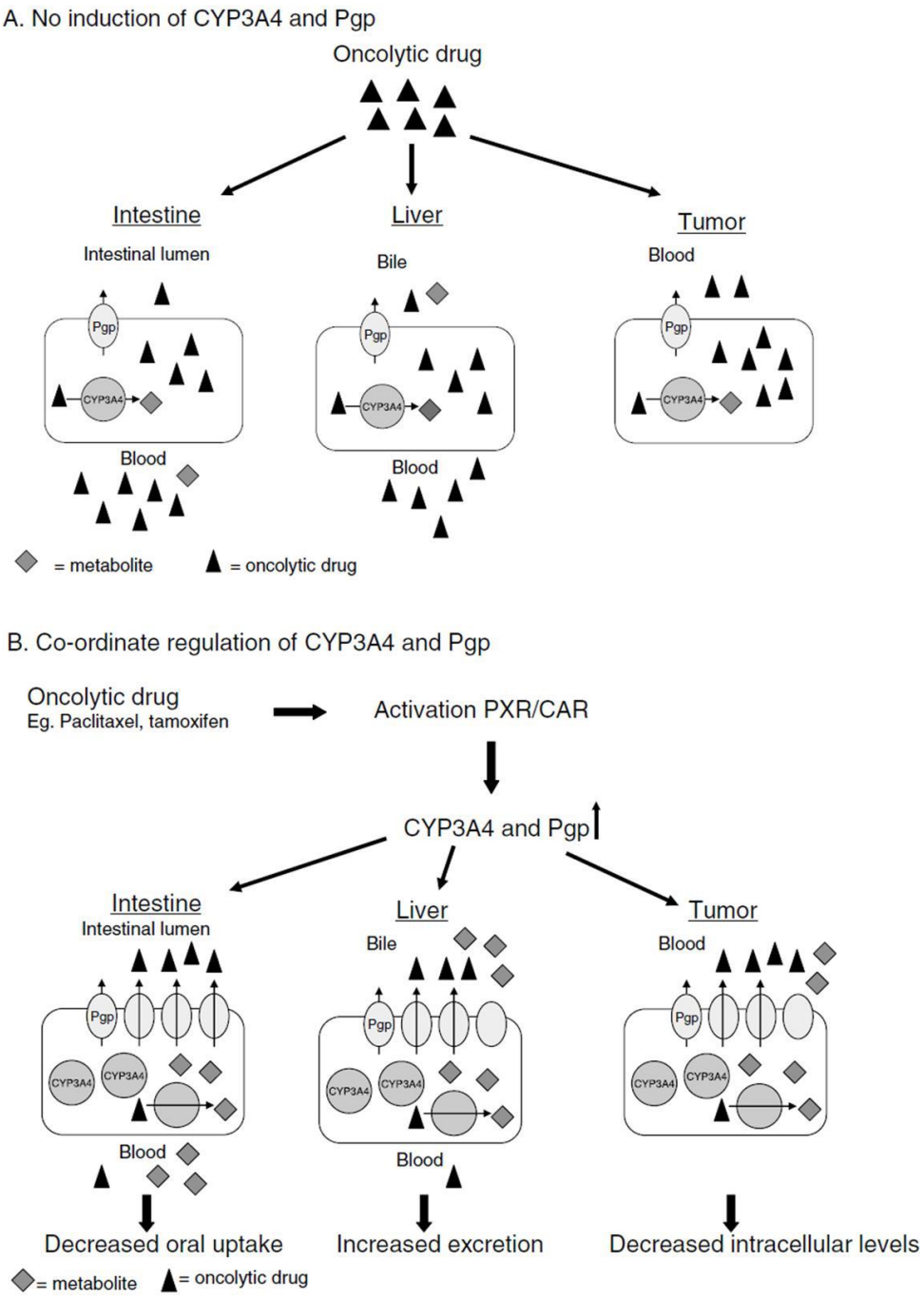

Figure 5. Role of nuclear receptors in mediating drug resistance *via* upregulation of efflux transporters and metabolizing enzymes. Reproduced with permission from reference [18].

In nucleus, it forms a heterodimeric complex with 9-cis retinoic acid receptor/retinoid X receptor (RXR) [159]. Various transcriptional coactivators including steroid receptor coactivator 1 (SRC1), glucocorticoid receptor interacting protein 1 (GRIP1), activator for thyroid hormone and retinoid receptors (ACTR), and peroxisome proliferator activated receptor coactivator 1-alpha (PGC1α) are recruited [161-163]. Subsequently, PXR promotes the transcription of various target genes by binding to (A/G)G(T/G)TCA in a sequence-specific manner [164]. The target genes include Phase I DMEs (CYPs, carboxylesterases, aldehyde and alcohol dehydrogenases), Phase II DMEs (glucoronosyltransferases, glutathione-S-transferases and sulfotransferases) and ABC efflux transporters (MDR1, MRPs and BCRP) [165-169] (**Figure 6**).

Tissue localization: PXR is highly expressed in liver, intestine and kidney. Other tissues including adrenal gland, blood-brain barrier, breast, bone marrow, colon, heart, osteoclasts, ovary, placenta, peripheral blood monocytes, stomach and uterus also express PXR [158, 170-174]. The expression of PXR has also been detected in breast, colon, endometrial, osteosarcoma, ovarian, and prostate cancers [175-180].

MDR Modulators: Potential Strategy for Overcoming MDR

A potential approach to overcome MDR is to administer compounds that would inhibit ABC transporters at the substrate binding sites. These compounds are referred as MDR inhibitors/modulators/reversal agents or chemosensitizers. They may modulate the activity of more than one ABC transporter. The modulators are categorized into first, second and third generations. First generation modulators include verapamil, cyclosporine A and erythromycin. These modulators exhibited low affinity towards ABC transporters, thus necessitating the use of high dose which often lead to undesirable side effects and toxicity. The second generation modulators such as dexverapamil and valspodar were developed and found to be potent.

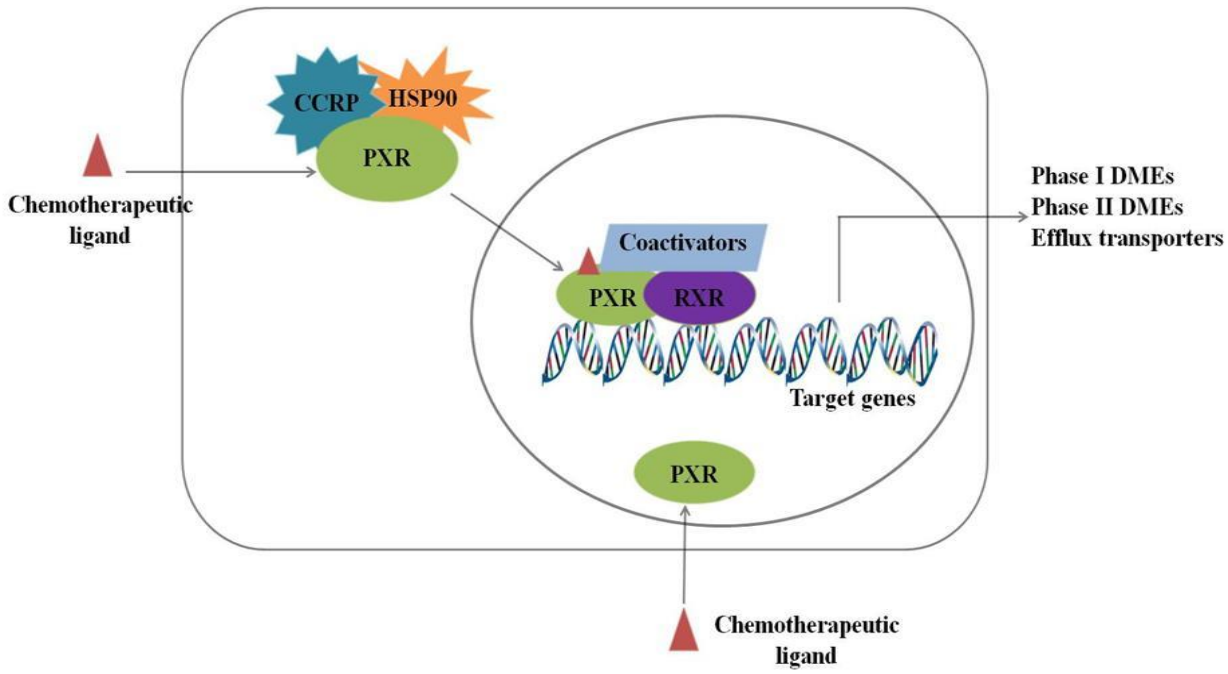

Figure 6. Mechanism of action of pregnane X receptor (PXR).

However, the inhibition of CYP450-mediated drug metabolism resulted in unpredictable pharmacokinetics of coadministered drugs, often raising their systemic toxicity. Third generation modulators such as tariquidar and zosuquidar are designed with the aid of combinatorial chemistry to overcome the limitations of the other generation MDR modulators. Clinical trials demonstrated no encouraging results so far [181, 182].

The small molecule tyrosine kinase inhibitor - cabozantinib, has entered into a phase III clinical trial for the treatment of hepatocellular carcinoma. Xiang Q et al., assessed the effect of this molecule on inhibiting MDR. Results demonstrated that cabozantinib increased the inhibitory effect of doxorubicin in xenograft nude mice model *via* inhibition of P-gp [183]. A similar study was performed by Liu KJ and colleagues using saracatinib (AZD0530), a highly potent dual kinase inhibitor, currently under investigation for the treatment of ovarian cancer. Their studies have concluded that this molecule reverses P-gp mediated drug resistance in cancer and enhances the chemotherapeutic efficacy of paclitaxel in mice xenografts [184]. All these studies suggest that efflux transporter inhibition may serve as a viable therapeutic approach for overcoming MDR in cancer.

Also, many anticancer drugs currently used in clinical setting can activate PXR. The concept of downregulating PXR to sensitize cancer cells to anticancer agents has been thoroughly investigated. In a study by Chen et al., expression of PXR in normal and cancerous prostate cancer (PC-3) cells has been examined [185]. PC-3 cells treated with PXR agonist SR12813 elevated MDR1 expression, enabling PC-3 cells resistant to paclitaxel and vinblastine. Knock-down of PXR with short hairpin RNA (shRNA) augmented sensitivity of PC-3 cells to anticancer drugs. Results from this investigation suggest that blockade of PXR activity enhances the effectiveness of anticancer drugs, particularly in PXR-positive cancers.

In a similar study, downregulation of PXR by small interfering RNA (siRNA) in HEC-1, an endometrial cancer cell line, reduced MDR1 expression and sensitized cells to anticancer agents such as paclitaxel and cisplatin [186]. Similarly, activation of PXR reduced the effectiveness of etoposide in treatment of osteosarcoma [179]. All these observations suggest that downregulation of PXR could be a novel therapeutic intervention to augment sensitivity of anticancer drugs and/or overcome resistance. PXR mediated chemoresistance may be surpassed with the use of appropriate antagonists (**Figure 7**) [154].

Ecteinascidin-743 (ET-743), a novel antineoplastic agent inhibits the proliferation of various cancer cells and human xenografts with half-maximal inhibitory (IC_{50}) concentrations ranging from 1 to 100 nM. This compound was one of the first PXR antagonists to be reported. Dose-dependent inhibition studies on transient transfected cells with SXR revealed that ET-743 inhibited PXR at nanomolar concentrations, with IC_{50} of approximately 3 nM [187]. Takeshita et al., reported inhibition of steroid-induced PXR mediated transcription of CYP3A4 by ketoconazole [188]. The exact mechanism of this inhibition was subsequently demonstrated by Huang et al [189]. Ketoconazole disrupted the interaction of PXR with coactivator SRC1, resulting in decreased activation of this NR and delayed metabolism of tribromoethanol in mice. A-792611, a novel HIV protease inhibitor exhibited downregulation of constitutive CYP3A4 expression. Also, this compound attenuated activation of PXR and resulted in decreased expression of PXR target genes in human hepatocytes. Cell based transactivation studies demonstrated that A-792611 inhibits activity of human PXR but not other NRs (such as CAR, farnesoid X receptor, vitamin D receptor, and peroxisome proliferator-activated receptor alpha) that regulate CYP3A4. Such unique property suggests that this compound may be used to prevent PXR mediated drug resistance [190].

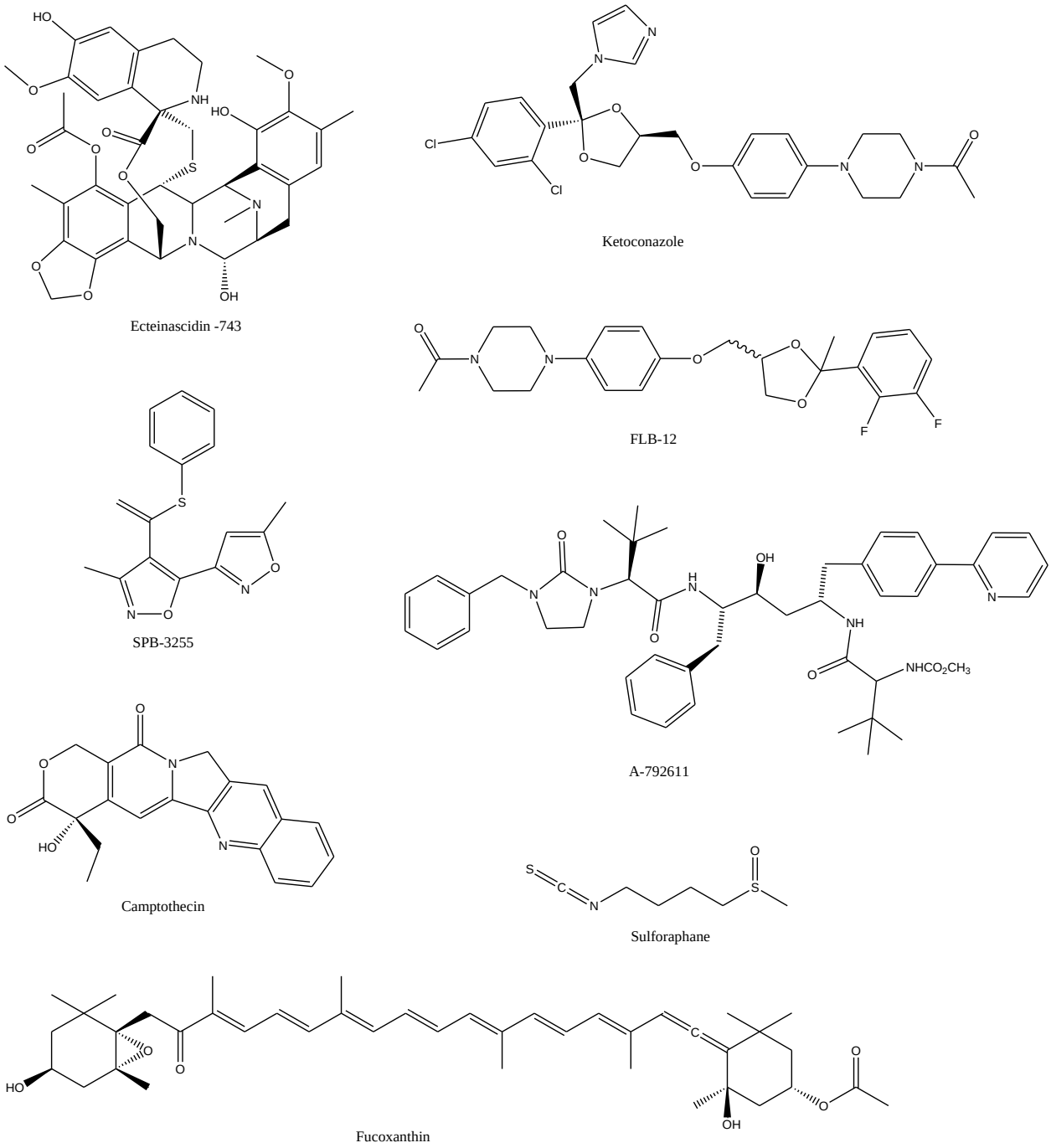

Figure 7. Structure of PXR antagonists.

Sulforaphane (SFN), a biologically active phytochemical, is an inhibitor of histone deacetylases and an inducer of phase II DMEs such as glutathione S-transferases. SFN significantly downregulates CYP3A4 expression in human primary hepatocytes. Cell based assays demonstrated that SFN is a PXR antagonist and inhibits PXR mediated augmentation of drug clearance. SFN can bind directly to PXR, inhibit coactivator recruitment, and efficiently prevent PXR activity [191]. PXR activation reduced the chemosensitivity of colorectal cancer cells to irinotecan. However, SFN reversed this effect by increasing the chemosensitivity of anticancer drug [192]. Camptothecin, a Top I inhibitor, inhibits PXR mediated transcriptional activation of CYP3A4. This compound acts by disrupting PXR interaction with SRC1 and do not compete with the agonist for binding to PXR. However, the effect of camptothecin is not particularly specific for PXR, since this compound also inhibits CAR mediated transactivation [193].

Fucoxanthin, a major non-provitamin A carotenoid, is a putative chemopreventive agent. Recently Lu et al. examined if fucoxanthin could overcome drug resistance through downregulation of PXR mediated pathways in hepatoma (HepG2) and colon adenocarcinoma (LS174T) cells. Fucoxanthin significantly decreased rifampin induced CYP3A4, MDR1 mRNA and CYP3A4 protein expression levels. PXR mediated CYP3A4 promoter activity was strongly attenuated with this compound in HepG2 cells. Also, CAR and rPXR mediated CYP3A4 promoter activity was attenuated in the same cell line. Mammalian two-hybrid assay revealed that this compound inhibited the interaction between PXR and SRC1. These interesting results could lead to development of new therapeutic and dietary approaches to lessen the incidence of adverse drug reactions [194].

Several attempts have been made to identify chemical scaffolds essential for PXR antagonist activity. The impact of functional groups on azole scaffold for PXR antagonist activity was studied. Fifteen analogs of ketoconazole were designed and SAR was predicted by studying dose-dependent inhibition of PXR mediated transcription in transfected cells. This study has identified that the two aromatic rings on ketoconazole and the five membered ring joining them are essential. The analogs lacking these functional groups did not exhibit any PXR antagonistic activity. SAR study facilitated identification of a new analog of ketoconazole, FLB-12, with lower IC_{50} value for PXR antagonism. FLB-12 was significantly less toxic than ketoconazole in all cell lines tested [195]. Also, FLB-12 antagonized PXR mediated SN-38 drug resistance in colon cancer cells [196]. These studies demonstrated the potential for developing PXR antagonists in overcoming MDR. A second scaffold for PXR antagonism was identified by screening several analogs of SPB-3255. The promising lead was SPB-3255 itself with an IC_{50} value of 850 nM compared to its analogs which exhibited PXR antagonism in the range of 1.5-16 μM. Two adjacent aromatic rings along with a second aromatic group connected by a two-atom linker were considered to be critical for PXR antagonistic activity [197, 198]. High throughput studies identified coumestrol, a phytoestrogen as a PXR antagonist with an IC_{50} value of 11 μM. These studies suggested that hydrogen bond donors are an important pharmacophore in PXR antagonist activity. Analogs devoid of hydroxyl group at the compound's termini were found to be inactive [199]. It is clearly evident that SAR studies may enable accelerated discovery of PXR antagonists with implications in attenuating drug resistance in cancer cells.

Conclusions and Future Perspectives

Drug resistance entails several mechanisms and different targets, thus impeding the possibility of overcoming drug resistance by targeting one protein. Efflux transporters and

metabolizing enzymes play a predominant role in drug disposition and acquisition of resistance. The use of MDR modulators demonstrated limited success due to toxicity issues. With increasing reports, it is clearly evident that the expression of efflux transporters and metabolizing enzymes is regulated by PXR, an orphan xenobiotic receptor. This striking evidence enabled discovery of PXR antagonists which can prevent drug mediated overexpression of efflux transporters and metabolizing enzymes. PXR inhibitors or antagonists currently under investigation possess activities other than inhibiting PXR. Future studies are warranted to identify specific PXR antagonists that do not interfere with the basal levels of PXR and may cause minimal/no side effects. It may be difficult for pharmaceutical scientists to design novel PXR antagonists owing to the ligand promiscuity, complexity of biology and lack of proper understanding. Better understanding of antagonist or allosteric sites on PXR that could modulate activity and application of computational approaches could lead to development of new molecules that could selectively fit into these sites. SAR studies amalgamated with high throughput screening studies will provide an effective and successful approach to identify and develop novel non-toxic, specific and potent PXR antagonists. Successful development of PXR antagonists may selectively target neoplastic cells or disrupt undesirable PXR mediated upregulation of drug metabolism. Such specific PXR antagonists may have wide applications in overcoming drug resistance including treatment of several cancers.

CHAPTER 2

DIFFERENTIAL EFFECT OF MDR1 AND MRP2 ON CELLULAR TRANSLOCATION OF GEMIFLOXACIN

Rationale

Fluoroquinolones are broad spectrum antibiotics widely indicated in both human and animal diseases. These compounds demonstrate excellent bactericidal activity and concentration-dependent killing effect [200-203]. Nalidixic acid and oxolinic acid were the earliest quinolone antibacterial drugs to be introduced. Emergence of resistance coupled with undesirable side effects has driven researchers to develop newer analogs, leading to the discovery of second, third and fourth generation fluoroquinolones. These agents have been widely indicated in the treatment of bacterial diseases including systemic infections [204-207]. The newer fluoroquinolone, gemifloxacin was approved by the United States Food and Drug Administration (US FDA) in 2003. It is recommended for the treatment of mild to moderate community acquired pneumonia (CAP), acute bacterial exacerbations of chronic bronchitis (ABECB) and urinary tract infections [208, 209].

The structure of gemifloxacin is depicted in **Figure 8**. It is a fluoronaphthyridine molecule having a side chain with cyclopropanyl group at position-1 and 3-aminomethyl-4-syn-methoxyimino-1-pyrrolidinyl substituent at C-7 position. Substitution of the carbon at C-8 position with nitrogen enhanced antimicrobial activity. Gemifloxacin acts by forming a ternary complex with both DNA gyrase and topoisomerase IV. Formation of this complex is responsible for the blockade of DNA replication and transcription, resulting in chromosomal disruption and bacterial cell death [205, 208]. Most fluoroquinolones acquire resistance due to two chromosomal mutations in the quinolone resistance determining regions (QRDR).

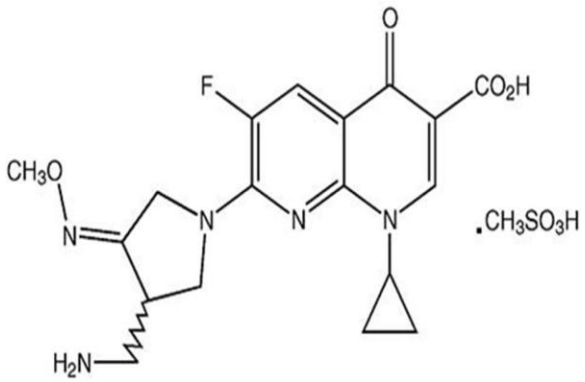

Figure 8. Structure of gemifloxacin mesylate.

Gemifloxacin has often shown retained activity despite two-step mutations in the QRDR [210, 211]. Unlike ciprofloxacin and levofloxacin, reduced photosensitivity and phototoxicity was observed with gemifloxacin [212-215]. Gemifloxacin exhibits a broad spectrum of activity against gram-positive and gram-negative bacteria [216-219]. The compound showed excellent antimicrobial activity with low minimum inhibitory concentrations (MIC_{90}) of 0.03, 0.06 and 0.008 µg/mL against *Streptococcus pneumoniae*, *S. pyrogenes* and *Haemophilus influenzae*, respectively [220-224]. Potent antibacterial activity against clinical isolates and reference strains was observed with gemifloxacin in both *in vitro* as well as *in vivo* infectious animal models [209, 217, 225, 226]. Upon oral administration, gemifloxacin is rapidly absorbed with peak concentration reaching within 0.5-2 hours. The absolute bioavailability (71%) was found to be lower than that of gatifloxacin (96%) and levofloxacin (99%) [201, 227]. This limitation could be due to efflux of fluoroquinolones by ATP-binding cassette (ABC) transporters [204].

ABC transporters i.e. P-glycoprotein (P-gp/MDR1), multidrug resistance protein 2 (MRP2) and breast cancer resistant protein (BCRP) are responsible for the efflux of several drugs, altering their absorption, distribution and excretion [228-232]. These efflux transporters are one of the leading membrane bound protein families in both prokaryotes and eukaryotes. MDR1, a 170 kDa transmembrane protein, is expressed on the apical membrane of many epithelial and endothelial cells. It acts as a biological barrier by extruding toxins and xenobiotics into extracellular environment [233]. MRP family consists of 190 kDa proteins responsible for the transport of drugs across lipid membranes. These proteins are similar to MDR1 with regard to function and localization, but may differ in substrate specificity. These efflux pumps derive their energy from ATP hydrolysis and expel antimicrobial drugs out of cell, thus reducing intracellular drug accumulation. This process may eventually lead to suboptimal eradication of

microorganisms. Interaction of gemifloxacin with efflux transporters in short term could possibly reduce bioavailability and consequently drug efficacy, which may also augment the risk of resistance development. Better understanding of these mechanistic interactions may aid in the development of newer strategies to achieve adequate therapeutic levels and higher bioavailability. Therefore, the primary objective of this study was to assess the short term affinity of gemifloxacin towards efflux transporters using polarized canine MDCKII-MDR1, MDCKII-MRP2 cells.

Materials and Methods

Materials

Gemifloxacin mesylate was obtained from Bosche Scientific LLC (New Brunswick, NJ). Madin-Darby Canine Kidney (MDCK) type II cells over expressing human MDR1 and MRP2 proteins (MDCKII-MDR1, MDCKII-MRP2) were a generous gift from Drs. A. Schinkel and P. Borst (The Netherlands Cancer Institute, Amsterdam). [^{14}C] Erythromycin (specific activity: 51.3 mCi/mMol) was procured from Moravek Biochemicals (Brea, CA). Dulbecco's modified eagle's medium (DMEM), trypsin replacement (TrypLE™ Express), non-essential amino acids, TRIzol® and ATP determination kit were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 and 25 cm^2 growth area), 12-well plates (3.8 cm^2 growth area per well), polyester transwells (pore size of 0.4 µm) and 96-well plates (0.32 cm^2 growth area per well) were procured from Corning Costar Corp. (Cambridge, MA). CellTiter 96® aqueous non-radioactive cell proliferation assay was obtained from Promega Corporation (Madison, WI). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO) and used without further purification.

Cell Culture

MDCK cells are the most studied epithelial cells with respect to genetics, lipid and protein composition [234]. MDCK type-II cells transfected with human MDR1 and MRP2 genes have been considered the best models to study various substrates for their efflux mechanisms [235, 236]. MDCKII-MDR1, MDCKII-MRP2 cells of passage numbers 5-10, 5-15 respectively were used for the studies. These cells were maintained with DMEM medium supplemented with 10% FBS (heat inactivated), 1% non-essential amino acids, 29 mM sodium bicarbonate, 100 µg/mL of penicillin and streptomycin each at 37°C. Cells were grown in T-75 flasks, passaged using TrypLE™ Express and plated at a density of 250,000 cells/well for uptake and transport studies. Medium was changed every alternate day and studies were conducted 5-7 days post seeding, unless specified.

Uptake Studies

Uptake studies were performed on cells grown on 12-well plates. [^{14}C] erythromycin (0.25 µCi/mL) was employed to study MDR1 and MRP2 mediated efflux on MDCKII-MDR1 and MDCKII-MRP2 cells [237, 238]. Cellular accumulation of [^{14}C] erythromycin was determined alone, and in the presence of gemifloxacin as well as quinidine and MK-571, specific inhibitors for MDR1 and MRP2 respectively. After reaching confluency, the medium was removed and cells were washed thrice with 1 mL of Dulbecco's phosphate buffered saline (DPBS) (130 mM NaCl, 0.03 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 20 mM HEPES and 5 mM glucose). Later 500 µL of the test solution was added. Cells were then incubated for 30 min at 37°C. Later, uptake was arrested with ice cold PBS and 1 mL of lysis solution (0.1% (v/v) Triton-X in 0.3 N NaOH) was added. Cells were lysed overnight and the cell lysate was then quantified for radioactivity with scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) in scintillation counter (BeckmanCounter, Fullerton, CA). The

protein concentration in each well was estimated by Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA) and used to normalize the radioactivity. Following a similar procedure, dose-dependent inhibition studies were performed. [^{14}C] erythromycin was spiked with various concentrations of gemifloxacin ranging from 1 µM to 1000 µM. Studies were performed in a similar way and the data was fitted to calculate the half maximal inhibitory concentration (IC_{50}) according to a published method [239].

Cellular Accumulation of Calcein-AM

Calcein-AM is an acetoxymethyl ester derivative of calcein, a cell impermeable compound. This ester derivative is cell permeable and upon translocation is metabolized by intracellular esterases into calcein. Calcein-AM is a substrate of MDR1 and calcein is a substrate of MRP2 [240, 241]. Inhibition of these efflux proteins causes an increase in intracellular accumulation of calcein, which produces fluorescence. This assay was performed on cells cultured in 96-well plates at a density of 10,000 cells/well. After washing, cells were exposed to 100 µL of calcein-AM (0.05 µM) alone, in the presence of specific inhibitor and gemifloxacin for 15 min. The test solution was immediately removed and replaced with 100 µL of DPBS. Fluorescence associated with the cells was quantified with a 96-well micro titer plate reader (SpectraFluor Plus, Maennedorf, Switzerland) at an excitation and emission wavelengths of 495 and 515 nm respectively.

Permeability Studies

Transport studies were performed on cells grown on transwells. The donor chamber contained test solution where as the receiver chamber contained DPBS. Apparent permeability of [^{14}C] erythromycin was determined from apical to basolateral (AP-BL) and basolateral to apical (BL-AP) directions across MDCKII-MDR1 and MDCKII-MRP2 cells. At predetermined

intervals (0, 15, 30, 45, 60, 90, 120, 150 min); samples were withdrawn from the receiver chamber and replaced with same amount of DPBS to maintain sink conditions. The samples were then quantified for [^{14}C] erythromycin as described previously. Cumulative amount transported was plotted with time to determine apparent permeability.

ATP Assay

The efflux proteins require ATP to extrude xenobiotics out of the cell [242]. Any change in the intrinsic levels of ATP may alter the functional activity of efflux proteins. Hence ATP activity assay was performed to examine if gemifloxacin alters ATP activity in cells. MDCKII-MDR1 and MRP2 cells grown in 96-well plates were treated with 100 µL of various concentrations of gemifloxacin for 150 min. Later 100 µL of lysis solution was added and cell lysate was used for the quantification of ATP with a kit (Invitrogen, Carlsbad, CA) following manufacturer's protocol.

Cell Proliferative Assay

MTS assay is used to determine if concentrations of gemifloxacin used are toxic to the cells. Cells were exposed to various concentrations of gemifloxacin for 150 min. The solutions were aspirated and 100 µL of serum free media was added. Twenty microliters of MTS and PMS (CellTiter 96® aqueous non-radioactive cell proliferation assay) reagent were then added to wells. After incubating for 4 hours, the quantity of formazan formed from MTS, is measured at 490 nm with a plate reader. The amount of formazan is directly proportional to the number of viable cells.

Data Analysis

Cellular accumulation of [^{14}C] erythromycin was determined with Eq. 1.

$$C_{sample} = \frac{DPM_{sample}}{DPM_{donor}} * C_{donor}$$ - Eq. 1

Disintegrations per minute (DPM) of sample and donor are represented as DPM_{sample} and DPM_{donor} respectively. The concentration of donor used is represented C_{donor} and C_{sample} represents the concentration of sample thus obtained.

The half maximal inhibitory concentration (IC_{50}) of [^{14}C] erythromycin by gemifloxacin was calculated with Eq. 2.

$$Y = min + \frac{max - min}{1 + 10^{(Log\,IC50 - X)}}$$ - Eq. 2

Y is the cellular accumulation of [^{14}C] erythromycin and X represents the logarithm of the gemifloxacin concentration used. Data was fitted to Eq. 2 with a transformed nonlinear regression curve analysis program (GraphPad Prism Version 4.0; GraphPad Software, San Diego, CA) to calculate IC_{50}.

The amount of [^{14}C] erythromycin transported across the cell monolayers over a specific interval (dM/dt) divided by the cross-sectional area of the transwell (A) generates the flux (J). Flux is thus estimated by Eq. 3.

$$J = \frac{dM/dt}{A}$$ - Eq. 3

Apparent permeability (P) was then obtained by normalizing flux to donor concentration, as described in Eq. 4.

$$P = \frac{J}{C_{donor}}$$ - Eq. 4

The efflux ratio was then obtained by dividing the BL-AP permeability by AP-BL permeability as described in Eq.5.

$$Efflux\ ratio = \frac{P_{(BL-AP)}}{P_{(AP-BL)}} \quad \text{- Eq. 5}$$

Statistical Analysis

All experiments were performed atleast in quadruplicate (n=4). The results were represented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) or student's t test (GraphPad InStat Version 4.0) was performed to determine statistical difference from the control. A P-value of <0.05 is considered to be statistically significant.

Results

Uptake Studies

Accumulation of [^{14}C] erythromycin on MDCKII-MDR1 cells was significantly enhanced (1.7 fold) in the presence of quinidine (100 µM), suggesting the inhibition of MDR1 efflux protein. A similar increase was also observed in the presence of gemifloxacin (250 µM) (**Figure 9**). Cellular accumulation of [^{14}C] erythromycin was also performed on MDCKII-MRP2 cells. Uptake of [^{14}C] erythromycin was elevated by almost 1.8 fold in the presence of MK-571 (100 µM). A similar rise was also observed in presence of gemifloxacin (250 µM) (**Figure 10**). Dose-dependent inhibition of [^{14}C] erythromycin excretion was performed on MDCKII-MDR1 cells with varying concentrations of gemifloxacin (1 µM to 1000 µM). IC_{50} value of gemifloxacin from the dose-response curve was calculated to be 123 ± 2 µM (**Figure 11**). Similar inhibition studies were also performed on MDCKII-MRP2 cells to delineate the inhibitory potential of gemifloxacin on [^{14}C] erythromycin uptake. IC_{50} value of gemifloxacin from the dose-response curve was calculated to be 16 ± 2 µM (**Figure 12**).

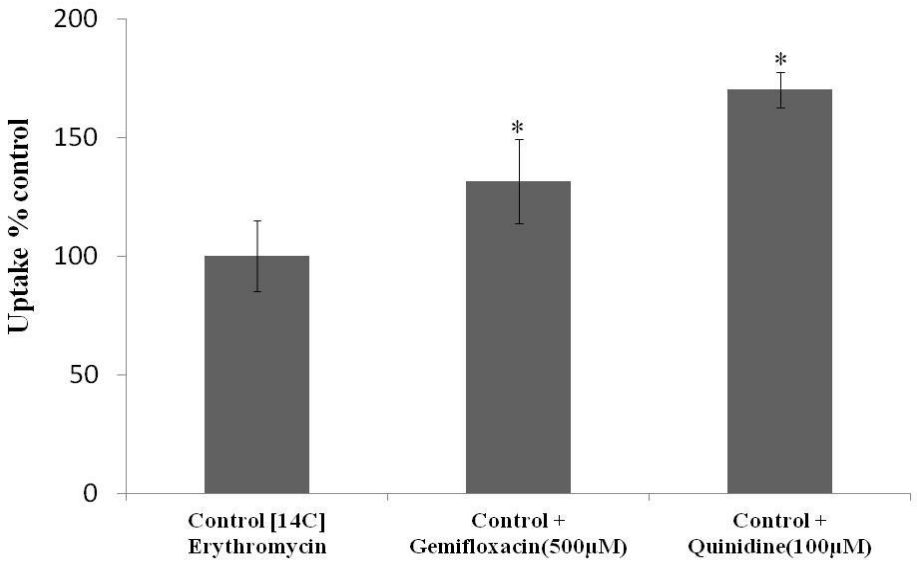

Figure 9. Cellular accumulation of [^{14}C] erythromycin alone, in presence of gemifloxacin (250 µM) and quinidine (100 µM) in MDCKII-MDR1 cells. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

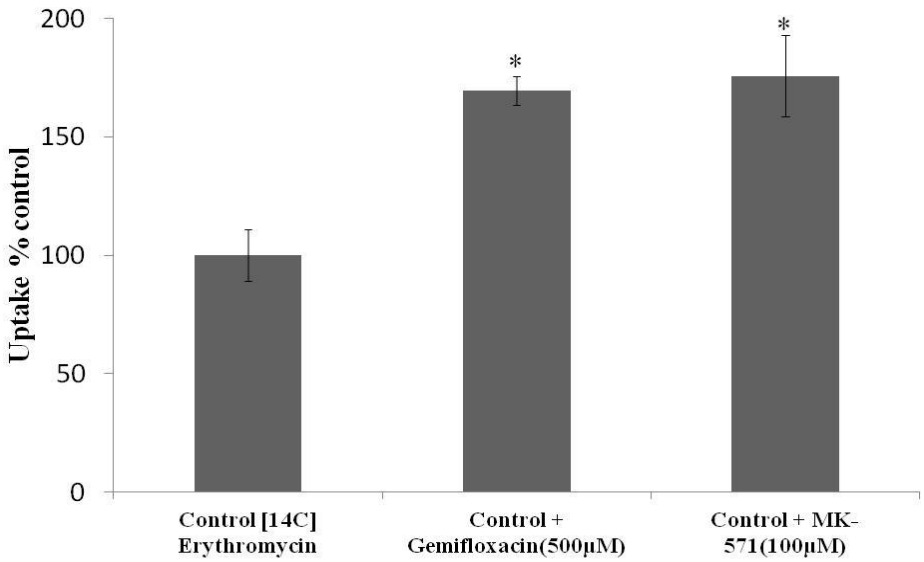

Figure 10. Cellular accumulation of [^{14}C] erythromycin alone, in presence of gemifloxacin (250 µM) and MK-571 (100 µM) in MDCKII-MRP2 cells. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

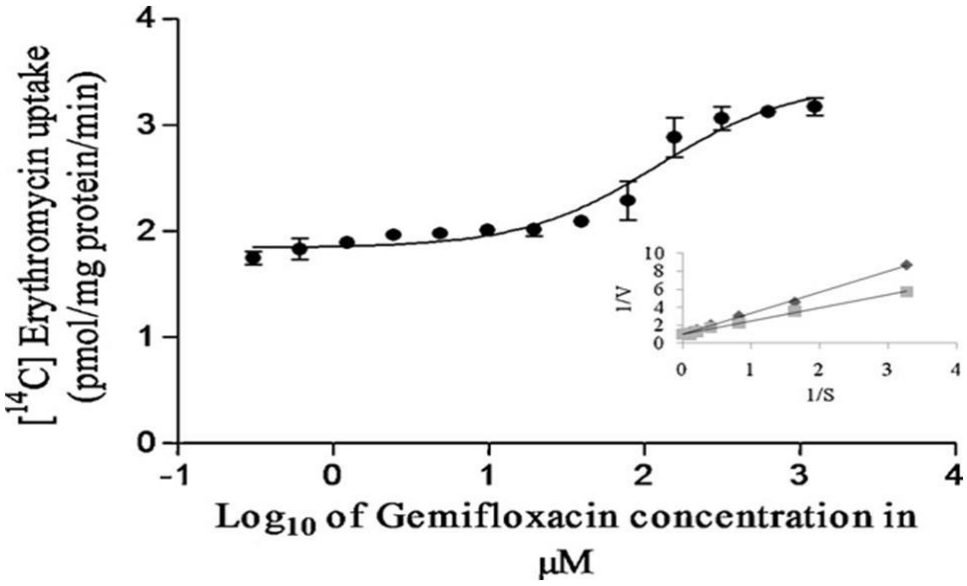

Figure 11. Dose-dependent inhibition of [^{14}C] erythromycin uptake in MDCKII-MDR1 cells in presence of varying concentrations of gemifloxacin (1 µM to1000 µM). Values represent mean ± SD. Inset shows the Lineweaver-Burk transformation of the data; ♦ represents gemifloxacin and ■ represents erythromycin.

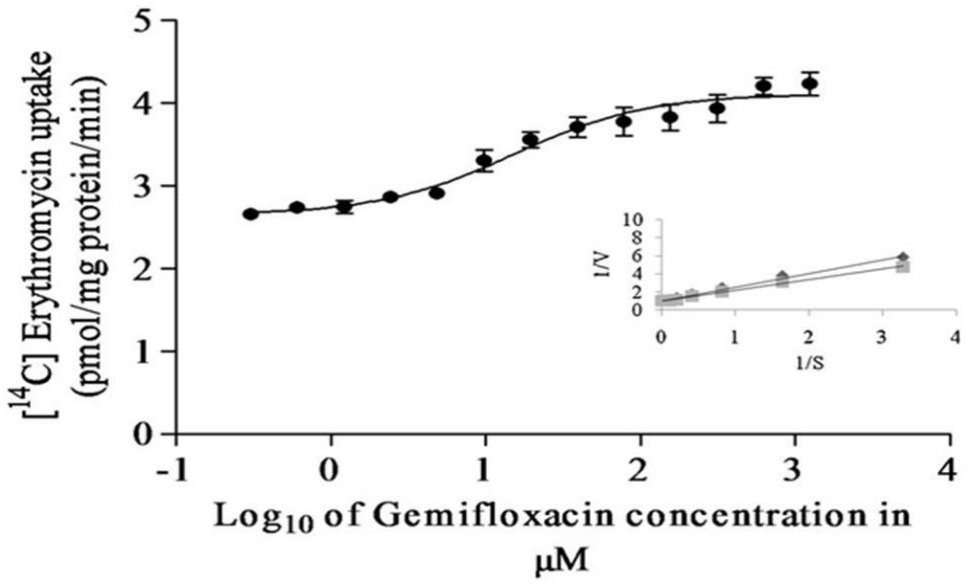

Figure 12. Dose-dependent inhibition of [^{14}C] erythromycin uptake in MDCKII-MRP2 cells in presence of varying concentrations of gemifloxacin (1 µM to1000 µM). Values represent mean ± SD. Inset shows the Lineweaver-Burk transformation of the data; ♦ represents gemifloxacin and ■ represents erythromycin.

Cellular Calcein Fluorescence

Intracellular fluorescence in MDCKII-MDR1 cells is doubled in the presence of quinidine (100 µM), suggesting increased accumulation of calcein-AM through inhibition of MDR1. Similar increase in fluorescence was also observed in the presence of gemifloxacin (250 µM) (**Figure 13**). This study was also performed on MDCKII-MRP2 cells to delineate calcein efflux by MRP2. Intracellular calcein fluorescence did significantly rise (2.5 fold) in presence of MK-571 (100 µM), probably through reduction in calcein efflux by MRP2. Accumulation of calcein was also significantly higher in the presence of gemifloxacin (250 µM) and was also confirmed by fluorescent microscopic images (**Figure 14**).

Permeability Studies

Transport of [^{14}C] erythromycin across MDCKII-MDR1 cells was assessed in both AP-BL and BL-AP directions. Since these efflux transporters are polarized on the apical side, permeability of [^{14}C] erythromycin in AP-BL is lower than BL-AP direction. Apparent permeability of [^{14}C] erythromycin from AP-BL direction was observed to be $2.96 \pm 0.37 \times 10^{-6}$ cm/sec. This permeability significantly ascended to $6.37 \pm 0.74 \times 10^{-6}$ cm/sec in the presence of quinidine (100 µM), probably due to inhibition of MDR1. A similar rise in the permeability of [^{14}C] erythromycin to $5.24 \pm 0.52 \times 10^{-6}$ cm/sec was also observed in the presence of gemifloxacin (250 µM). Permeability of [^{14}C] erythromycin was obtained as $10.56 \pm 0.86 \times 10^{-6}$ cm/sec from BL-AP direction. Such BL-AP permeability was reduced in the presence of quinidine (100 µM) and gemifloxacin (250 µM) to 8.56 ± 0.43 and $7.38 \pm 0.72 \times 10^{-6}$ cm/sec, respectively (**Figure 15**). Efflux ratio of [^{14}C] erythromycin on MDCKII-MDR1 cells as calculated by ratio of permeability in BL-AP to AP-BL direction was found to significantly diminish in the presence of quinidine (100 µM) and gemifloxacin (250 µM).

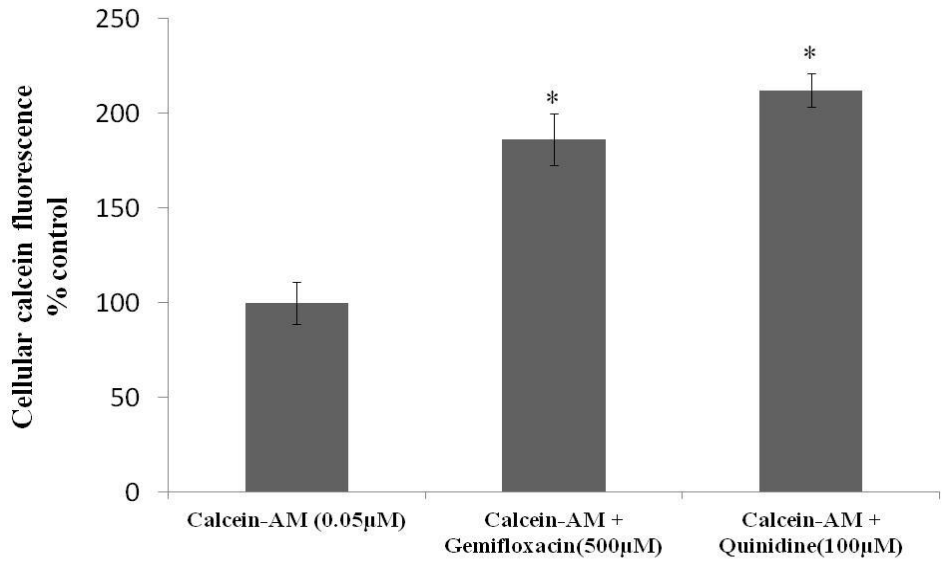

Figure 13. Cellular accumulation of calcein-AM alone, in presence of gemifloxacin (250 µM) and quinidine (100 µM) in MDCKII-MDR1 cells. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

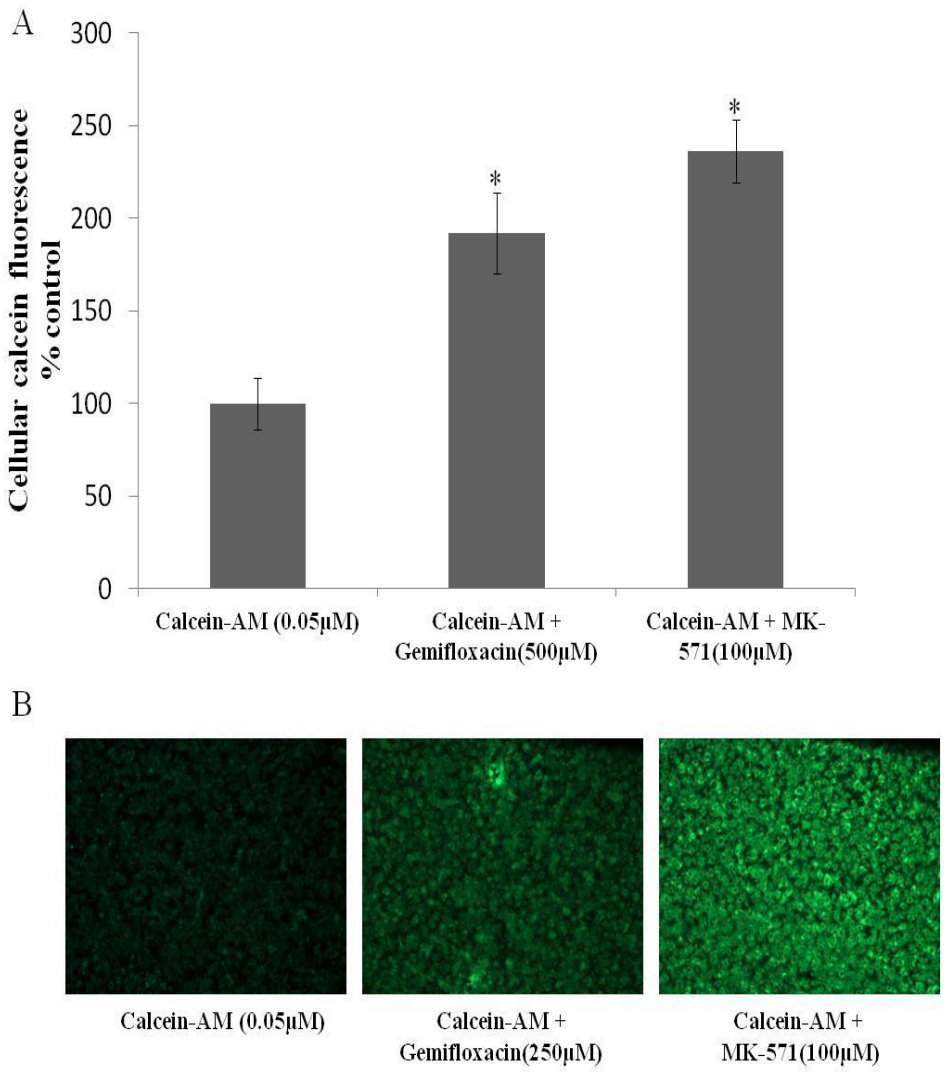

Figure 14. (A) Cellular accumulation of calcein-AM alone, in presence of gemifloxacin (250 µM) and MK-571 (100 µM) in MDCKII-MRP2 cells as measured by intrinsic calcein fluorescence. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05. (B) Cellular images of two independent experiments.

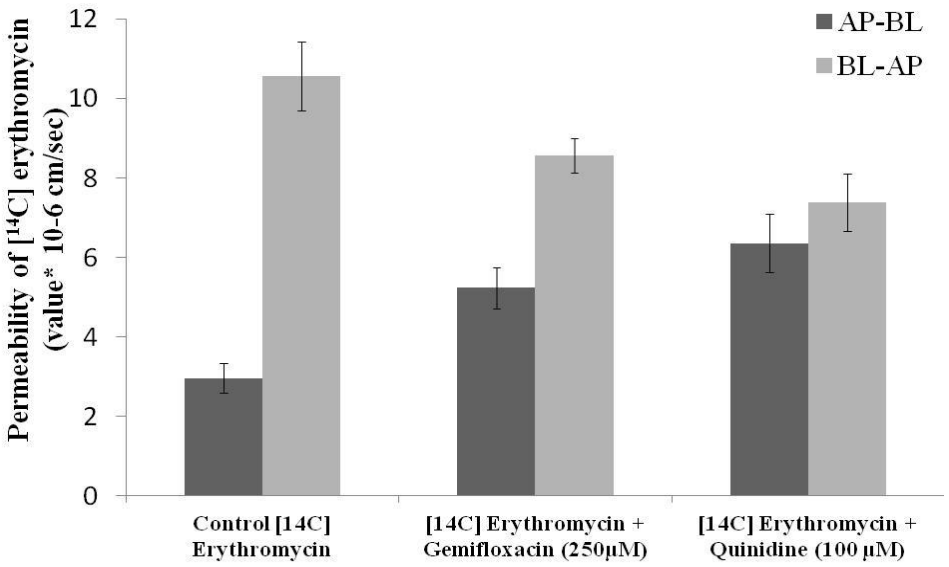

Figure 15: Transepithelial transport of [^{14}C] erythromycin alone, in presence of gemifloxacin (250 µM) and quinidine (100 µM) in MDCKII-MDR1 cells from AP-BL and BL-AP directions. Values represent mean ± SD.

Transport of [^{14}C] erythromycin across MDCKII-MRP2 cells was similarly assessed in both AP-BL and BL-AP directions. Apparent permeability of [^{14}C] erythromycin in AP-BL direction was found to be $3.76 \pm 1.37 \times 10^{-6}$ cm/sec. This permeability significantly rises in the presence of MK-571 (100 µM) to $10.61 \pm 2.20 \times 10^{-6}$ cm/sec, probably indicating the inhibition of MRP2 efflux pump. In a similar manner, permeability was also enhanced in the presence of gemifloxacin (250 µM) to $8.80 \pm 0.68 \times 10^{-6}$ cm/sec. In a similar manner, permeability of [^{14}C] erythromycin decreased from BL-AP in the presence of MK-571 and gemifloxacin to 10.38 ± 1.34 and $11.05 \pm 0.93 \times 10^{-6}$ cm/sec respectively, relative to control with $18.53 \pm 1.94 \times 10^{-6}$ cm/sec (**Figure 16**).

ATP Activity Assay

ATP activity assay was performed post treatment with gemifloxacin for 150 min on MDCKII-MDR1 and MRP2 cells. All concentrations of gemifloxacin used (1 µM to 1000 µM) except 1000 µM, did not significantly alter ATP levels compared to control. Gemifloxacin (1000 µM) increased ATP level by almost 15% and 10% in MDCKII-MDR1 and MRP2 cells, respectively (**Figure 17**).

Cell Proliferation Assay

MTS assay was performed to examine the cytotoxicity of various concentrations of gemifloxacin (1 µM to 1000 µM) post treatment over 150 min. All concentrations of gemifloxacin used (1 µM to 1000 µM) except 1000 µM, did not significantly alter cell viability compared to control. Gemifloxacin (1000 µM) decreased cell viability by almost 25% and 20% on both MDCKII-MDR1 and MRP2 cells. All the other concentrations used were found to be non-cytotoxic (**Figure 18**).

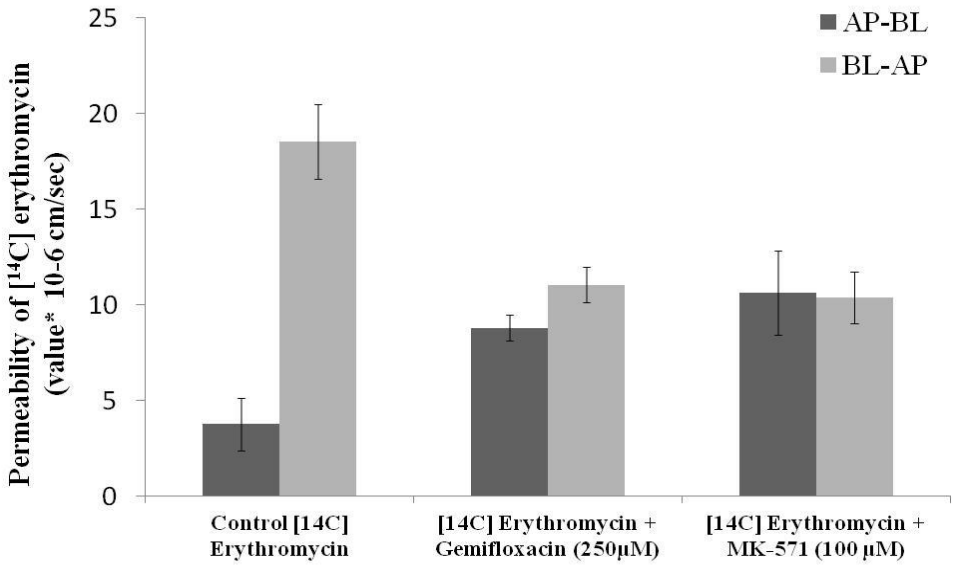

Figure 16: Transepithelial transport of [^{14}C] erythromycin alone, in presence of gemifloxacin (250 µM) and MK-571 (100 µM) in MDCKII-MRP2 cells from AP-BL and BL-AP directions. Values represent mean ± SD.

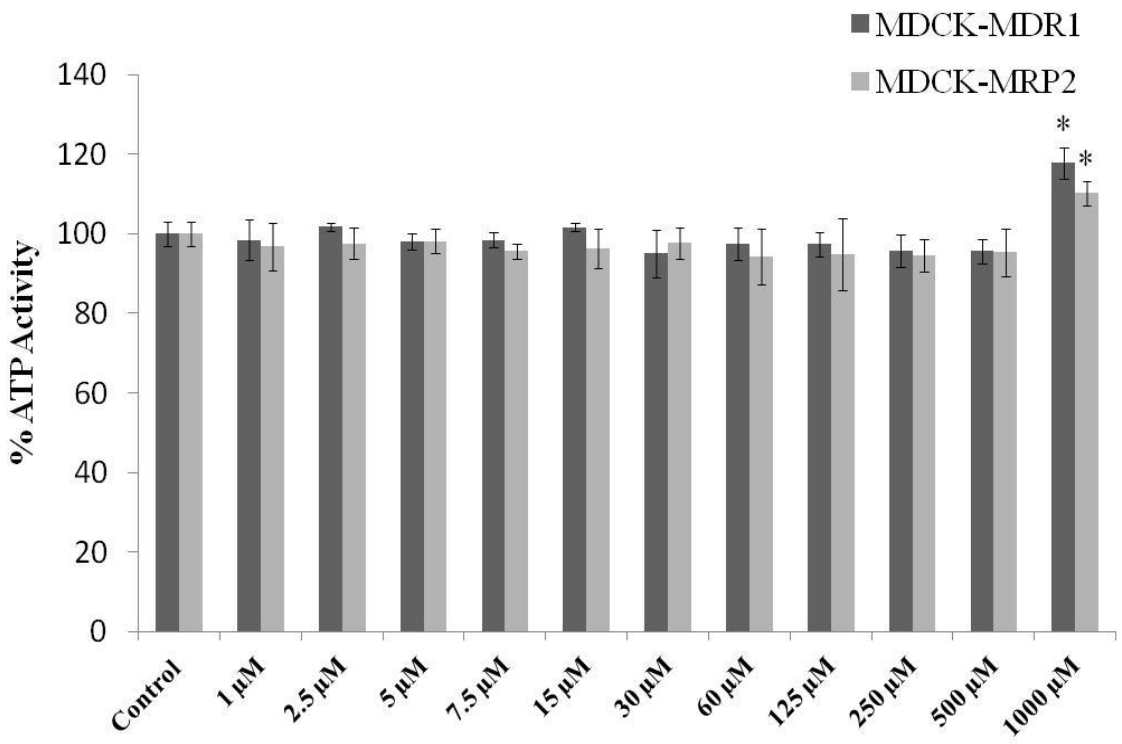

Figure 17. ATP assay in the presence of varying concentrations of gemifloxacin (1 µM to1000 µM) in MDCKII-MDR1 and MDCKII-MRP2 cells for 150 min. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

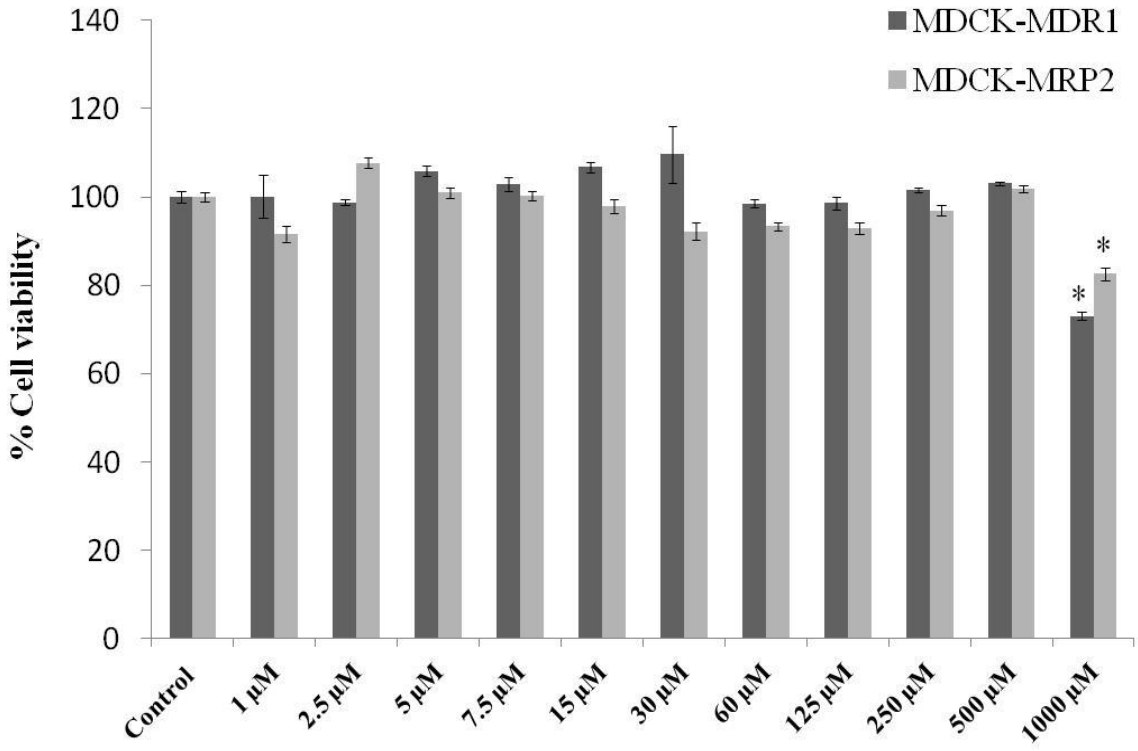

Figure 18. Cytotoxicity assay in the presence of varying concentrations of gemifloxacin (1 µM to1000 µM) in MDCKII-MDR1 and MDCKII-MRP2 cells for 150 min. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Discussion

Gemifloxacin is a new fluoroquinolone antibiotic approved by US FDA for treatment of both gram positive and negative bacterial infections. It has broad spectrum of activity and inhibits both bacterial DNA gyrase and topoisomerase IV. Most fluoroquinolones, being substrates of efflux transporters, are generally extruded out from cellular matrix, significantly reducing intracellular accumulation and thereby altering drug bioavailability [204]. The primary objective of this study was to evaluate the effect of efflux transporters on the cellular translocation of gemifloxacin.

Cellular accumulation of [^{14}C] erythromycin in the presence of quinidine (a known substrate and/or inhibitor) appears to be significantly higher than the control, confirming the presence of MDR1 in MDCKII-MDR1 cells [229, 231]. A significantly elevated cellular accumulation of [^{14}C] erythromycin in the presence of gemifloxacin is also observed. This result indicates that this fluoroquinolone interacts with MDR1, suggesting gemifloxacin may be a substrate. Similarly, cellular accumulation of [^{14}C] erythromycin in the presence of MK-571 (a known inhibitor of MRPs) [229, 243] was found to be significantly higher than the control, confirming the presence of MRP2 in MDCKII-MRP2 cells. A significantly elevated cellular accumulation of [^{14}C] erythromycin in the presence of gemifloxacin suggests that this fluoroquinolone may also be a substrate of MRP2. A significant rise in [^{14}C] erythromycin uptake in the presence of quinidine and MK571 is consistent with our earlier results that both MDR1 and MRP2 are functionally active in MDCKII-MDR1 and MDCKII-MRP2 cells, respectively [229, 244].

Dose-dependent inhibition studies suggest high affinity of gemifloxacin towards both MDR1 and MRP2. Gemifloxacin inhibited both MDR1 and MRP2 mediated efflux of [^{14}C]

erythromycin in a dose-dependent manner with an IC_{50} value of 123 ± 2 μM and 16 ± 2 μM, respectively. A comparison of IC_{50} values indicates that gemifloxacin probably has a much higher affinity towards MRP2 relative to MDR1. IC_{50} value of gemifloxacin was also found to be significantly lower than another fourth generation fluoroquinolone, gatifloxacin previously reported from our laboratory [228]. These lower IC_{50} values represent that gemifloxacin is a better substrate of efflux transporters as compared to gatifloxacin. This further explains the difference in their bioavailability values (gatifloxacin - 97% as compared to gemifloxacin - 71%). Lineweaver-Burk transformation of the above data revealed that the inhibitory mode of gemifloxacin for MDR1 and MRP2 mediated excretion of [^{14}C] erythromycin was a competitive type. This analysis suggests that gemifloxacin and erythromycin may share a common binding site in both the efflux transporters. Further evidence of functional activity of MDR1 and MRP2 on the cellular translocation of gemifloxacin has been studied with calcein-AM. Calcein-AM is a substrate of MDR1 and calcein is a substrate of MRP2 [240, 241, 245]. A rise in intracellular calcein fluorescence in presence of quinidine and MK-571 confirms inhibition of both MDR1 and MRP2 functional activities. A similar elevation in calcein fluorescence in presence of gemifloxacin in MDCKII-MDR1 and MRP2 cells demonstrates its substrate specificity for both MDR1 and MRP2. Such increase in calcein accumulation in MDCKII-MRP2 cells is further confirmed by fluorescent microscopic images.

The efflux ratio (ratio of basolateral to apical versus apical to basolateral permeability) is considered as one of the indicators for identifying MDR1 and MRP2 substrates [246]. Since these efflux transporters (MDR1 and MRP2) are localized on the apical direction, [^{14}C] erythromycin alone exhibited much higher transport in the BL-AP direction relative to AP-BL direction. When this ratio of apparent permeability approaches 1.0 transport equals in both the

directions. Efflux ratios of [^{14}C] erythromycin were found to be 3.56 and 4.93 in MDCKII-MDR1 and MRP2 cells, respectively. A significant reduction in efflux ratio to 1 (complete inhibition) was evident in the presence of quinidine and MK-571 confirming inhibition of MDR1 and MRP2 functional activities. Furthermore, the presence of gemifloxacin also affected bi-directional permeability of [^{14}C] erythromycin. The efflux ratio is lowered to 1.63 and 1.26 in MDCKII-MDR1 and MRP2 cells. This significant reduction further confirms the substrate specificity of gemifloxacin towards MDR1 and MRP2.

Since the efflux pumps require ATP for their activation, it is postulated that any change in the amount or activity of ATP may signify sensitivity of the substrate for the transporter. ATP determination assay was performed in MDCKII-MDR1 and MRP2 cells to examine ATP involvement in cellular translocation of gemifloxacin. No significant change in the ATP activity was observed at various gemifloxacin concentrations. This result suggests that MDR1 and MRP2 mediated efflux by gemifloxacin is not affected by ATP activity. Though not to a large extent, a minor increase in ATP levels was observed in the presence of 1000 µM concentration of gemifloxacin. This aberration may be due to stress response generated with the usage of a very high concentration. A cell proliferation assay is carried out to determine if the concentrations used are cytotoxic. Results obtained from this assay indicated that gemifloxacin tested at various concentrations do not elicit any cytotoxic effects in MDCKII-MDR1 and MRP2 cells. However, the highest concentration (1000 µM) tested was found to reduce the number of viable cells. This high concentration leads to cytotoxicity which may be one of the factors responsible for elevated ATP levels [229, 247].

Conclusions

In conclusion, this report provides direct evidence that gemifloxacin is effluxed by both MDR1 and MRP2. This report provides an insight onto the mechanism of gemifloxacin interaction with efflux transporters. It also offers strategy for the development of new therapeutic delivery systems. This study also indicates that simultaneous administration of macrolides and fluoroquinolones may be more effective against various strains of *Streptococci* [231]. Expression of efflux transporters is regulated by the ligand activated transcription factor, pregnane X receptor (PXR, NR1I2) [168, 169, 248, 249]. PXR is considered to play an important role in regulating response to various drugs, thereby regulating their physiological disposition. Prolonged administration of fluoroquinolones may induce the expression of MDR1 and MRP2, which may contribute to the development of drug resistance. This induction in the expression of efflux transporters may affect intracellular drug accumulation, limiting oral bioavailability. However, further studies are required to confirm this hypothesis.

CHAPTER 3

PXR MEDIATED INDUCTION OF EFFLUX TRANSPORTERS BY FLUOROQUINOLONES: A POSSIBLE MECHANISM FOR DEVELOPMENT OF MULTIDRUG RESISTANCE

Rationale

Fluoroquinolones represent an important class in the armamentarium of antimicrobial agents. Because of their broad spectrum activity, they are widely indicated in the treatment of human and veterinary diseases [250]. Structural modifications of the basic 4-quinolone skeleton resulted in the development of newer analogs i.e., second, third and fourth generation fluoroquinolones [251]. These molecules have been widely indicated for the treatment of systemic bacterial infections. Fluoroquinolones such as ofloxacin, levofloxacin, gatifloxacin and gemifloxacin were reported to be substrates of efflux transporters when investigated during short-term interaction studies [252-255]. Such substrate specificity of fluoroquinolones towards efflux transporters may be one of the major factors accounting for their alterations in oral bioavailability. A vast amount of literature is documented on the pharmacokinetic properties of fluoroquinolones [256]. However, little information is available on the molecular determinants (nuclear receptors) of their cellular absorption and viable potential for acquisition of multidrug resistance (MDR).

Development of MDR is a complex cellular signaling process and reduces efficacy of therapeutic drug molecules. Development of MDR can occur at various levels including drug delivery, drug detoxification and cellular microenvironment. Significant research from the last two decades has demonstrated that acquisition of MDR is predominantly due to the elevated expression and activity of ATP-binding cassette (ABC) proteins [257]. ABC transporters

represent a super family of membrane bound proteins found in both prokaryotes and eukaryotes. These integral proteins translocate a wide variety of exogenous and endogenous substances across concentration gradient utilizing the energy derived from ATP hydrolysis. To date, forty-eight efflux transporters have been identified and fifteen of them are associated with the development of drug resistance. Of these ABC proteins, P-glycoprotein (P-gp/MDR1) and multidrug resistance protein 2 (MRP2) plays a predominant role in efflux of several drugs altering their absorption, distribution and excretion [258].

MDR1 is a 170 kDa transmembrane protein expressed on the apical membrane of many epithelial and endothelial cells. This protein exhibits twelve transmembrane domains (TMDs) and two ATP binding sites. MRP family is the largest branch of the ABC transporters consisting of thirteen different proteins (MRP 1-10, CFTR SUR1 and SUR2), primarily responsible for drug transport across lipid membranes. Similar to P-gp, MRP proteins also possess twelve TMDs and two ATP binding regions. However, few MRP transporters (MRP 1, 2, 3, 6 and 7) possess an additional hydrophobic region composed of five TMDs at the NH2-terminal end totaling 17 TMDs [73, 259]. Expression of these efflux transporters is not static. Rather, it is controlled dynamically *via* transcriptional regulators by certain nuclear receptors or xenosensors. The ligand activated transcription factor, pregnane X receptor (PXR; NR1I2), is considered a master regulator of "homeland" defense and plays vital role in modulating response to various xenobiotics, thereby regulating drug disposition kinetics [249]. Upon transcriptional activation, PXR present in the cytoplasm translocates to the nucleus and forms a heterodimer with retinoid X receptor (RXR; NR2B1). This heterodimeric complex binds to the promoter region of target genes, thereby increasing transcription. The target genes include phase I drug metabolizing enzymes (DMEs) including a number of cytochrome P450 enzymes (CYPs), carboxylesterases,

aldehyde and alcohol dehydrogenases, phase II DMEs such as UGTs, GSTs, and SULTs and phase III ABC drug efflux transporters [165, 260].

The clinical effectiveness of fluoroquinolones may depend on the activation of nuclear receptors and development of MDR. Therefore, the objective of this study was to examine the interaction of fluoroquinolones with nuclear receptor and efflux transporters upon long term treatment. Such information on functional and molecular aspects may assist in the development of alternative and newer strategies to overcome/prevent drug-drug interactions, adjust dose, achieve adequate therapeutic levels and most importantly reduce drug resistance.

Materials and Methods

Materials

Gemifloxacin and gatifloxacin were procured from Bosche Scientific LLC (New Brunswick, NJ). Levofloxacin and ofloxacin were obtained from TCI America (Portland, OR). LS-180 cells were obtained from American Type Culture Collection (Manassas, VA). Dulbecco's modified eagle's medium (DMEM), TrypLE™ Express (stable trypsin replacement), non-essential amino acids, TRIzol®, Lipofectamine™, Opti-MEM® and NuPAGE® Novex 4-12% bis-tris gels were procured from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 and 25 cm^2 growth area); 12-well plates (3.8 cm^2 growth area per well), and 96-well plates (0.32 cm^2 growth area per well) were procured from Corning Costar Corp. (Cambridge, MA). The chemicals and buffers for cDNA synthesis (oligodT, $MgCl_2$, dNTP, M-MLV reverse transcriptase), Dual-Glo® luciferase assay system and CellTiter 96® aqueous non-radioactive cell proliferation assay kit were obtained from Promega (Madison, WI). Light Cycler 480® SYBR I green master mix was purchased from Roche Applied Science (Indianapolis, IN). [^{3}H] Digoxin (specific activity: 0.1

Ci/mMol) and [^{14}C] erythromycin (specific activity: 51.3 mCi/mMol) were procured from Moravek Biochemicals (Brea, CA). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO) and used without further purification.

Plasmids

The pCDG-hPXR expression vector was generously provided by Dr. Ronald M. Evans (Salk Institute for Biological Studies, La Jolla, CA). The pGL3-MDR1 and pGL3-MRP2 luciferase reporter constructs were generously donated by Dr. Ahmad R. Safa (Indiana University Simon Cancer Center, Indianapolis, IN) and Dr. Ishtiaq Qadri (NUST Center of Virology & Immunology, Rawalpindi, Pakistan). The renilla luciferase control plasmid pRL-TK was obtained from Promega. All these plasmids were checked by enzyme restriction, followed by agarose gel electrophoresis.

Cell Culture

LS-180 cells of passage numbers 65-70 were selected for the studies. Cells were maintained using DMEM medium supplemented with 10% FBS (heat inactivated), 1% non-essential amino acids, 29 mM sodium bicarbonate, 100 µg/mL of penicillin and streptomycin each at 37°C. Cells were grown in T-75 flasks, passaged using TrypLE™ Express and plated at a density of one million cells in T-25 flasks, 250,000 cells/well in 12-well plates and 10,000 cells/well in 96-well plates for further studies. After reaching 70-80% confluency, the growth medium was replaced with medium containing fluoroquinolones at three different concentrations (2.5, 5 and 7.5 µM). The concentrations of fluoroquinolones were determined from clinically relevant plasma concentrations in human volunteers [261-263]. Four different fluoroquinolones (gemifloxacin, gatifloxacin, levofloxacin, ofloxacin) were selected for our studies. Rifampin (7.5 µM), a well-known inducer of PXR and efflux transporters, was included as a positive control in

all the experiments [264]. Stocks of respective drugs were prepared either in sterile water or dimethyl sulfoxide (DMSO). Stocks were diluted appropriately with medium to achieve the desired concentration. DMSO concentration did not exceed 0.5% in all the drug solutions. Cells were treated for 72 hours and processed for respective experiments accordingly.

Quantitative Gene Expression Analysis

LS-180 cells were lysed in TRIzol®, phase separated using chloroform and the aqueous layer containing RNA was precipitated using isopropanol. The obtained RNA was washed twice with 75% ethanol and dissolved in DNase/RNase-free water. Concentration was determined by measuring the absorbance at 260 nm using Nanodrop (Thermo Fisher Scientific, Wilmington, DE). RNA was reverse transcribed into complementary DNA (cDNA) with oligodT as a template and M-MLV reverse transcriptase. The conditions for reverse transcription were initial denaturation at 70°C for 5 min, reverse transcription at 42°C for 1 hour and final extension at 72°C for 5 min. For quantitative gene expression analysis, 80 µg of cDNA was amplified using 10 µM of forward and reverse primers and SYBR I green master mix on ABI Prism 5700 Sequence Detection System (Applied Biosystems, CA). Primer-Blast tool (PubMed) was used for designing the primers and sequences are summarized in **Table 4.** All the primers were designed such that 100-150 bp long amplicons will be generated to increase the efficiency of amplification. The reactions were carried out with initial denaturation at 70°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 10 sec. The specificity of polymerase chain reaction (PCR) products was confirmed by melting curve analysis. To normalize the amount of cDNA in all the samples, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal standard.

Table 4. Primers used in quantitative gene expression analysis. Sequence is given from 5'->3'.

Gene	Forward primer	Reverse primer
GAPDH	ATCCCTCCAAAATCAAGTGG	GTTGTCATGGATGACCTTGG
PXR	GCAGGTGGCTTCCAGCAACT	GGGCGGTCTGGGGAGAAGAG
MDR1	CTTATGCTCTGGCCTTCTGG	TGCTTCAATGCTTGGAGATG
MRP2	AAATATTTTGCCTGGGAACC	TGTGACCACAGATACCAGGA

Reporter Activity Assays

MDR1 and MRP2 reporter activity assays were performed as described in previous published protocols [265, 266]. Briefly, LS-180 cells plated in 96-well plates were transfected with 75 ng/well of nuclear receptor expression vector (pCDG-hPXR), 200 ng/well of respective gene firefly luciferase reporter construct (pGL3-MDR1 or pGL3-MRP2) and 15ng/well of control renilla luciferase expression vector (pRL-TK), using Lipofectamine™ 2000 for 24 hours in reduced media. Later, the transfection media was aspirated and cells were washed with sterile phosphate buffered saline (PBS - 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl and 135 mM NaCl). Following drug exposure for 72 hours, the firefly and renilla luciferase activities were measured using Dual-Glo® luciferase assay system following manufacturer's protocol. The ratio of firefly luminescence to renilla luminescence is used to normalize the transfection efficiency in all the samples.

Immunoblot Assay

Cells were washed with PBS and lysed in PBS containing 1% Triton X-100 and protease inhibitor cocktail. The lysate was collected following centrifugation at 12,000 rpm for 15 min and quantified using Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA) as an internal standard. Proteins were denatured and separated using poly acrylamide gel electrophoresis (PAGE). Later, the protein was transferred onto polyvinylidene difluoride (PVDF) membrane and blocked overnight at 4°C with 2% non-fat dry milk and 0.25% BSA in 0.05 M tris buffered saline (TBS). The blot was exposed to primary antibody at 25°C for 4 hours. After subsequent washes (3×10 min), the blot was exposed to the secondary antibody at 25°C for 1.5 hours. Finally the blot was visualized for the protein using chemiluminescent substrate with chemiImager 8900 (Alpha Innotech, San Leandro, CA).

Uptake Study

The effect of long term exposure of fluoroquinolones on the intracellular accumulation of [^{3}H] digoxin and [^{14}C] erythromycin was studied on LS-180 cells. Digoxin and erythromycin were used as model substrates to study MDR1 and MRP2 mediated efflux, respectively. Following treatment with fluoroquinolones, the medium was aspirated and cells were washed with 1 mL of PBS (3×10 min). Later, 500 µL of respective substrate was added and cells were incubated for 30 min at 37°C. Uptake was terminated with ice cold PBS and 1 mL of lysis solution (0.3 N NaOH containing 0.1% (v/v) Triton-X) was added. Followed by overnight lysis, the cell lysate was analyzed for radioactivity using a scintillation cocktail (3 mL) (MP Biomedicals, Santa Ana, CA) in scintillation counter (BeckmanCounter, Fullerton, CA). Protein concentration was used to normalize the uptake values.

Cell Viability Assay

Cytotoxicity assay was carried out with a Cell Titer 96® aqueous non-radioactive cell proliferation assay kit. This assay was performed to determine if the highest concentrations of each drug used was toxic to the cells. Following drug treatment in a 96-well plate, solutions were aspirated and 100 µL of reduced serum media was added. Twenty microliters of MTS and PMS reagent were then added to the wells and incubated for 4 hours at 37°C. The amount of formazan formed from MTS is measured at 490 nm using a plate spectrophotometer (SpectraFluor Plus, Maennedorf, Switzerland). As the amount of formazan formed is directly proportional to the number of viable cells, the toxicity of treatment can be estimated.

Statistical Analysis

For experiments performed as biological duplicate and experimental quadruplicates, results were expressed as mean ± standard error (SE). For experiments performed as

experimental quadruplicates, results were expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) or student's t test (Graph Pad InStat Version 4.0) was performed to confirm statistical significance of the experimental values. A P-value of less than 0.05 was considered to be statistically significant.

Results

Quantitative Gene Expression Analysis

All concentrations (2, 5 and 7.5 µM) of gemifloxacin significantly induced PXR by almost 18 fold. Gatifloxacin showed a gradual increase in induction of PXR by 6-14 fold. Levofloxacin and ofloxacin exhibited 10-15 fold increase in PXR levels, with no significant difference observed between 5 and 7.5 µM concentrations. Rifampin induced PXR by almost 15 fold (**Figure 19**). Upon treatment with fluoroquinolones, the direct transcriptional downstream targets of PXR, such as MDR1 and MRP2, were also measured. All three concentrations of gemifloxacin (2, 5 and 7.5 µM) induced MDR1 by 5-7 fold. The other three fluoroquinolones demonstrated a concentration-dependent induction of MDR1 mRNA levels from 3-7 fold. Rifampin induced MDR1 by 9 fold (**Figure 20**). Similar changes in mRNA levels of MRP2 were also noticed in LS-180 cells treated with all the fluoroquinolones tested in the study. Surprisingly, all fluoroquinolones and rifampin induced MRP2 to a similar extent (~ 5-6 fold) at various concentrations tested (**Figure 21**).

Reporter Activity Assays

Post treatment with fluoroquinolones at three different concentrations, the cell lysate was quantified for simultaneous detection of both firefly and renilla luciferase luminescence signals. All the fluoroquinolones studied demonstrated a concentration-dependent increase in the relative luminescence values (RLVs) when transfected with MDR1 reporter construct (**Figure 22**).

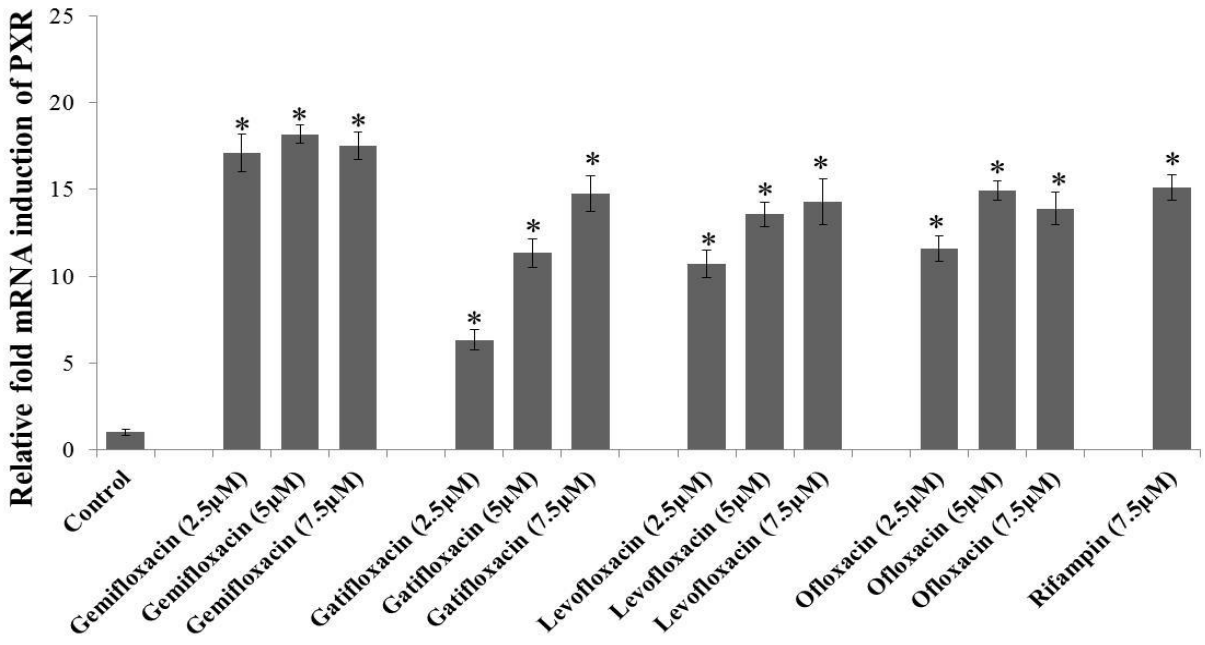

Figure 19. Relative fold induction of PXR mRNA in LS-180 cells treated with gemifloxacin, gatifloxacin, levofloxacin, ofloxacin (2.5, 5 and 7.5 µM) and rifampin (7.5 µM) for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

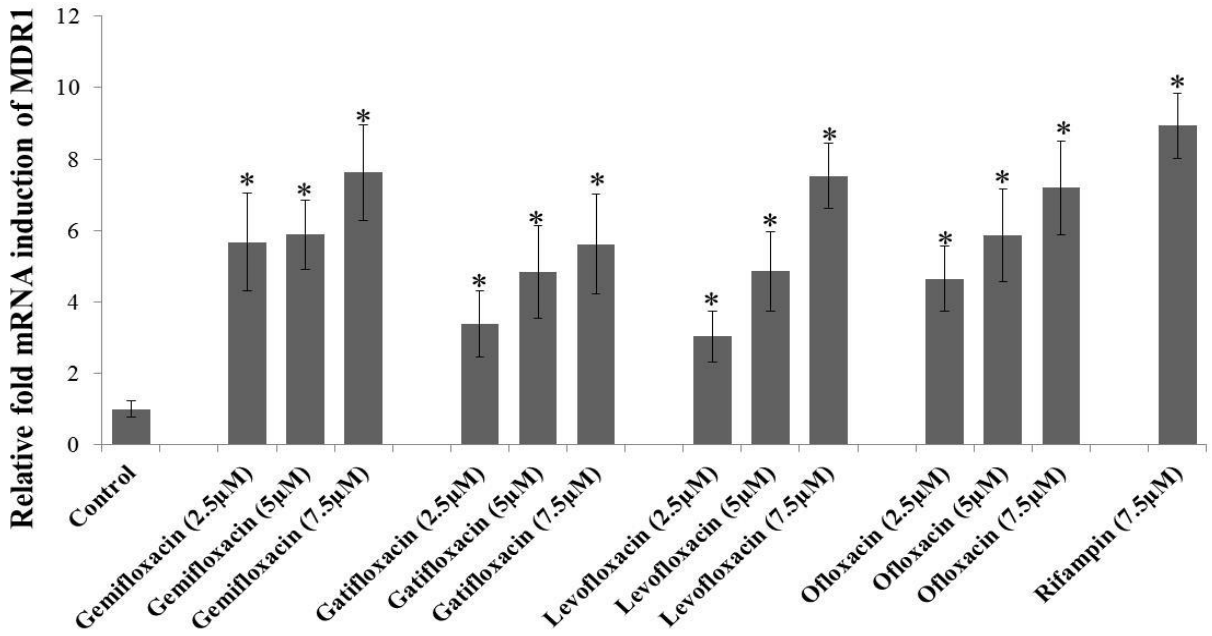

Figure 20. Relative fold induction of MDR1 mRNA in LS-180 cells treated with gemifloxacin, gatifloxacin, levofloxacin, ofloxacin (2.5, 5 and 7.5 µM) and rifampin (7.5 µM) for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

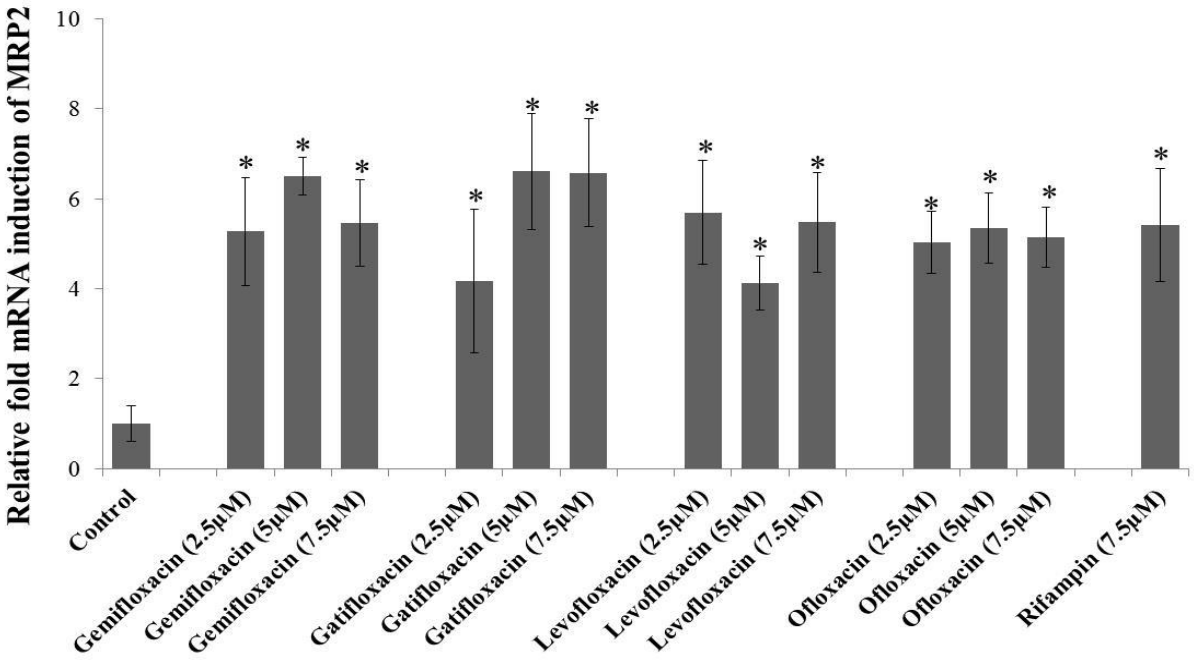

Figure 21. Relative fold induction of MRP2 mRNA in LS-180 cells treated with gemifloxacin, gatifloxacin, levofloxacin, ofloxacin (2.5, 5 and 7.5 μM) and rifampin (7.5 μM) for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

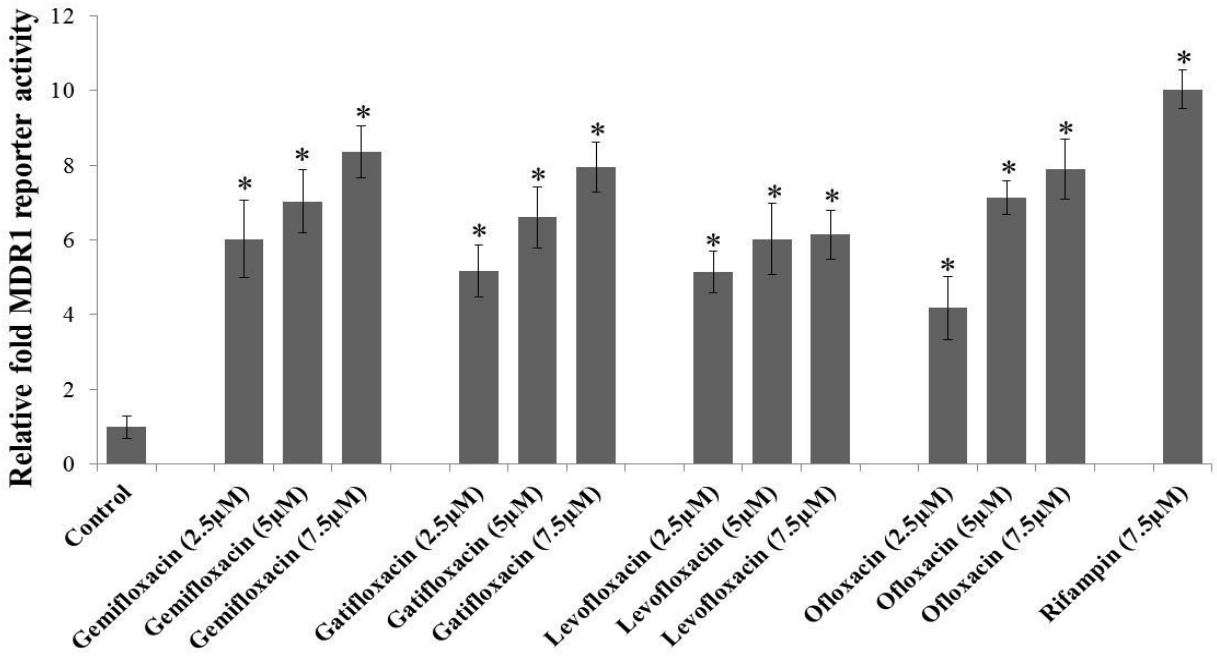

Figure 22. Relative fold expression of MDR1 reporter activity values in LS-180 cells treated with gemifloxacin, gatifloxacin, levofloxacin, ofloxacin (2.5, 5 and 7.5 μM) and rifampin (7.5 μM) for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

With the MRP2 reporter construct, gemifloxacin and gatifloxacin showed a linear increase in reporter activity while the other two drugs (levofloxacin and ofloxacin) had a similar rise (~ 6 fold) with all the concentrations (**Figure 23**). Rifampin exhibited a 10 fold rise in RLVs with both the reporter constructs.

Relative Fold Expression of MDR1 and MRP2 Protein Levels

LS-180 cells were treated with the highest concentration (7.5 µM) of each fluoroquinolone and rifampin (7.5 µM). Post treatment, cell lysate was extracted and the relative levels of MDR1 and MRP2 proteins were monitored. Protein expression was quantified by spot densitometry using AlphaEase software (AlphaInnotech, San Leandro, CA). All the fluoroquinolones significantly induced the protein expression of MDR1. Gemifloxacin and gatifloxacin showed a marked increase in MDR1 protein expression by 2 fold. Levofloxacin and ofloxacin showed 1.9 and 1.6 fold rise in MDR1 protein expression levels (**Figure 24**). A similar increase in MRP2 protein levels was also evident when treated with these drug molecules. Gemifloxacin and gatifloxacin induced MRP2 protein level (~ 2.3 fold) as that of the positive control rifampin. Levofloxacin and ofloxacin also showed 2 fold induction in the protein expression of MRP2 (**Figure 25**).

Cellular Accumulation of MDR1 and MRP2 Substrates

Uptake of [^{3}H] digoxin and [^{14}C] erythromycin was assessed to examine if the induction in MDR1 and MRP2 mRNA and protein levels further translates to functional activity. A significant decrease in the uptake of [^{3}H] digoxin was evident in treated cells relative to control cells. Gemifloxacin and gatifloxacin diminished digoxin uptake by almost 30% and 25% respectively, while the other two fluoroquinolones and rifampin lowered the uptake by 20% (**Figure 26**).

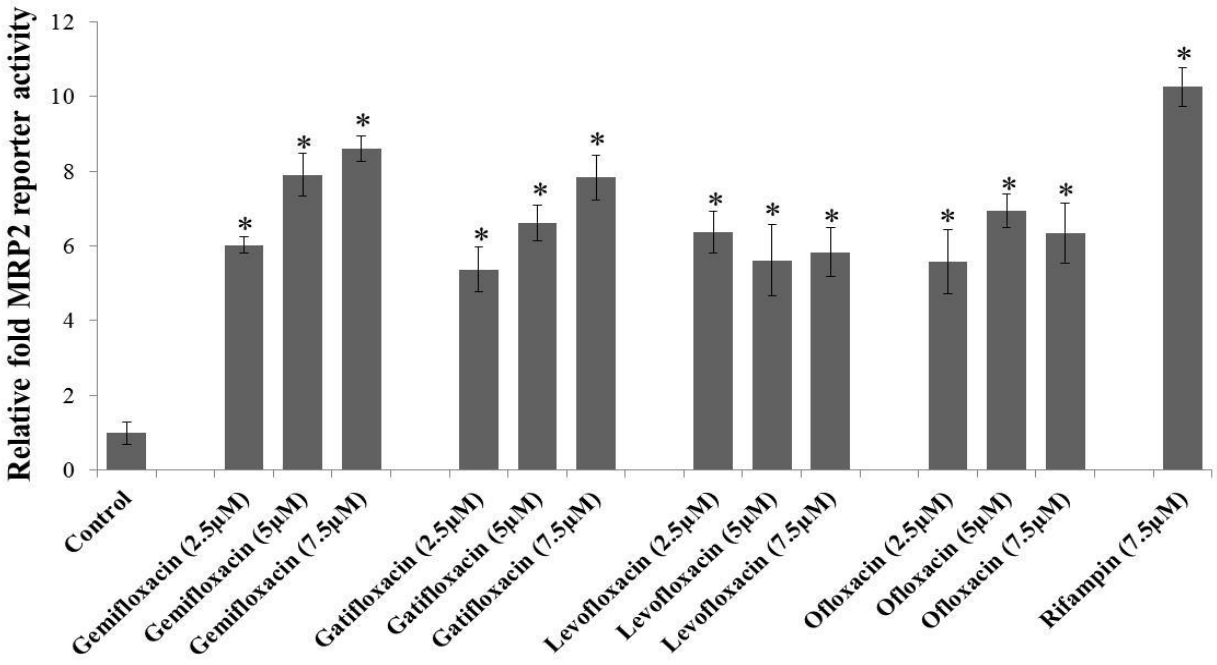

Figure 23. Relative fold expression of MRP2 reporter activity values in LS-180 cells treated with gemifloxacin, gatifloxacin, levofloxacin, ofloxacin (2.5, 5 and 7.5 μM) and rifampin (7.5 μM) for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

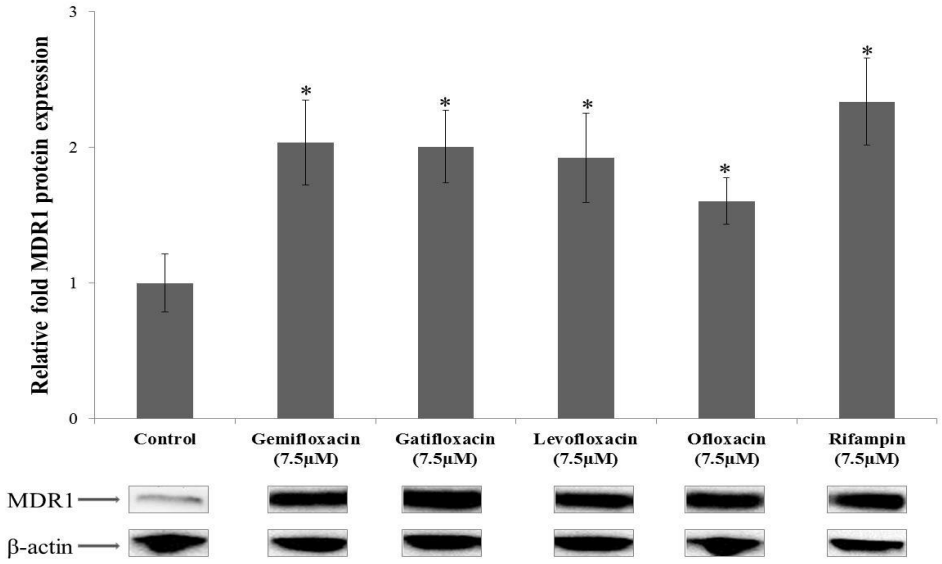

Figure 24. Relative fold induction of MDR1 protein levels in LS-180 cells treated with 7.5 µM of gemifloxacin, gatifloxacin, levofloxacin, ofloxacin and rifampin for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

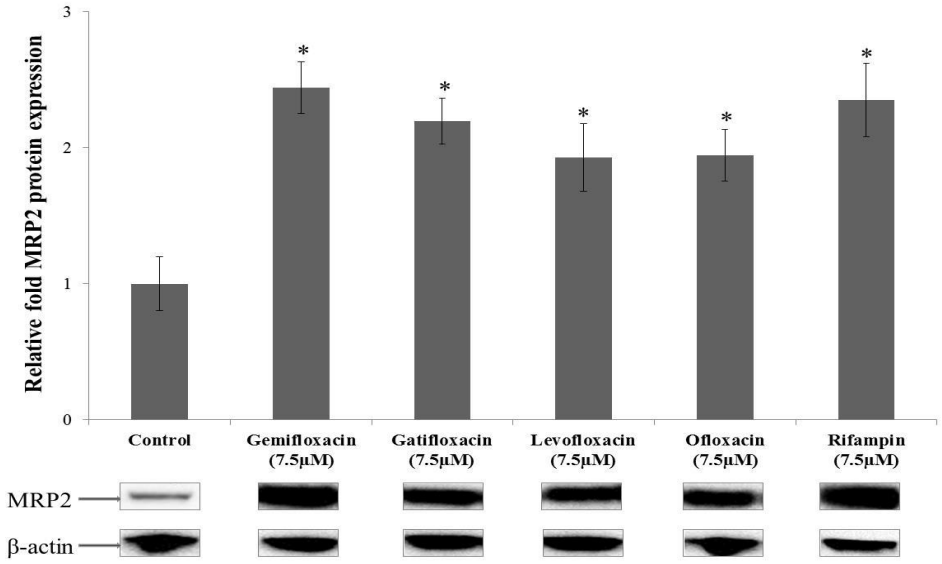

Figure 25. Relative fold induction of MRP2 protein levels in LS-180 cells treated with 7.5 µM of gemifloxacin, gatifloxacin, levofloxacin, ofloxacin and rifampin for 72 hours. Values represent mean ± SE. * represents statistical significance from control at P-value of <0.05.

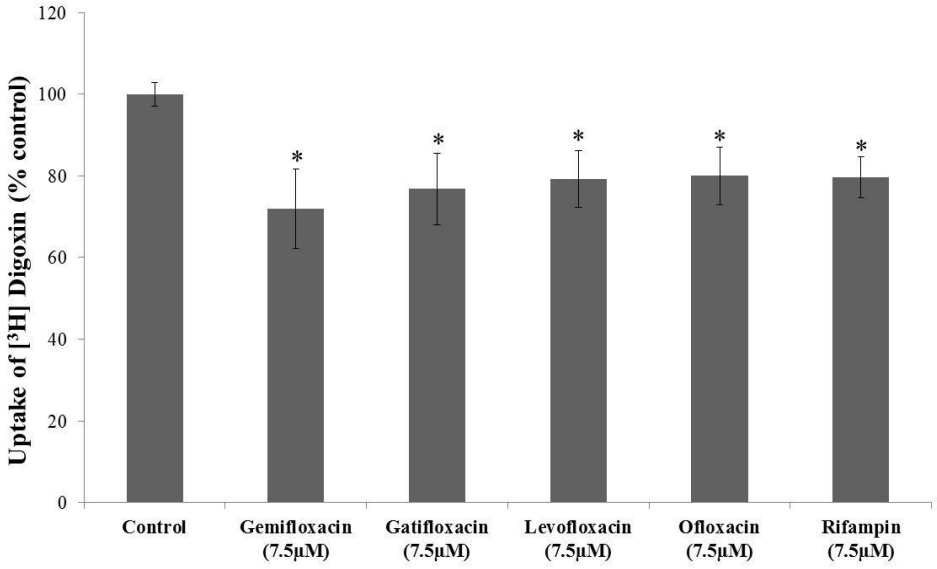

Figure 26. Cellular accumulation of [^{3}H] digoxin in LS-180 cells treated with 7.5 µM of gemifloxacin, gatifloxacin, levofloxacin, ofloxacin and rifampin for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Similarly, the uptake of [^{14}C] erythromycin was also assessed post treatment on LS-180 cells. All the fluoroquinolones and rifampin reduced the uptake of this substrate by ~20% compared to control/untreated cells (**Figure 27**).

Cell Viability Assay

A cell viability assay was performed to evaluate the cytotoxicity of the highest concentration (7.5 µM) of each fluoroquinolone and rifampin post treatment for 72 hours on LS-180 cells. The highest concentration tested was found to be non-cytotoxic to the cells as evident by the number of viable cells (**Figure 28**). This assay indicated that the highest concentration of all fluoroquinolones did not elicit any cytotoxic effects or cause any toxic stress to LS-180 cells when treated for 72 hours.

Discussion

Fluoroquinolones (fluorinated 6-quinolones) represent a class of antibacterial agents widely indicated in the treatment of various bacterial infections including ocular, lower respiratory, urinary tract, biliary-tract, bone and joint infections. However, their clinical utility has been impended due to their widespread misuse and overuse, leading to the development of resistance [267]. A major determinant for development of resistance is the induction of ABC efflux transporters regulated by the nuclear receptor PXR. Hence, the primary objective of this study was to assess the differential expression pattern and functional activity of nuclear receptor (PXR) and efflux transporters (MDR1 and MRP2) upon long term treatment with fluoroquinolones. LS-180 cells were selected for our studies since they are considered as high throughput cell culture models to study PXR mediated induction of efflux transporters [268, 269].

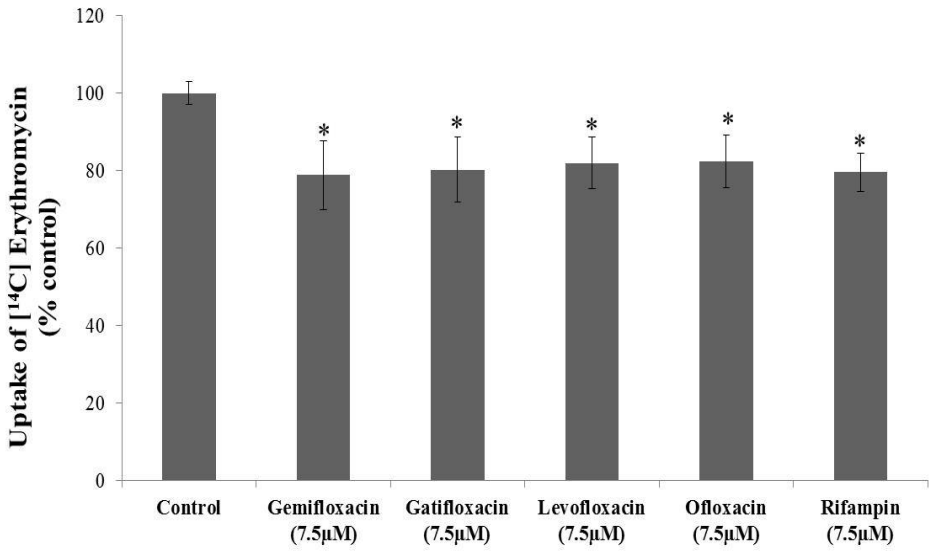

Figure 27. Cellular accumulation of [^{14}C] erythromycin in LS-180 cells treated with 7.5 µM of gemifloxacin, gatifloxacin, levofloxacin, ofloxacin and rifampin for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

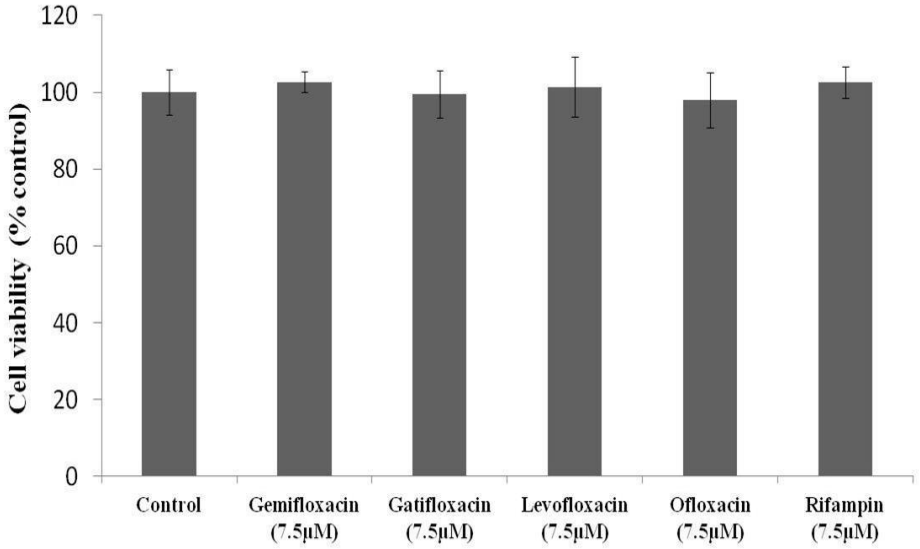

Figure 28. Cell viability assay on LS-180 cells treated with 7.5 µM of gemifloxacin, gatifloxacin, levofloxacin, ofloxacin and rifampin for 72 hours. Values represent mean ± SD.

All four fluoroquinolones tested in the study demonstrated a significant induction in the RNA levels of PXR. This study confirms the promiscuous nature of PXR and the ability of different molecules to activate PXR. Upon transcriptional activation, PXR heterodimerizes with RXR and promotes the transcription of various target genes by binding to a specific region (A/G)G(T/G)TCA in the promoter sequence [164]. Hence, the RNA levels of downstream target genes of PXR such as MDR1 and MRP2 were also examined. All the fluoroquinolones demonstrated a variable rise in MDR1 mRNA levels, with gemifloxacin showing the highest induction. Surprisingly, mRNA levels of MRP2 were induced to a similar extent with all the concentrations of fluoroquinolones studied. This variable degree of activation possibly arises from crosstalk between different nuclear receptors and various signaling mechanisms [270].

In order to confirm PXR mediated activation of efflux transporters by fluoroquinolones, cell based reporter assays have been performed as an alternative to study animal based *in vivo* PXR activation. The relative luminescence values (RLVs) showed a concentration-dependent increase when transfected with the pGL3-MDR1 vector. Both the gene expression analysis and reporter studies demonstrate that fluoroquinolones exhibit a concentration-dependent activation of MDR1 *via* PXR, with gemifloxacin demonstrating the highest level of induction. Similarly, LS-180 cells co-transfected with the pGL3-MRP2 vector demonstrated that only gemifloxacin and gatifloxacin produced a concentration-dependent activation of MRP2. Levofloxacin and ofloxacin showed similar rise in reporter activity values with all the three different concentrations studied. This study has further confirmed PXR mediated activation of MRP2 by all the fluoroquinolones.

Results from immunoblot analysis suggest that elevated RNA levels of MDR1 and MRP2 efflux transporters are being translated to protein expression. Though the mRNA expression

levels were induced to a greater extent, the protein expression levels were not comparably higher. Several possible biological reasons can account for such poor correlation in expression levels between mRNA and protein. These include post transcriptional and translational modifications, along with the half-lives of respective proteins [271]. Further, research has shown that only 20-40% of corresponding mRNA levels translate to protein concentration [272]. Uptake studies were performed to assess if increased protein expression levels translate into increased functional activity. Reduced cellular accumulation of [^{3}H] digoxin and [^{14}C] erythromycin was the result of elevated functional activity of both the efflux proteins post long term treatment with fluoroquinolones. Results from this study are consistent with Vallet et. al who studied the cellular accumulation of rhodamine-123 in J774 macrophage wild-type cells and moxifloxacin treated cells. Cellular accumulation of rhodamine-123 was significantly lower in moxifloxacin treated macrophage cells compared to wild-type cells. This study also suggested overexpression of efflux transporters upon prolonged treatment with moxifloxacin [273].

Conclusions

In summary, this study provides an evidence-based scientific explanation that all the fluoroquinolones tested induce the expression of efflux proteins (MDR1 and MRP2) *via* activation of nuclear factor (PXR). The activated 'master xenosensor' affects the cellular uptake, pharmacokinetics, toxicity and drug-drug interactions of many xenobiotics. Induction of efflux transporters alters drug disposition and consequently affects the plasma drug concentrations. This study provides an insight onto the mechanism of development of MDR with long term usage of these drugs and warrants their misuse.

CHAPTER 4

RITONAVIR: A NOVEL THERAPEUTIC FOR OVERCOMING DRUG RESISTANCE IN CANCER CHEMOTHERAPY

Rationale

Chemotherapy represents the most common treatment modalities for cancer. However, a major impediment to successful chemotherapy is the development of multidrug resistance (MDR). MDR is described as a phenomenon where resistance to one drug often confers resistance to several structurally and functionally unrelated drugs. Resistance to chemotherapy can either be *intrinsic* (resistance exists before the drug treatment is initiated) or *acquired* (resistance develops during treatment) [2]. Several possible mechanisms and molecular alterations have been implicated in the development of MDR, including activation of efflux transporters and metabolizing enzymes, mutation of drug targets, tumor microenvironment conditions (hypoxic conditions leading to vascularization) and altered cellular repair mechanisms (activation of DNA repair, mutant p53 and impaired apoptosis) [3].

Induction of ATP-binding cassette (ABC) efflux transporters and drug metabolizing enzymes was found to be the major cause of MDR development in response to chemotherapy [18, 88]. Among the different ABC transporters, P-glycoprotein (P-gp/MDR1), multidrug resistance protein 2 (MRP2) and breast cancer resistant protein (BCRP/MXR) play a predominant role in acquisition of drug resistance. P-gp for example exports several anticancer agents (doxorubicin, paclitaxel, dasatinib, erlotinib, gefitinib, vinblastine and vincristine) and its overexpression is correlated with drug resistance and treatment failure. Similarly, overproduction of MRP2 is responsible for reducing effect of several chemotherapeutics including etoposide, mitoxantrone and valsartan; while BCRP overexpression has been attributed to resistance for

topoisomerase I inhibitors, anthracyclines and mitoxantrone [73]. Also, metabolizing enzymes such as cytochrome P450 (CYP450) are induced in response to many anticancer drugs causing rapid biotransformation of active molecule, resulting in treatment failure [147, 148]. Also, many drug molecules require enzymatic biotransformation to exert their cytotoxic effect. For example, cytarabine, a nucleoside drug extensively administered for leukemia, requires phosphorylation by deoxycytidine kinase to active form cytarabine triphosphate. Cancer cells acquire resistance *via* downregulation/mutation of involved metabolic enzyme [274]. Two recent review articles have discussed mechanisms and insights of drug resistance in cancer [257, 275].

Drug resistance in HIV patients is also well known. Application of highly active antiretroviral therapy (HAART) played an instrumental role in reducing AIDS related mortality and morbidity. A prototypical HAART combination includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a boosted protease inhibitor (PI). Ritonavir, a first generation PI is considered as a powerful boosting drug. Inhibition of CYP3A4 and efflux transporters by ritonavir forms the basis for enhancing the concentration of concomitantly administered drugs. Pharmacokinetic 'boosting' by ritonavir enables higher sustained levels of the coadministered drug. Such boosted regimens have been utilized to overcome resistance, allowing simplified dosage regimen and potentially improving patient adherence. This combination is clinically proven beneficial for individuals with HIV infection [276]. Therefore, a hypothesis has been tested to overcome drug resistance in cancer employing ritonavir and anticancer drug combination. This hypothesis has been examined *in vitro* using model intestinal cells (LS-180). These cells have been selected to study expression levels of efflux transporters (MDR1, MRP2) and metabolizing enzyme (CYP3A4). Various efficacy studies including cell proliferation, migration and apoptosis have also been performed on human breast

adenocarcinoma cells (T47D) and prostate cancer cells (PC-3) due to their aggressive phenotypic nature.

Materials and Methods

Materials

Doxorubicin, paclitaxel, tamoxifen, vinblastine and ritonavir were obtained from Sigma Chemicals (St. Louis, MO). LS-180, T47D and PC-3 cells were procured from American Type Culture Collection (Manassas, VA). [^{3}H] Lopinavir (specific activity: 0.5 Ci/mMol) was purchased from Moravek Biochemicals (Brea, CA). Dulbecco's modified eagle medium (DMEM), RPMI 1640, stable trypsin replacement (TrypLE™ Express), non-essential amino acids and Vivid™ CYP450 screening kit were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 and 25 cm^2 growth area), 12-well plates (3.8 cm^2 growth area per well) and 96-well plates (0.32 cm^2 growth area per well) were purchased from Corning Costar Corp. (Cambridge, MA). The buffers for cDNA synthesis (oligodT, dNTP, $MgCl_2$, M-MLV reverse transcriptase), CellTiter 96® aqueous non-radioactive cell proliferation assay and ApoONE® homogeneous caspase-3/7 assay kit were obtained from Promega Corporation (Madison, WI). Light Cycler 480® SYBR I green master mix was obtained from Roche Applied Science (Indianapolis, IN). QCM™ 24-well colorimetric cell migration assay kit was purchased from Millipore (Billerica, MA). BCA protein assay reagent was purchased from Thermo Fisher Scientific Inc (Rockford, IL). TRI Reagent® and all other chemicals were products of commercial grade purchased from Sigma Chemicals and used without further purification.

Cell Culture

LS-180 and T47D cells were maintained in T-75 flasks at 37°C in a humidified atmosphere of 5% CO_2 and 90% relative humidity with DMEM medium supplemented with 10% FBS (heat inactivated), 1% non-essential amino acids, 20 mM HEPES, 29 mM sodium bicarbonate and 100 µg/mL of penicillin and streptomycin each. PC-3 cells were maintained in RPMI 1640, supplemented with 10% FBS (non-heat inactivated), 15 mM HEPES, 24 mM sodium bicarbonate and 100 µg/mL of each penicillin and streptomycin. The medium was replaced every alternate day. After reaching 80-90% confluency, cells were passaged using TrypLE™ Express and seeded at a density of one million cells in 25 cm^2 flasks, 250,000 cells/well in 12-well plates or 10,000 cells/well in 96-well plates. Drug solution stocks were prepared in dimethyl sulfoxide (DMSO) and sterile filtered using 0.22 µm filters. Prior to initiation of a treatment; stocks were diluted in appropriate volume of the medium and final concentration of DMSO did not exceed 0.5% (v/v). LS-180 cells were used for gene/protein expression and functional activity studies. T47D and PC-3 cells were used for efficacy studies upon cotreatment.

Quantitative Gene Expression Studies

LS-180 cells were treated with drug solutions for 72 hours. Following treatment, RNA was extracted from cells using TRI® reagent following manufacturer's protocol. Cells were lysed in phenol and chloroform was added for phase separation. The aqueous phase containing RNA was collected and 100% isopropanol was added to precipitate RNA. The pellet was washed with 75% ethanol and dissolved in DNase/RNase-free water. RNA was quantified with Nanodrop (Thermo Fisher Scientific, Wilmington, DE). Two microgram of RNA was reverse transcribed with oligodT as template using M-MLV reverse transcriptase. The conditions for reverse transcription were initial denaturation at 70°C for 5 min, reverse transcription at 42°C for 1 hour

and final extension at 72°C for 5 min. For quantitative gene expression analysis, 80 µg of cDNA was amplified using SYBR-green master mix on ABI Prism 5700 Sequence Detection System (Applied Biosystems). Primers were designed with Primer-Blast tool from PubMed and are summarized in **Table 5**. The reactions were carried out with initial denaturation at 70°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 10 sec. PCR specificity was confirmed by melting curve analysis. Relative amount of RNA levels was normalized to the internal standard GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in each sample. Any difference in RNA levels in treated vs control samples was quantified using modified ddCT method [277].

Immunoblot Analysis

After treatment with respective drug solutions, cells were washed with PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl and 135 mM NaCl) and lysed in PBS with 1% Triton X-100 and protease inhibitor cocktail maintained at pH 7.4, for 15-20 min on ice. Upon homogenization, cells were centrifuged at 12,000 rpm for 10 min and the protein lysate was collected. The amount of protein in the lysate was determined using BCA protein assay reagent. An equivalent amount of protein (25 µg) in all samples was separated with poly acrylamide gel electrophoresis (PAGE). The protein was then transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked overnight at 4°C with 2% non-fat dry milk and 0.25% BSA prepared in 0.05 M tris buffered saline. The blot was then exposed to primary P-gp antibody (Abcam, MA) at 37°C for 4 hours. After subsequent washes the blot was then exposed to secondary antibody-goat anti rabbit HRP conjugate (Santa Cruz Biotechnology, Inc., CA). Finally the blot was visualized for the protein using chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) in chemiImager 8900 (AlphaInnotech, San Leandro, CA).

Table 5. Primers used in quantitative gene expression analysis. Sequence is given from 5'->3'.

Gene	Forward primer	Reverse primer
GAPDH	ATCCCTCCAAAATCAAGTGG	GTTGTCATGGATGACCTTGG
MDR1	CTTATGCTCTGGCCTTCTGG	TGCTTCAATGCTTGGAGATG
MRP2	AAATATTTTGCCTGGGAACC	TGTGACCACAGATACCAGGA
CYP3A4	ACCGTGACCCAAAGTACTGG	GTTTCTGGGTCCACTTCCAA

Uptake Study

Uptake studies with [^{3}H] lopinavir were conducted to confirm the functional activity of the transcribed genes. This study was performed on LS-180 cells plated in 12-well plates. After treatment, the drug solutions were aspirated and cells were washed twice with 2 mL of DPBS (30 mM NaCl, 2.5 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose and 20 mM HEPES) at pH 7.4 for 10 min. Uptake was initiated by adding 0.5 mL of [^{3}H] lopinavir (0.5 µCi/mL) into the wells. After 30 min of incubation, solution was removed and uptake was terminated with ice-cold stop solution (200 mM KCl and 2 mM HEPES). Cells were lysed overnight at 25°C using lysis solution (0.1% (v/v) Triton-X in 0.3 N NaOH). Cell lysate was then quantified for radioactivity with a scintillation counter (BeckmanCounter, Fullerton, CA). The amount of radioactivity was normalized to protein count and analyzed following a previously published method [252].

Vivid™ CYP3A4 Assay

This assay was performed on cells plated in 96-wells. Cells treated with 100 µL of drug solutions were subsequently lysed with 100 µL of lysis solution. Forty microliters of cell lysate was then added to 60 µL of master mix containing Vivid™ Red Substrate. The metabolite formed is highly fluorescent and dependent upon cellular CYP3A4 levels. After 30 min incubation, the fluorescent readings were monitored at an excitation wavelength of 530 nm and an emission wavelength of 585 nm by a microtiter plate reader (SpectraFluor Plus, Maennedorf, Switzerland). The readings were then normalized to protein count as described earlier.

Cell Proliferative Assay

This study was performed on T47D and PC-3 cells to determine the IC_{50} value (half-maximal inhibitory concentration of tumor cells growth) of chemotherapeutic drugs

(doxorubicin, paclitaxel, tamoxifen and vinblstine) alone, and in the presence of ritonavir. T47D /PC-3cells were seeded in 96-well plates and cultured for 24 hours, before the initiation of treatment. Later, medium was aspirated and cells were treated with various concentrations of drug alone and in presence of ritonavir for 72 hours. Following treatment, the drug solutions were aspirated and 100 µL of serum free media and 20 µL of MTS and PMS reagents were added to the wells. After incubating for 3 hours, the quantity of formazan product was measured at 490 nm with a plate reader. The amount of formazan formed from dehydrogenase enzymes is directly proportional to the number of viable cells. Data was fitted to a transformed nonlinear regression curve analysis program (GraphPad Prism Version 4.0; GraphPad Software, San Diego, CA) to calculate IC_{50} value.

Migration Assay

This assay was performed in modified Boyden's insert, where cells from an upper compartment were allowed to migrate into a lower compartment across the microporous membrane in the presence of a chemoattractant. Briefly, T47D and PC-3 cells pretreated with drugs were serum starved for 16 hours. Later, 300 µL of cell suspension (1 million/ml) in serum free media was seeded in the upper compartment and 500 µL of 10% serum media was added to the lower compartment. This gradient in serum concentration acts as a driving force for cells to migrate. The cells that had migrated into lower compartment were then incubated with a cell stain solution, subsequently extracted using an extraction reagent and quantified by measuring the absorbance at 560 nm.

Caspase-3/7 Assay

This assay was used to determine the apoptotic induction of chemotherapeutic drugs alone and in presence of ritonavir. The assay consists of a pro-fluorescent substrate which when

cleaved by caspases-3/7 produces fluorescent rhodamine. The amount of fluorescence produced is proportional to the extent of apoptosis in cells. This assay was performed in 96-well plates. After treating the cells, drug solutions were aspirated and 100 µL of serum free media and equal volume of caspase buffer with substrate were added. The contents were gently mixed in a plate shaker for 1 min and incubated for 2 hours at room temperature. Later, the fluorescent readings were monitored at an excitation wavelength of 485 nm and an emission wavelength of 527 nm with a microtiter plate reader.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) or student's t test (Graph Pad InStat Version 4.0) was performed to confirm statistical significance of the experimental values. A P-value of less than 0.05 was considered to be statistically significant. Post-hoc analysis was performed using Scheffe test.

Results

RNA Expression Analysis

LS-180 cells were treated with vinblastine alone and in presence of different concentrations of ritonavir. The concentration of vinblastine was determined from previously published results [265]. Ritonavir concentrations ranging from 1/8th -1/2nd of vinblastine dose were selected, to determine the optimal booster dose. Vinblastine (0.1 µM) alone showed 7 fold induction in MDR1, which was significantly lowered in the presence of ritonavir (25 nM, 30 nM and 35 nM). The minimal concentration of ritonavir (25 nM) equivalent to 1/4th of vinblastine dose significantly lowered MDR1 to the basal level (**Figure 29**). Levene's statistic to test the homogeneity of variance for this data is as follows: $F(6,21) = 2.24$, $p = 0.08$. This concentration was selected as optimal booster dose for all further experiments. Vinblastine exhibited 8 fold

induction in MRP2 levels which significantly diminished in the presence of ritonavir. Vinblastine is a well-known inducer of CYP3A4. Vinblastine alone produced 40 fold induction in CYP3A4 mRNA levels which was completely abolished in presence of ritonavir (Figure **30**).

Protein Expression Analysis

LS-180 cells were treated with vinblastine alone and in the presence of optimal ritonavir dose (25nM) for 72 hours. The relative fold induction of MDR1 protein expression was determined. Vinblastine showed induction in MDR1 protein level which was neutralized when cells were treated with vinblastine and ritonavir (**Figure 31**).

Uptake Study

Lopinavir is a well-known substrate for both MDR1 and MRP2 efflux pumps [244]. To evaluate the functional activity of the efflux pumps upon treatment, uptake of [^{3}H] lopinavir was quantified on LS-180 cells. In vinblastine treated cells, the uptake of [^{3}H] lopinavir was significantly lower compared to control suggesting enhanced activity of efflux pumps. However, in cells treated with vinblastine and ritonavir, the uptake was comparable to control, showing reduced activity of efflux pumps upon cotreatment (Figure **32**).

Vivid™ CYP3A4 Assay

To study the functional activity of CYP3A4 gene, vivid assay was performed following drug treatment. The amount of metabolite formed from the substrate is dependent on levels of CYP3A4 activity in the cells. Compared to control cells, vinblastine treated cells showed 80% increase in metabolite formation which confirms high CYP3A4 activity. However, the cells cotreated with vinblastine and ritonavir, such CYP3A4 activity was significantly diminished, comparable to control value (Figure **33**).

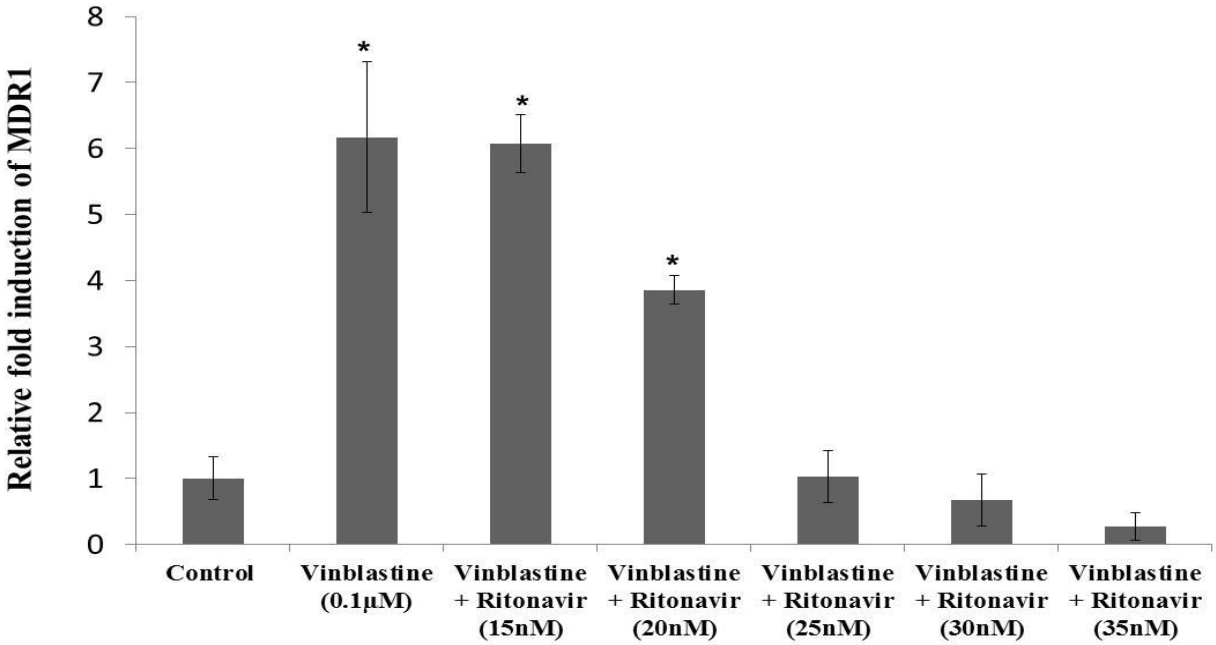

Figure 29. Relative fold induction of MDR1 mRNA in LS-180 cells treated with vinblastine (0.1 μM) alone and in presence of different concentrations of ritonavir for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

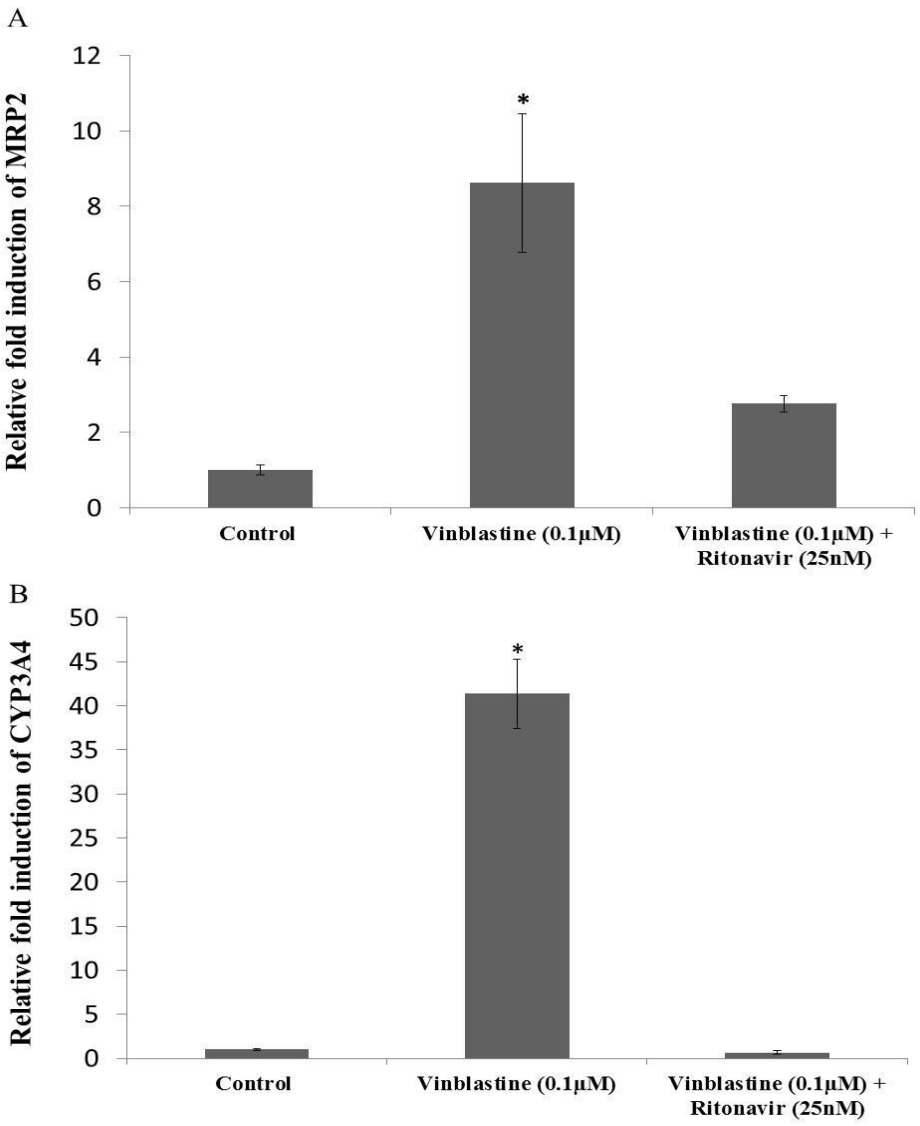

Figure 30. Relative fold induction of (A) MRP2 and (B) CYP3A4 mRNA in LS-180 cells treated with vinblastine (0.1 µM) alone and optimized concentration of ritonavir (25 nM) for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

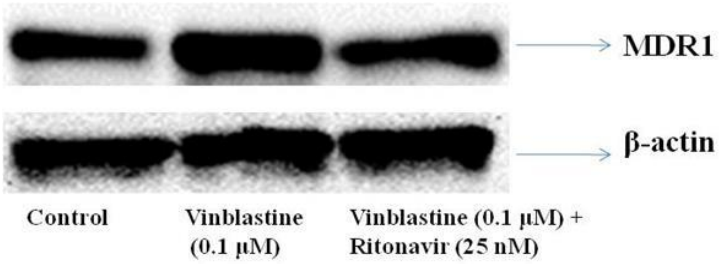

Figure 31. Immunoblot analysis showing the protein expression of MDR1 post treatment with vinblastine (0.1 µM) alone and optimized concentration of ritonavir (25 nM) on LS-180 cells for 72 hours. β-actin was used as an internal control.

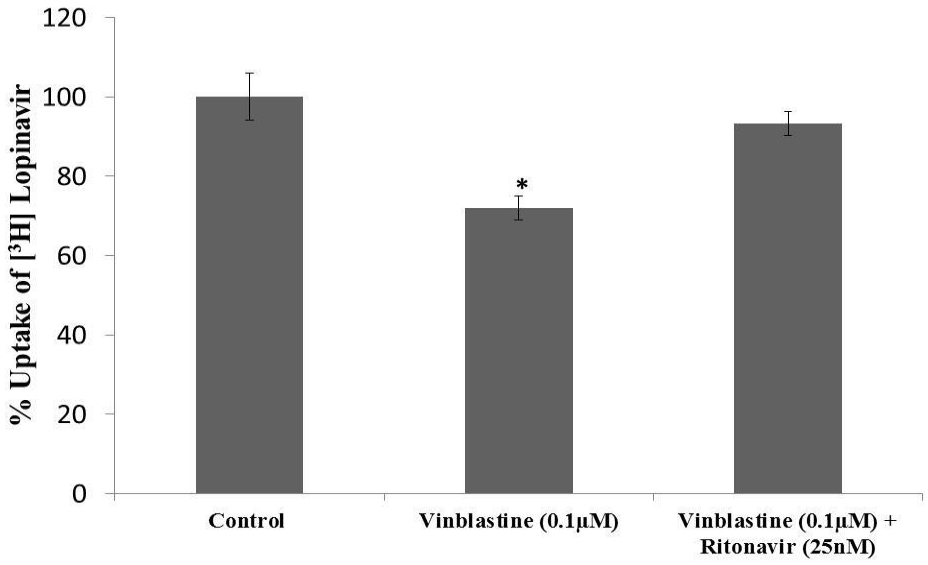

Figure 32. Uptake of [^{3}H] lopinavir post treatment with vinblastine alone (0.1 µM) and optimized concentration of ritonavir (25 nM) on LS-180 cells for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

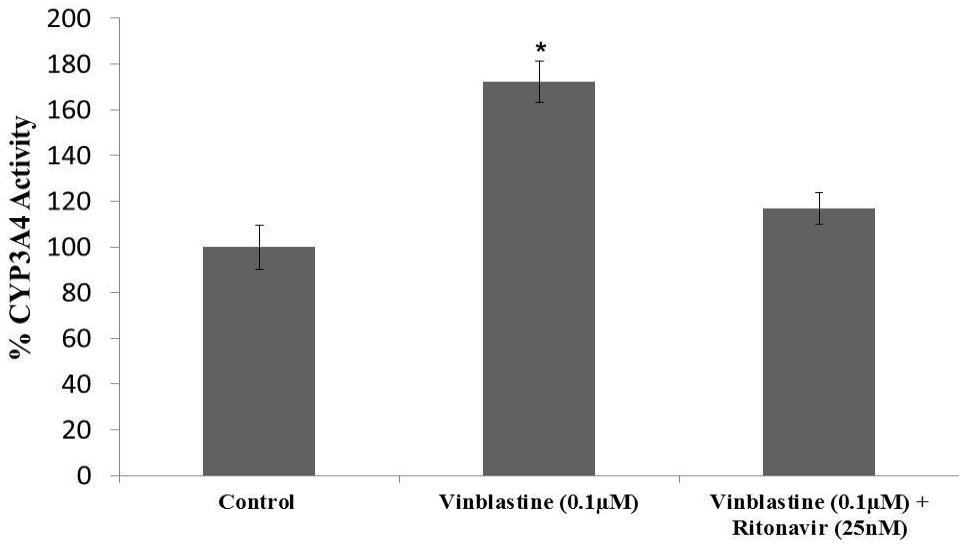

Figure 33. Percentage CYP3A4 activity post treatment with vinblastine alone (0.1 µM) and optimized concentration of ritonavir (25 nM) on LS-180 cells for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Cell Proliferation Assay

MTS assay was performed to determine inhibitory effect of chemotherapeutic agents (doxorubicin, paclitaxel, tamoxifen, and vinblastine) alone and in the presence of ritonavir in T47D cells. IC_{50} values of doxorubicin and paclitaxel (478 ± 13 nM and 3.3 ± 1.2 nM) were reduced by almost four fold in the presence of ritonavir to 167 ± 14 nM and 0.9 ± 0.4 nM, respectively (Figure **34**). Similarly, presence of ritonavir lowered IC_{50} of tamoxifen (3.5 ± 1.1 μM) by almost three fold (1.1 ± 0.6 μM). IC_{50} of vinblastine was observed to be 10.81 ± 1.38 nM. This IC_{50} value was diminished by seven fold in the presence of ritonavir to 1.44 ± 0.45 nM (**Figure 35**). These results show that ritonavir enhanced the ability of all chemotherapeutics to inhibit cell growth.

Migration Assay

T47D cells pretreated with drug solutions were serum starved for 16 hours. The relative numbers of cells that had migrated towards 10% serum media were estimated by a spectrophotometer. The numbers of cells that have migrated was reduced by 10 - 40% in cells treated with chemotherapeutics alone. This reduction was further enhanced to almost 1.5 - 2 fold in cells cotreated with ritonavir (**Figure 36** and **Figure 37**).

Caspase-3/7 Assay

This assay was performed to determine levels of apoptosis in T47D cells in the presence of anticancer agents alone and with ritonavir. The chemotherapeutics alone raised apoptosis by approximately 3 - 4.5 fold and this value was further enhanced by almost two fold in the presence of ritonavir (Figure **38** and Figure **39**).

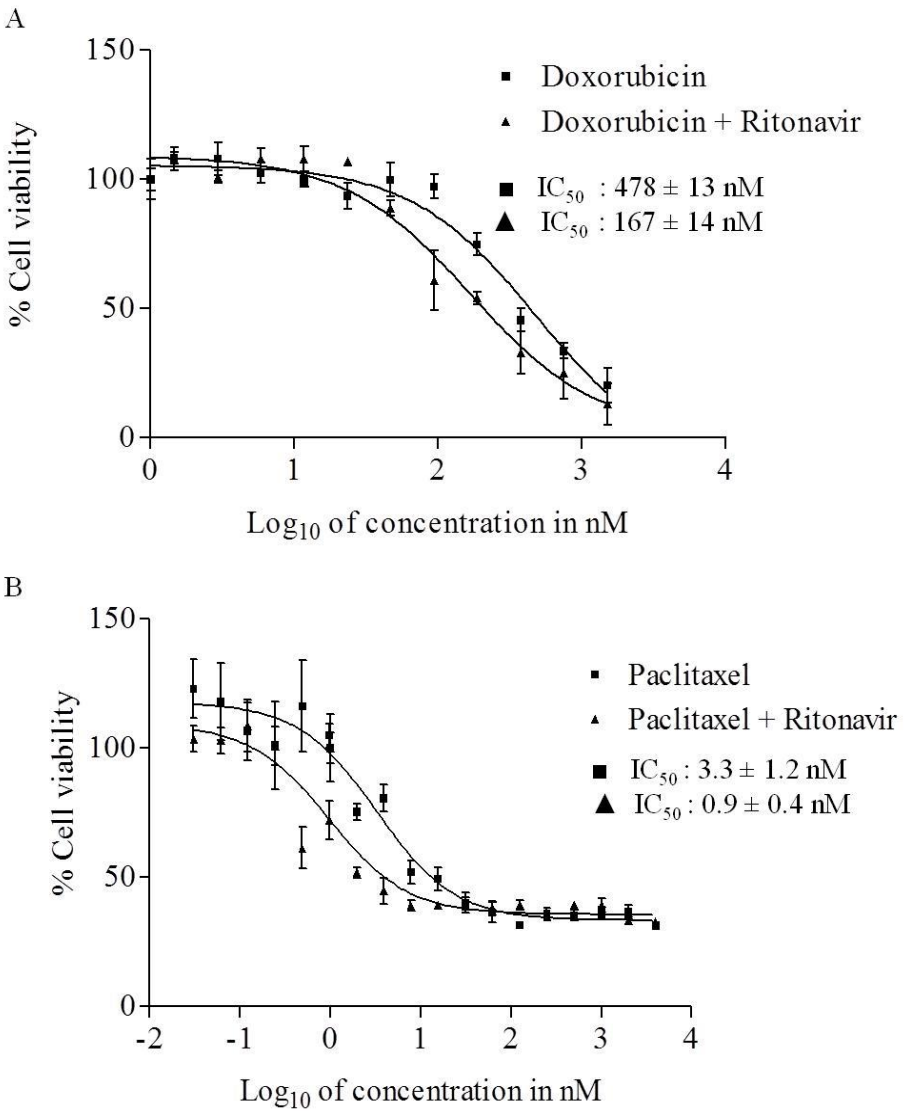

Figure 34. Dose-dependent percentage viability of T47D cells treated with different concentrations of (A) doxorubicin (1.5 µM - 1.5 nM) and (B) paclitaxel (4 µM - 0.03 nM) alone and in presence of ritonavir for 72 hours. Values represent mean ± SD.

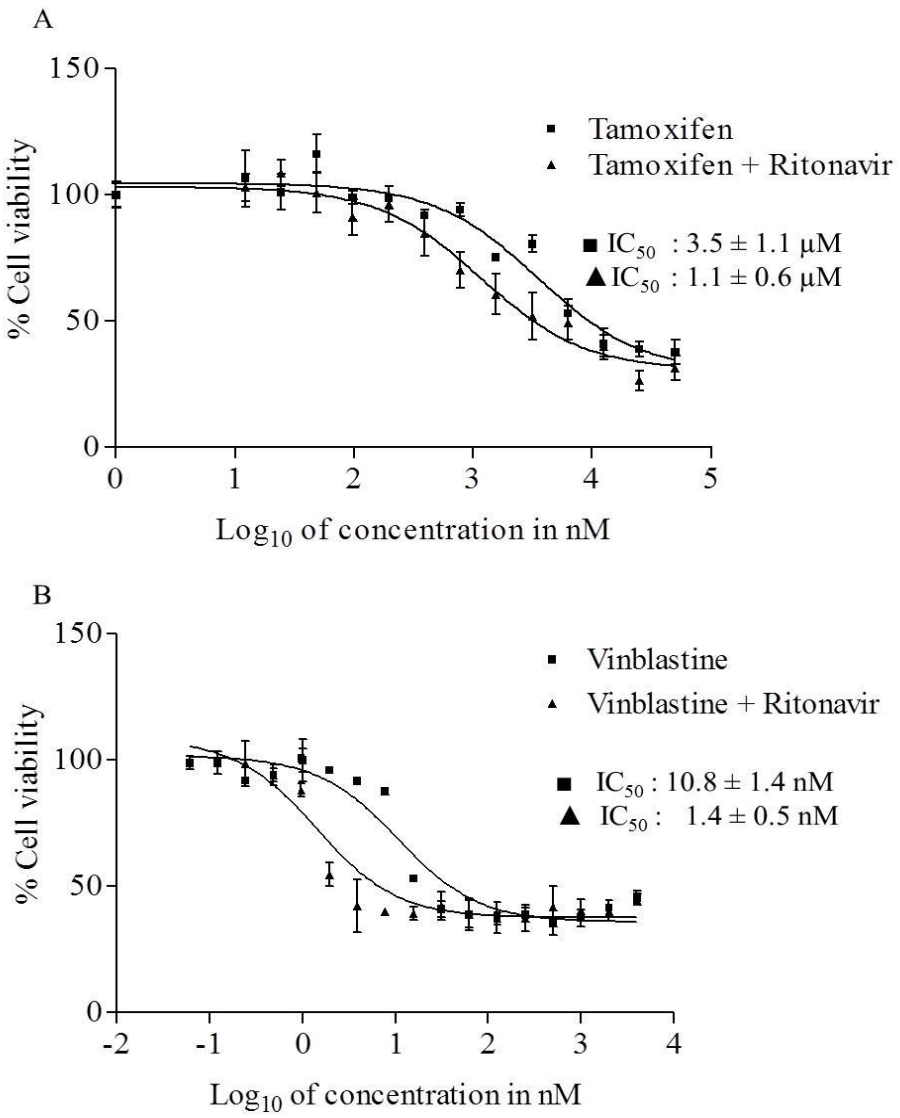

Figure 35. Dose-dependent percentage viability of T47D cells treated with different concentrations of (A) tamoxifen (50 µM - 12 nM) and (B) vinblastine (4 µM - 0.03 nM) alone and in presence of ritonavir for 72 hours. Values represent mean ± SD.

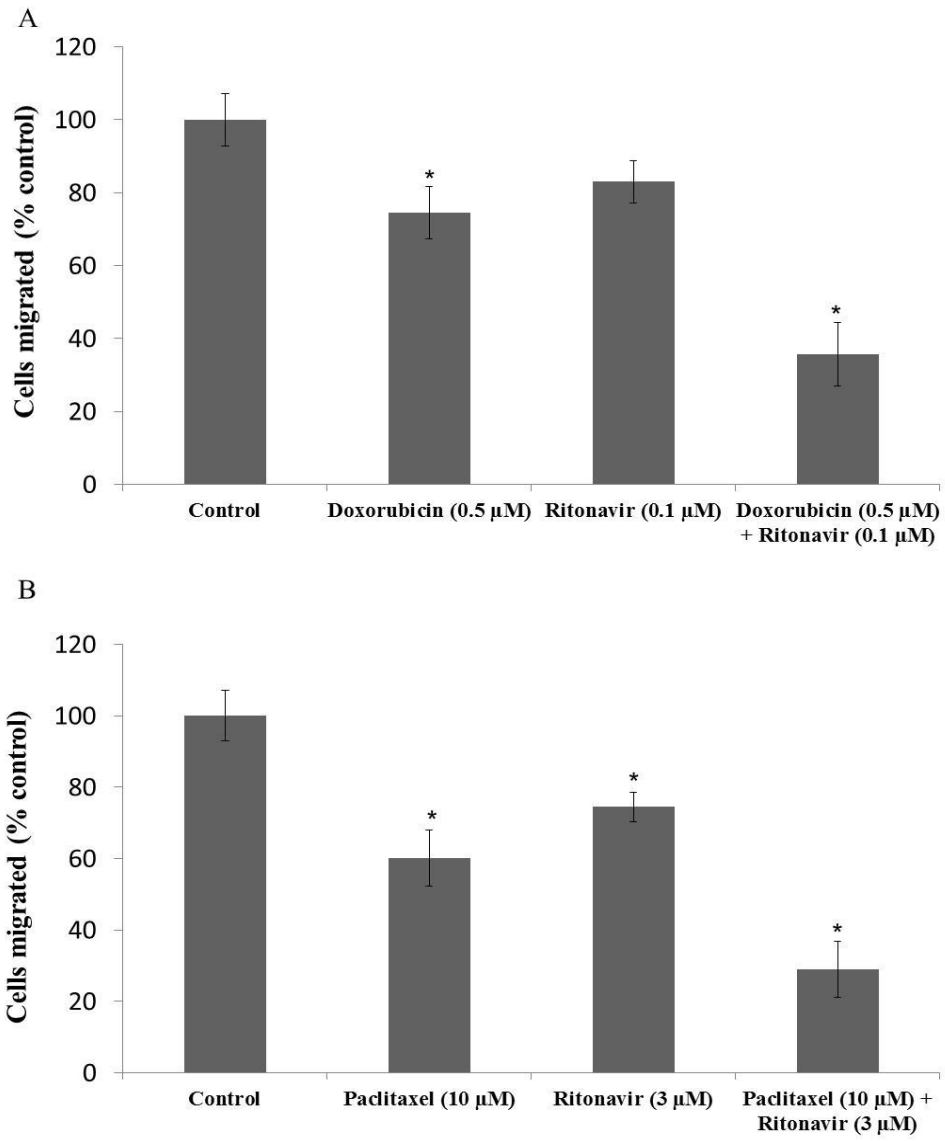

Figure 36. Relative number of T47D cells migrated towards 10% serum media post treatment with (A) doxorubicin and (B) paclitaxel alone and in presence of optimized concentration of ritonavir for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

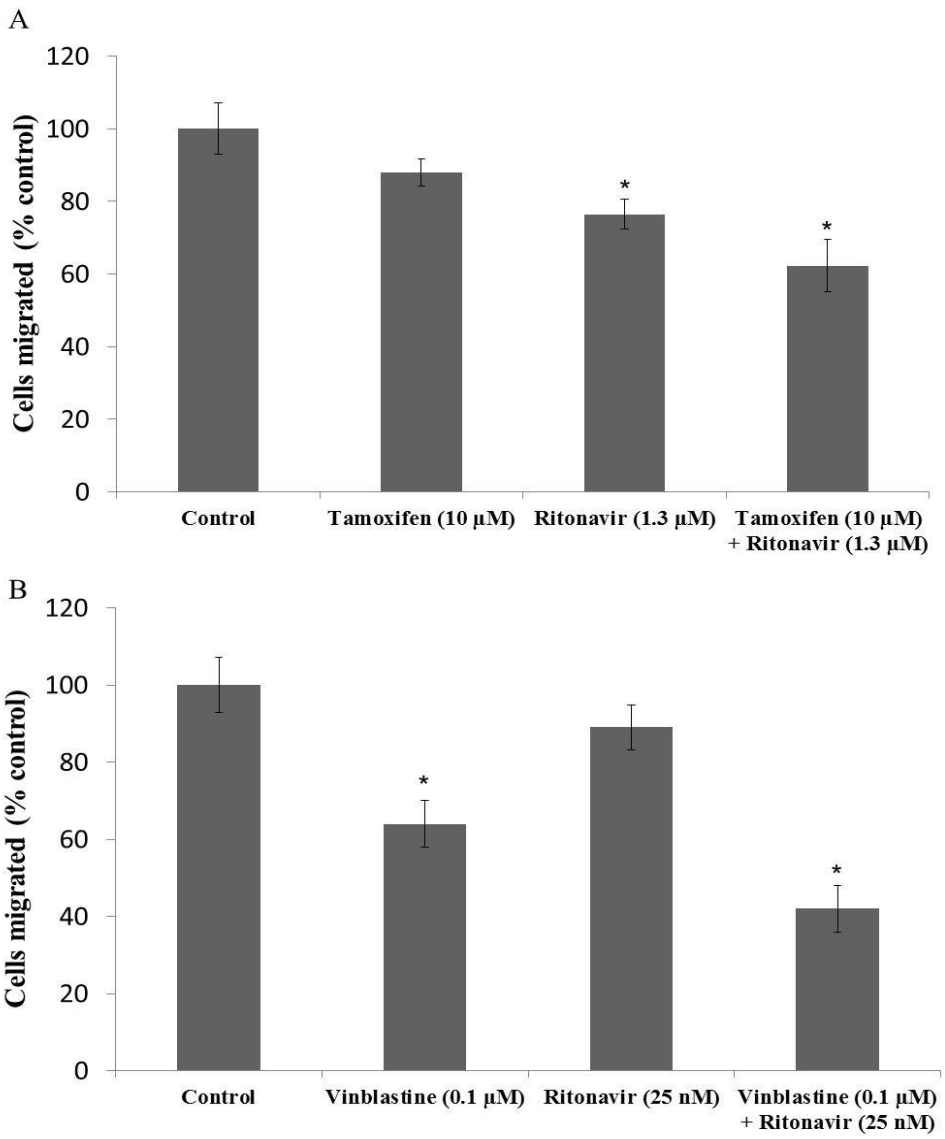

Figure 37. Relative number of T47D cells migrated towards 10% serum media post treatment with (A) tamoxifen and (B) vinblastine alone and in presence of optimized concentration of ritonavir for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

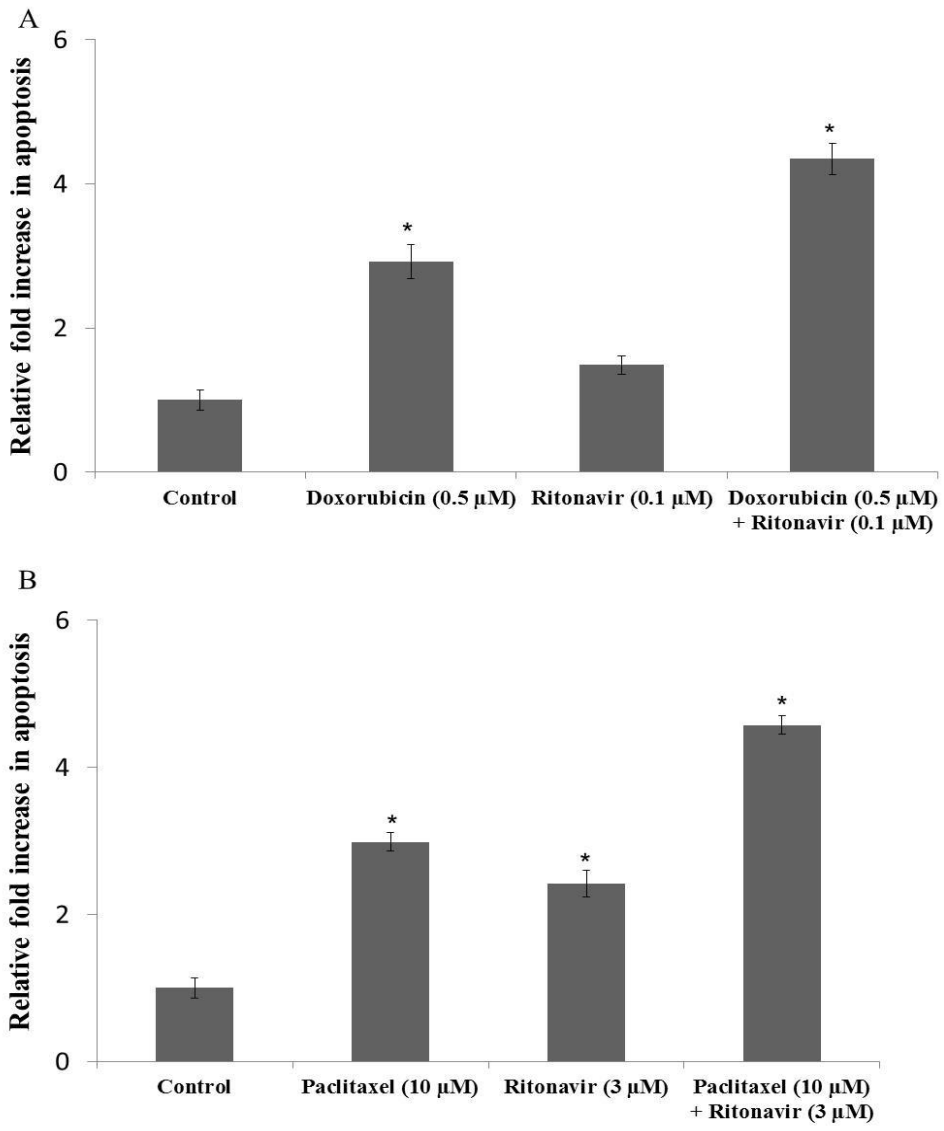

Figure 38. Relative fold apoptosis in T47D cells post treatment with (A) doxorubicin and (B) paclitaxel alone and in presence of optimized concentration of ritonavir for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

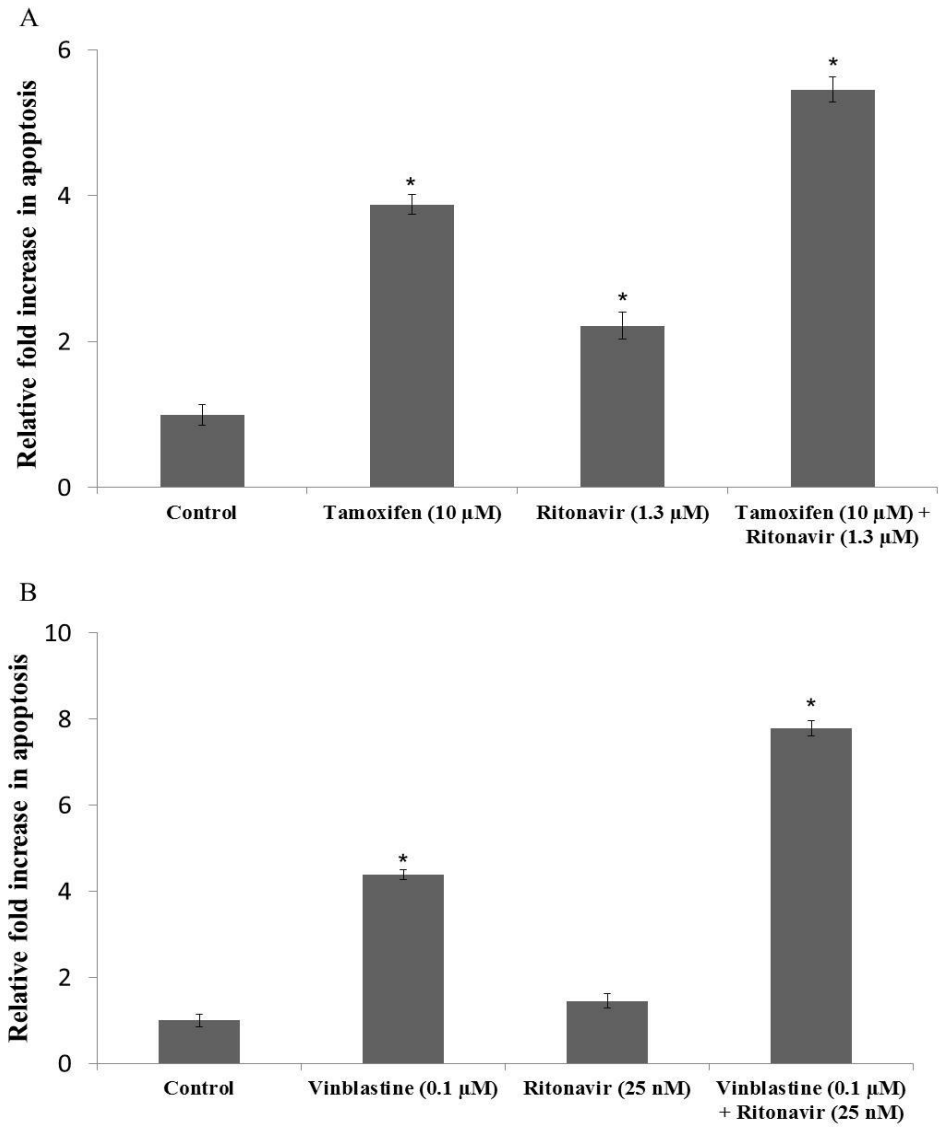

Figure 39. Relative fold apoptosis in T47D cells post treatment with (A) tamoxifen and (B) vinblastine alone and in presence of optimized concentration of ritonavir for 72 hours. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

The relative levels of cell proliferation, migration and apoptosis in T47D and PC-3 cells treated with different chemotherapeutics alone or in presence of ritonavir were summarized in **Table 6** and **Table 7**, respectively.

Discussion

Development of MDR in cancer therapy necessitates progressive increase in dosing of anticancer drugs which in turn may lead to systemic toxicity and adverse side effects. Various mechanisms of drug resistance, including reduced cellular drug accumulation, higher detoxification, hypoxic conditions leading to neovascularization, altered enzymatic activities, downregulation of target and enhanced DNA repair can be considered [149]. The primary objective of this study was to develop strategy for overcoming acquired drug resistance and enhance antitumor activity. LS-180 cells were employed as an in vitro cell culture model to study drug resistance. These cells have been shown to be an excellent model to predict long term drug-drug interactions based on inductions of efflux transporters and metabolizing enzymes [269].

Vinblastine, substrate of both efflux transporters and metabolizing enzymes, was chosen as a model anticancer drug. Prolonged exposure to vinblastine is known to induce efflux transporters and metabolizing enzymes [265]. Overexpression of MDR1 is considered to be a major contributor to drug resistance. In order to neutralize the vinblastine mediated inductions of efflux transporters and metabolizing enzymes, ritonavir, which is a known substrate and inhibitor was selected. Dose dependent studies were performed to optimize ritonavir dose and to evaluate the induction potential of MDR1 gene upon cotreatment. Presence of ritonavir at various concentrations (25nM, 30nM and 35nM) demonstrated a significant lowering of MDR1 overexpression caused by vinblastine. The lowest concentration i.e. 25nM which was adequate to completely overcome this overexpression of MDR1 was selected for all further studies. Upon

treatment with vinblastine alone and in the presence of ritonavir for 72 hours on LS-180 cells, gene expression analysis of MRP2, and CYP3A4 was studied with real time PCR. Vinblastine induced overexpression of MRP2 (8 fold) and CYP3A4 (40 fold) was neutralized in the presence of ritonavir and the gene expression levels almost reached to the endogenous levels of their respective control genes. Results from these experiments clearly suggest that ritonavir can inhibit the ability of vinblastine to induce expression of MDR1, MRP2, and CYP3A4 at the transcriptional level.

To evaluate whether induction of efflux transporters and metabolizing enzyme at the transcriptional level correlates to the protein expression and activity, immunoblot and functional activity studies were performed, respectively. Immunoblot analysis further confirmed the induction of MDR1 by vinblastine alone. This induction was diminished when cells were cotreated with vinblastine and ritonavir. Reduced intracellular accumulation of [^{3}H] lopinavir in vinblastine treated cells confirmed the functional activity of transcribed efflux transporters. On the other hand, no change in [^{3}H] lopinavir uptake in the presence of vinblastine and ritonavir suggest that ritonavir can completely block the functional activity of efflux transporters. CYP3A4 activity was assessed by using Vivid™ assay. An increase in CYP3A4 activity for vinblastine treated cells was found to correlate well with the mRNA levels qualitatively but not quantitatively. Functional activity was elevated by about 1.7 times while mRNA was induced by almost 40 fold. Induced expression of CYP3A4 significantly increased the formation of metabolite confirming elevated CYP3A4 activity. Cotreatment of vinblastine with ritonavir significantly neutralized such increased activity.

Table 6. Ratio of cell proliferation IC_{50} reduction, migration inhibition and apoptosis increase of chemotherapeutics in the presence of ritonavir to chemotherapeutics alone in T47D cells.

Drug	Ratio of enhanced efficacy in presence of ritonavir		
	Cell Proliferation (IC_{50}) ↓	Migration ↓	Apoptosis ↑
Doxorubicin	2.9	2.1	1.5
Paclitaxel	3.7	2.1	1.5
Tamoxifen	3.2	1.4	1.4
Vinblastine	7.7	1.5	1.8

Table 7. Ratio of cell proliferation IC_{50} reduction, migration inhibition and apoptosis increase of chemotherapeutics in the presence of ritonavir to chemotherapeutics alone in PC-3 cells

Drug	Ratio of enhanced efficacy in presence of ritonavir		
	Cell Proliferation (IC_{50}) ↓	Migration ↓	Apoptosis ↑
Doxorubicin	3.9	3.0	2.8
Paclitaxel	4.3	3.6	2.0
Tamoxifen	3.0	2.2	2.5
Vinblastine	3.2	2.5	3.2

With the encouraging results from gene and protein expression analysis, three other chemotherapeutics (doxorubicin, paclitaxel and tamoxifen) were tested for different efficacy studies on T47D and PC-3 cells. Although gene and protein expression studies were conducted on LS-180 cells, efficacy studies were performed on T47D and PC-3 cells due to their aggressive phenotypic nature. Cell proliferative assay was performed in T47D breast cancer cells to evaluate the efficacy of anticancer drug and its cotreatment with ritonavir. IC_{50} value of all four drugs (doxorubicin, paclitaxel, tamoxifen and vinblastine) was lowered in the presence of ritonavir. This observation clearly shows that ritonavir enhances the antitumor efficacy, preventing the proliferation of cancer cells. Recent studies have shown that PIs also possess antiproliferative activity. Ritonavir can reduce proliferation of cancerous cells through inhibition of nuclear factor κB (NFκB) translocation and transcriptional activation [278-280].

Cell migration is another important cellular process involved in angiogenesis, wound healing and immune responses. Migration assay was performed to better correlate in vitro migratory potential of cancer cells to their in vivo invasive properties. This assay was performed to better understand tissue repair and regeneration during cancer progression. All the four drugs, except for tamoxifen, inhibited cell migration compared to control. Interestingly, cells cotreated with ritonavir significantly impaired cell migration by almost 1.5 fold. Thus cell proliferation and migration assays further confirmed that cellular accumulation of vinblastine increased over time in the presence of ritonavir. Apoptosis is a self-defense mechanism for causing cell death to maintain development, homeostasis or aging. A cascade of cleavage events by caspases may lead to apoptosis. The levels of apoptosis were further enhanced in presence of ritonavir. This significant increase in apoptotic cells may be due to enhanced cellular accumulation and also due

to ritonavir, which can also promote apoptosis via inhibition of various signal transducers [281, 282].

Conclusions

In summary, cotreatment with ritonavir neutralizes the induced gene expression of many drug resistant proteins and may overcome MDR. This promising data from our in vitro studies can form the basis for further development of a clinically applicable oral formulation of vinblastine or any other anticancer drug combined with ritonavir. The process of traditional drug development could be fastened since ritonavir is clinically approved for human use [283]. Its repositioning in cancer chemotherapy may provide an excellent platform to reverse and overcome drug resistance besides providing enhanced antitumor efficacy.

CHAPTER 5

HYPOXIA-INDUCIBLE FACTOR 1 (HIF-1): A POTENTIAL TARGET FOR INTERVENTION IN OCULAR NEOVASCULAR DISEASES

Introduction

A constant oxygen supply is essential for proper tissue development, homeostasis and function of all eukaryotic organisms. Cells require oxygen as an electron acceptor during oxidative phosphorylation for efficient ATP production. Oxidative phosphorylation produces higher energy (~18 fold) than glycolysis [284, 285]. Oxygen serves as a major element in regulating membrane transport, intracellular signaling, expression of many genes, and cell survival [286, 287]. Hypoxia (~1% O_2) occurs when tissue oxygenation demand exceeds the vascular supply. Response to reduced oxygen levels is mediated by the transcriptional regulator hypoxia-inducible factor 1 (HIF-1). HIF-1 was first discovered by its ability to induce expression of erythropoietin (EPO) in kidney and liver. Such production of EPO is inversely related to tissue oxygen concentration. In response to hypoxia, EPO is stimulated which in turn promotes red blood cell production and oxygen carrying capacity. This information led to the identification of a hypoxia response element (HRE; 5'-RCGTG-3') in the 3'-enhancer region of EPO [288, 289].

Structure and Regulation of HIF-1

A functional HIF-1 system is expressed in all metazoan species including the simplest animal *Trichoplax adhaerens* [290]. The *HIF1A* gene was mapped on 14q21-q24 human chromosome. HIF-1 is a heterodimeric complex consisting of an oxygen-dependent subunit (HIF-1α) and a constitutively expressed nuclear subunit (HIF-1β) [291]. HIF-1β is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). It was first identified as structural binding component of aryl hydrocarbon receptor (AHR), which induces the transcription of

Cyp1a1 metabolizing enzyme [292]. Both subunits are members of basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) protein family. In human, *HIF1A*, *EPAS1* and *HIF3A* genes encode three different isoforms of HIF-α (HIF-1α, HIF-2α and HIF-3α), respectively [293].

Structurally, HIF-1α exhibits bHLH and PAS domains at the N-terminal. The bHLH domain and N-terminal of PAS (PAS-A) (amino acids/aa 1-166) facilitate DNA dimerization while complete bHLH and PAS domains (aa 1-390) facilitate DNA binding [294, 295]. HIF-1α also exhibits an oxygen-dependent degradation (ODD) domain, two transactivating domains (TADs) and two nuclear localizing signals (NLS). The ODD domain (aa 401-575) located within central region plays a predominant role in regulating stability of HIF-1α with respect to oxygen concentration [296]. Two TADs (N-TAD; aa 531-575 and C-TAD; 786-826) help in recruiting various coactivators required for transcription of target genes. Bridged between them is an inhibitory domain (ID; aa 576-785) capable of repressing their transcriptional activity under normoxic conditions (~20% O_2) [297, 298]. N-NLS (aa 17-30) and C-NLS (aa 718-721) promote nuclear translocation of HIF-1α. However, studies have demonstrated that nuclear import is highly dependent on C-NLS [299]. HIF-1α is ubiquitously expressed in all human tissues, while the other related protein HIF-2α is primarily expressed in lung, endothelium and carotid artery [300-302]. HIF-2α shares 48% structural identity with HIF-1α. A third protein, HIF-3α, is also expressed in many tissues including adult thymus, lung, brain, heart, and kidney. This protein lacks C-TAD. However, the N-terminus of this protein shares 57% and 53% structural homology with HIF-1α and HIF-2α, respectively [303, 304]. A splice variant of HIF-3α is the inhibitory PAS domain (IPAS) protein, primarily expressed in Purkinje cells and corneal epithelium. This variant acts like a negative regulator of HIF-1 by binding to amino terminal region of HIF-1α, preventing transactivation. Further, this protein is also induced under hypoxia in heart and lung,

suggesting a negative feedback mechanism for HIF-1 activity [305, 306]. The domain organization of both the subunits (HIF-1α and HIF-1β) is depicted in **Figure 40**.

Functional activity of HIF-1 is regulated by levels of oxygen-dependent HIF-1α subunit. Although transcription of HIF-1α mRNA occurs at normoxic conditions, the protein is rapidly degraded *via* the ubiquitin proteasome pathway [307, 308]. HIF-1α protein has a very short half-life ($t_{1/2}$~5 min) and its stability is highly regulated by posttranscriptional modifications including hydroxylation, ubiquitination, acetylation, phosphorylation and nitrosation [309, 310].

Prolyl Hydroxylation

Hydroxylation of proline residues led to the identification of oxygen sensing mechanism of HIF-1α [311, 312]. It was considered a major breakthrough in delineating the signal transduction of HIF-1. Mutagenic studies substituting proline stabilized HIF-1α even under normal oxygen tension, demonstrate its importance in regulating transcriptional responses. Two proline residues (Pro/P 402 and 564) located within ODD domain are rapidly hydroxylated by 2-oxoglutarate (2-OG) dependent dioxygenases [313-315]. These dioxygenases recognize a conserved amino acid sequence 'LXXLAP', where X represents any amino acid. Human dioxygenases have been coined as prolyl hydroxylases (PHDs) or HIF- prolyl hydroxylases (HPHs) [316]. PHDs require oxygen for hydroxylation as well as ferrous ion (Fe^{2+}) and ascorbate as cofactors [317]. During hydroxylation an oxygen molecule is split so that one oxygen atom is transferred on to proline while the other reacts with 2-OG to produce succinate and CO_2 [313, 318]. Absolute requirement for Fe^{2+} ion stems from the observation that iron chelators or transition metal ions can suppress hydroxylation either by reducing the availability of Fe^{2+} or substituting Fe^{2+} at the active binding site [319, 320]. Ascorbate plays a very important role in regulating the activity of PHDs and maintaining the Fe^{2+} state of iron [318].

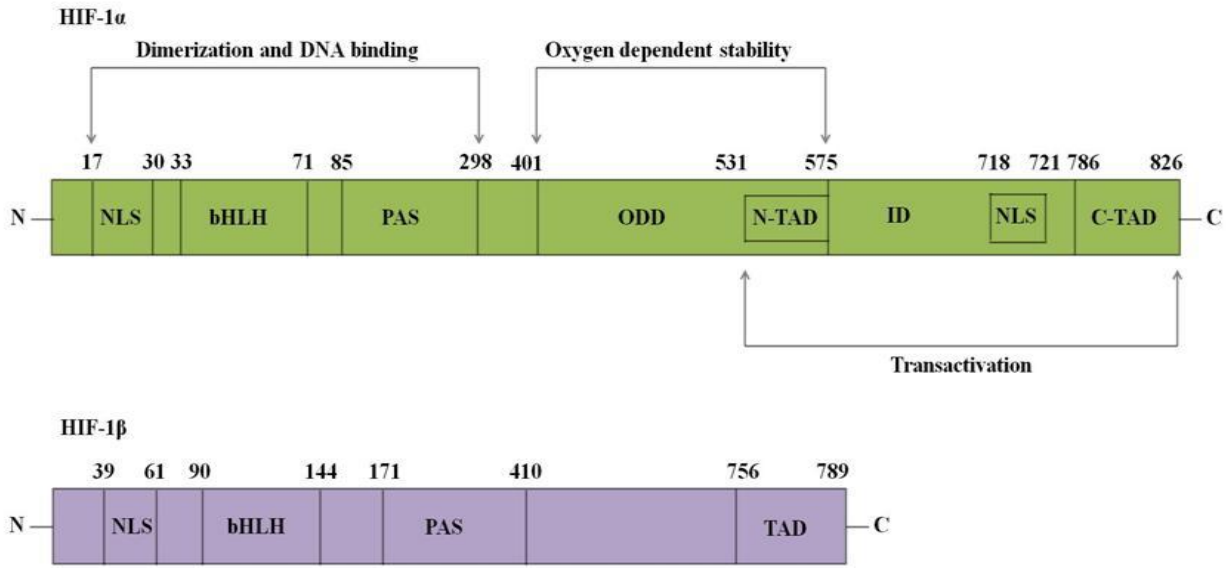

Figure 40. Structure of HIF-1 subunits depicting various domains.

Molecular cloning studies have identified three isoforms of PHDs (PHD 1, 2 and 3) [321]. All the three isoforms can hydroxylate HIF-1α, with the highest activity exhibited by PHD2. The relative *in vitro* hydroxylation activity can be demonstrated as PHD2 >> PHD3 > PHD1 [316, 322]. Subcellular localization of these isoforms varies. PHD1 is exclusively localized in the nucleus; while PHD2 is localized in the cytoplasm and PHD3 is found in both compartments. However, PHD2 is able to shuttle between cytoplasmic and nuclear components facilitating HIF-1α degradation in both compartments. Further studies have suggested that PHD2 and PHD3 mRNA expression is hypoxia-inducible, while PHD1 mRNA expression is not altered by hypoxia [323].

Polyubiquitination

Post hydroxylation, von Hippel-Lindau protein (pVHL) binds HIF-1α. X-ray crystallographic studies have demonstrated that hydroxyproline fits accurately into a pocket in pVHL hydrophobic core and this binding is highly specific [324, 325]. Moreover, pVHL associates with elongin C and this interaction is stabilized by elongin B. Cullin-2 and Rbx1 proteins are also recruited to form the VCB-Cul2 E3 ligase complex which facilitates polyubiquitination and degradation by the 26S proteasome [326, 327]. Although pVHL-E3 ligase complex is predominantly expressed in the cytoplasm, cytoplasmic-nuclear trafficking of the complex facilitates HIF-1α degradation in both compartments [328, 329]. pVHL thus plays a predominant role in the degradation of HIF-1α. Loss of activity or mutation of pVHL has been implicated in the development of many disease processes due to induction of hypoxia regulated genes [330-332].

Lysine Acetylation

Jeong et al., have identified a key lysine residue (Lys/K 532) that plays a crucial role in determining proteasomal degradation of HIF-1α. An acetyl group of acetyl-coA is transferred onto K532, located within ODD domain, by acetyltransferase ARD1. This modification further promotes interaction of HIF-1α with pVHL, in concert with proline hydroxylation. ARD1 is present in all the human tissues and its activity is not dependent on oxygen levels. However, the transcriptional and translational levels of ARD1 are reduced under hypoxia, causing decreased acetylation [333]. Replacing lysine with arginine enhances stability of HIF-1α while increasing acetylation promoted its degradation [334, 335].

Asparagine Hydroxylation

A third hydroxylation site on asparagine 803 (Asn/N 803) was identified on C-TAD of HIF-1α. This asparagyl residue is conserved on HIF-2α isoform (N851) [336, 337]. Unlike other posttranslational modifications already discussed, asparagine hydroxylation may not affect the stabilization of HIF. Rather, it promotes HIF activity *via* modulation of TADs. Under normoxic conditions, N803 is hydroxylated by a factor inhibiting HIF-1 (FIH-1), an oxygen-dependent 2-OG dioxygenase requiring Fe^{2+} and ascorbate as cofactors [338-340]. It is considered as a second oxygen sensor and is localized in cytoplasm. Transcription of FIH-1 is not dependent on oxygen concentration [323]. Hydroxylation on N803 prevents interaction of HIF-1α with its coactivators CREB binding protein (CBP)/p300 due to steric inhibition. This coactivator recruitment is essential for transactivation of HIF-1α [341, 342].

Phosphorylation

Phosphorylation of HIF-1α by mitogen-activated protein kinase (MAPK) pathway appears to play a crucial role in regulating its activity and function. HIF-1α is highly phosphorylated *in vitro* by p42/p44 and p38 kinases [343-345]. Such activation promotes

transcriptional activity of HIF-1. It is hypothesized that HIF-1β exhibits preferential binding to the phosphorylated HIF-1α protein [346]. Inhibitors of p42/44 protein kinases diminished hypoxia induced transcriptions of target genes, while their stimulation accelerates their translational activity [347]. Threonine (Thr/T) at residues 796 and 844 appear to be the potential phosphorylation sites in HIF-1α and HIF-2α, respectively [348].

Transactivation and Target Genes

In normoxic conditions, *de novo* synthesized cytoplasmic HIF-1α is rapidly hydroxylated (P402 and P564) and acetylated (L532). Later, HIF-1α is captured by pVHL and degraded by 26S proteasome [311, 325, 327]. However, in hypoxic conditions hydroxylation is inhibited. It becomes stabilized and then translocates into nucleus *via* NLS. The protein heterodimerizes with constitutively expressed HIF-1β, binds to the pentacore DNA binding sequence, recruits coactivators and activates transcription of various target genes (**Figure 41**) [349-351]. To date, more than hundred target genes of HIF-1 have been identified. The target genes play a key role in regulating angiogenesis, cell survival and proliferation, chemotherapy and radiation resistance, invasion and metastasis, genetic instability, immortalization, immune evasion, metabolism and stem cell maintenance [56-63]. Some important target genes have been listed in **Table 8**.

Angiogenesis is a complex signaling process involving multiple gene products [352]. Many of these genes are upregulated due to hypoxic insult [353-357]. Hypoxia is an important regulatory factor directing angiogenic switch, with HIF-1 playing a predominant role in "flipping the switch" *via* direct transcriptional upregulation of vascular endothelial cell growth factor (VEGF). VEGF is a potent endothelial-specific mitogen. It interacts with its receptor (VEGFR) localized on endothelial cells and stimulates endothelial cell proliferation [354, 358-360].

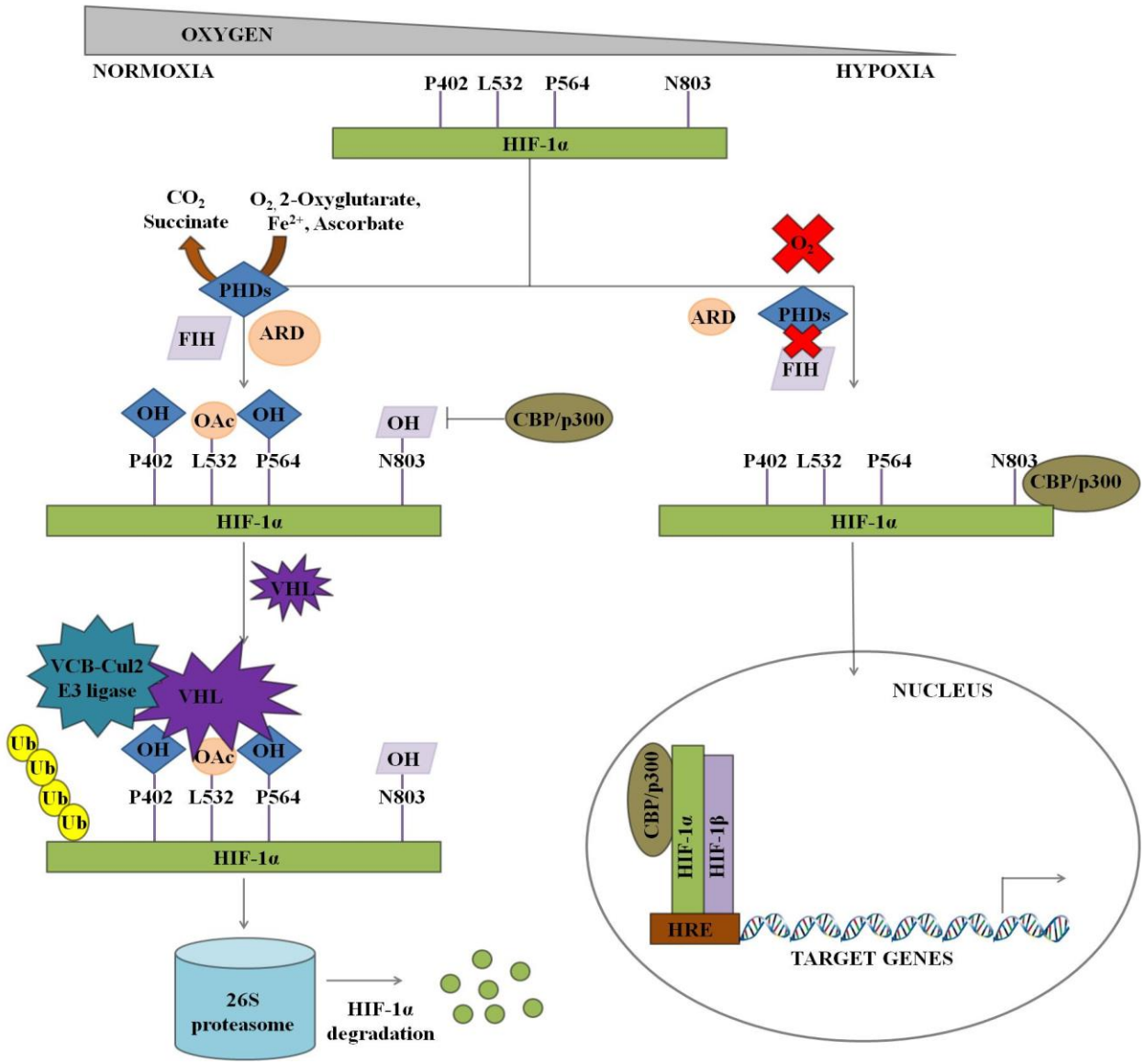

Figure 41. Schematic representation of oxygen-dependent regulation of HIF-1.

Table 8. Target genes of HIF-1 pathway.

Function	Oxygen Regulated Gene
Angiogenesis	Endocrine gland derived VEGF (EG-VEGF)
	Transforming growth factor-β3 (TGF- β3)
	Vascular endothelial growth factor (VEGF)
	VEGF receptor (VEGFR1/Flt-1)
Apoptosis	Nip3
Cell proliferation and survival	Insulin-like growth factor (IGF) 2
	IGF-binding protein (IGFBP) -1
	IGFBP-2
	IGFBP-3
	Transforming growth factor- α (TGF-α)
Dedifferentiation	Inhibitor of differentiation protein-2 (ID2)
Drug resistance	Breast cancer resistance protein (BCRP)
	P-glycoprotein (P-gp/MDR1)
Energy metabolism	Leptin
Erythropoiesis	Erythropoietin (EPO)
Genetic instability	MutSalpha (MSH2 and MSH6 complex)
Glucose metabolism	Aldolase-A
	Aldolase-C
	Enolase-1
	Glucose transporter-1 (GLUT-1)
	GLUT-3
	Hexokinase-1
	Hexokinase-2
	Lactate dehydrogenase A (LDHA)
	Phosphofructokinase L (PFKL)
	Phosphoglycerate kinase 1 (PGK1)
Histone modifiers	JMJD2B
Iron metabolism	Ceruloplasmin
	Transferrin
	Transferrin receptor
Matrix metabolism	Collagen prolyl hydroxylase
	Matrix metalloproteinases (MMPs)
	Plasminogen activator inhibitor -1 (PAI-1)
Migration/Invasion	$\alpha_v\beta_3$ integrin
	Chemokine receptor (CXCR4)
Nucleotide metabolism	Adenylate kinase-3

Apart from VEGF induction, many complex mechanisms are also involved in HIF-1 mediated angiogenic control. The expression of α_{1B}-adrenergic receptor, adrenomedulin (ADM), angiopoietin 2, endothelin-1 (ET1), heme oxygenease-1 (HO-1), nitric oxide synthase, placental growth factor (PGF), platelet derived growth factor-B (PDGF-B) and stromal derived growth factor-1 (SDF-1) is regulated by hypoxia [361-369]. Also, expression of collagen prolyl hydroxylase, matrix metalloproteinases (MMPs) and plasminogen activator receptors and inhibitors (PAIs) under hypoxic control regulates matrix metabolism and vessel maturation (**Figure 42**) [370-372].

Role of HIF-1 in Ocular Diseases

Retina, a light sensitive tissue, forms the inner lining of posterior ocular segment and is metabolically one of the most active tissues in human body [373]. Continuous oxygen supply to retina facilitates high energy demand for sensitive and efficient transduction of images to readable neuronal signals [374, 375]. This neuronal function is executed by five different cell types including photoreceptors, bipolar cells, amacrine cells, horizontal cells and ganglion cells. Photoreceptors (cones and rods) play a vital role in phototransduction process. Cones mediate vision in bright light while rods mediate in dim light [376, 377]. Number of rods outweighs the number of cones by ~20 fold. Under dark conditions, a single rod cell requires 10^8 ATP/sec for ion homeostasis and signal transduction machinery. However under light exposure due to reduction in ion influx, energy requirement falls by 75% [378]. The energy requirement is met by oxidative phosphorylation process occurring in mitochondria, located within inner segment of photoreceptors [379]. Thus, oxygen concentration tightly controls retinal function. In human a constant supply of oxygen is regulated *via* choroidal and retinal circulation. Since human retina is thick, these two separate and distinct systems act to facilitate diffusion.

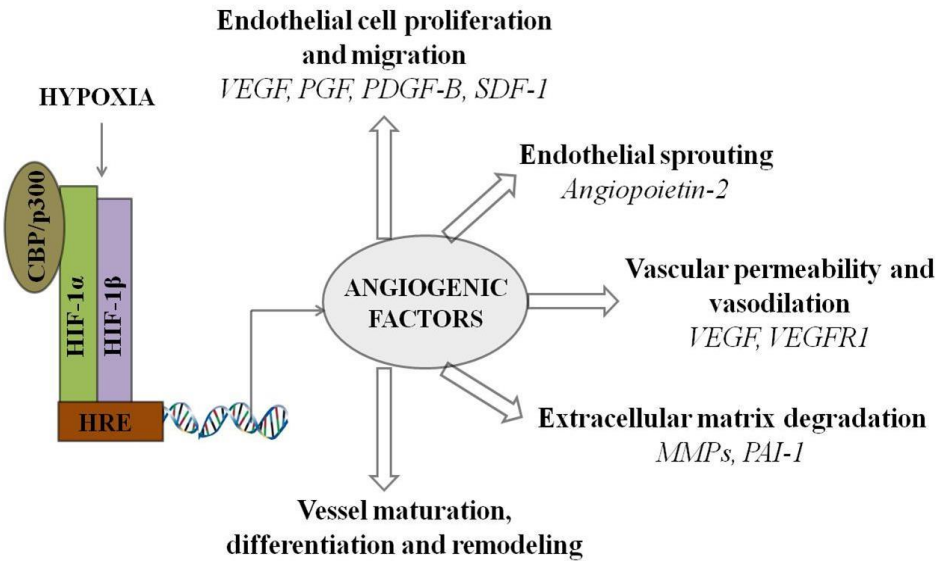

Figure 42. Schematic representation of angiogenic regulation by HIF-1.

Choroidal vasculature nourishes the outer retina including retinal pigment epithelium (RPE) and photoreceptors while retinal vasculature perfuses the inner retinal layers. Choroidal circulation is highly vascularized. It is under low autoregulation and requires strong sympathetic control. Retinal circulation is relatively sparse, controlled by autoregulation and lacks sympathetic control. Arteriovenous oxygen gradient is also different between the two vasculatures [380, 381]. Further, the choroidal vessels are fenestrated while the retinal vessels lack fenestrations and express tight junctions [382]. Both vasculatures play an important role in regulating retinal physiology. Lack of oxygen supply can lead to vision threatening pathologies such as diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration and glaucoma. Despite the fact that initiating events are different, hypoxia with subsequent neovascularization is a characteristic phenomenon noticed with all these vascular diseases.

Diabetic Retinopathy

Diabetic retinopathy (DR) is a frequent secondary microvascular complication in patients with diabetes mellitus. It is one of the four major causes of visual impairment often leading to blindness [383, 384]. Almost 25-50% of diabetic patients exhibit retinopathy symptoms within the first 10-15 years and this number approaches nearly 100% within 30 years of diabetic onset [385, 386]. DR is characterized by bi-phasic progression with an initial non-proliferative (vaso-obliterative) phase followed by a proliferative (vaso-proliferative) phase. During initial stages, a persistent rise in blood glucose levels leads to a loss of intramural pericyte function. As a result small saccular capillary outpouchings, known as microaneurysms appear [387]. Intraretinal microvascular abnormalities, hemorrhages, edematous thickening of basement membrane, soft exudates and cotton wool spots are also observed [388-391]. Changes in vasculature and perturbations in oxygen tension lead to development of hypoxia, elevating the expression of

angiogenic factors and subsequent neovascularization (proliferative phase) (**Figure 43**) [392]. The newly formed blood vessels are often fragile and permeable. Such vessels grow through the surface of retina into the vitreous and subsequent bleeding may lead to obstructed vision. Further, contraction of associated fibrovascular component may result in retinal detachment, vision loss and blindness [393-396].

The role of HIF-1 in the proliferative stage of DR has been clearly established. Mean oxygen tension is significantly lower in diabetics relative to non-diabetic patients in both lens (8.4 ± 0.7 mm Hg vs 10.7 ± 0.8 mm Hg) and vitreous cavity (5.7 ± 0.7 mm Hg vs 8.5 ± 0.6 mm Hg) [397]. Expression levels of HIF-1α and VEGF are elevated in diabetic preretinal membranes compared to non-diabetic idiopathic epiretinal membranes [398, 399]. Moreover, the production of VEGF and intercellular adhesion molecule (ICAM)-1 are diminished in a diabetic mice model lacking Hif-1α expression. It leads to much reduced vascular leakage and neovascularization in Hif-1α knockout mice relative to wild type mice [400]. These findings clearly suggest that alteration in HIF-1α pathway may be an attractive strategy for the treatment of DR.

Retinopathy of Prematurity

Retinopathy of prematurity (ROP), formerly known as retrolental fibroplasias, is the leading cause of visual impairment and blindness in children [401]. It was first described in early 1940s. This condition is associated with low gestational period, low birth weight and hyperoxia [402]. Human retinal vasculature begins to develop during 16^{th} week of gestation and concludes at 40^{th} week. Hence, premature infant's exhibit incompletely developed retinal vasculature and peripheral avascular zone. Oxygen supplementation (hyperoxia) is often needed in premature infants to overcome respiratory insufficiency. Such acute rise in oxygen tension can stimulate apoptosis of vascular endothelia cells and may cause vaso-obliteration *via* generation of reactive

oxygen species (ROS) (Phase I). Further, high perinatal levels of prostaglandins (PGD_2 and PGE_2) and nitric oxide (NO) accelerates oxidative metabolism. Reduced levels of antioxidants may induce complexity in disease pathology [403-406]. In a subsequent phase, the infant's vaso-obliterated retina undergoes hypoxic/ischemic stress. It triggers a series of events such as stabilization of HIF-1α and production of various proangiogenic factors resulting in neovascularization (Phase II) (**Figure 43**). In contrast to normal developmental vasculature, this pathological vasculature displays excessive, uncontrolled and misdirected growth towards vitreous and lens. It can cause fibrous scarring, retinal detachment and blindness [407-410].

Given the regulatory role of oxygen, it is evident that HIF plays a predominant role in the development and progression of ROP. In a mouse oxygen-induced retinopathy (OIR) model, the expression levels of HIF-1α and HIF-2α proteins peak after two hours of hypoxic exposure. However, HIF-1α is stabilized in neuronal cells and inner retinal layers whereas HIF-2α was upregulated in Muller glia and astrocytes [411]. Indeed, inhibition of Hif-1α and Vegf by gene therapy in mice ischemic retinopathy model inhibited neovascular tufts and nuclei compared to control hypoxia group [412]. Hence, altering the HIF-1α pathway may be beneficial than targeting other downstream factors.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in patients over the age of 65. World Health Organization (WHO) global eye disease survey has revealed that more than 50 million people are affected with AMD and atleast one-third of them are blind or severely visually impaired [413]. Development of AMD is multifactorial including aging, smoking, genetic factors, obesity, hypertension and hypercholesterolemia [414-418]. The disease is characterized by degeneration of central retina leading to disturbed fine and color vision.

AMD is classified into two clinical forms, non-exudative/dry AMD and exudative/wet AMD [419]. The dry phase accounts for 85 to 90% of the total cases. It is characterized by the presence of acellular polymorphous debris, termed as drusen, between the basal lamina of RPE and inner collagenous layers of Bruch's membrane (**Figure 43**). As AMD progresses geographical atrophy of photoreceptors and central retina are noticed [420, 421]. Although wet AMD represents 10 to 15% of total cases, it accounts for approximately 90% of vision loss. In certain cases, dry AMD progresses to wet AMD characterized by choroidal neovascularization (CNV). These abnormal blood vessels penetrate Bruch's membrane; grow into RPE and neural retina. This may lead to blurred vision, retinal detachment, fibrosis and complete vision loss [422, 423].

Cell and molecular biology studies have demonstrated that hypoxia, chronic oxidative stress and inflammation play key role in AMD [424-426]. Hypoxia may result from higher oxygen consumption, resulting from increased metabolic activity of the inflamed retina or due to poor circulation in central macula, resulting from vessel stenosis and microthrombosis [427-429]. Also, thickening of Bruch's membrane and drusen formation further stabilizes HIF [430]. ROS also causes elevation in HIF protein expression and results in increased transcriptional activity of hypoxia regulated genes [431, 432]. Such hypoxic milieu contributes to progression of exudative AMD and development of CNV. Infact, HIF-1α and HIF-2α expression were identified in endothelial cells and macrophages of choroidal neovascular membranes [433, 434].

Glaucoma

Glaucoma represents a multifactorial optic neuropathic disease [435]. It is classified into open and closed-angle depending on the anterior chamber angle. Open-angle glaucoma exhibits unobstructed and normal iridocorneal angle while the closed-angle glaucoma exhibits occlusion of the angle by the peripheral iris [436, 437]. Glaucoma is characterized by increased intraocular

pressure, degeneration of retinal ganglion cells and axons. However, the exact role of hypoxia in the development of glaucoma is still unknown. Clinical observations have demonstrated retinal vascular abnormalities and impaired blood flow at the optic nerve head which may result in stabilization of hypoxic factors leading to retinal ganglion cell death [438, 439]. The expression of HIF-1α in control and glaucomatous human donor eyes was studied. Indeed, higher expression of the protein was noticed in retina and optic nerve head of glaucomatous eyes [440]. These findings clearly suggest the importance of hypoxia signaling mechanism in the pathogenesis of glaucoma.

Existing Anti-VEGF Therapeutics

Overproduction of VEGF plays an important role in pathogenesis of DR, ROP, AMD, neovascular glaucoma, central and branch retinal vein occlusion [441-444]. The human *VEGFA* gene localized in chromosome 6p21.3 exhibits eight exons and four principal isoforms. The shorter isoform, $VEGF_{121}$, is an acidic polypeptide and lacks heparin binding domain. The larger isoforms, $VEGF_{189}$ and $VEGF_{206}$, are highly basic and exhibit high affinity binding to heparin. The shorter isoform is freely diffusible while the larger isoforms are completely sequestered in the extracellular matrix. $VEGF_{165}$ exhibits intermediate properties, existing in both diffusible and bound forms [445]. The primary sources of VEGF in retina are RPE cells, Muller cells and ganglion cells. VEGF binds to two types of protein kinase activating receptors, VEGFR1 and VEGFR2. These high affinity receptors have been localized on retinal endothelial cells and pericytes [446-449]. Currently, anti-VEGF therapeutics are indicated in the treatment of ocular neovascular diseases. These include Pegaptanib sodium (Macugen; Eyetech Pharmaceuticals/Pfizer, NY), Ranibizumab (Leucentis; Genentech, CA), Bevacizumab (Avastin; Genentech, CA) and Aflibercept (VEGF Trap-Eye; Regeneron, NY).

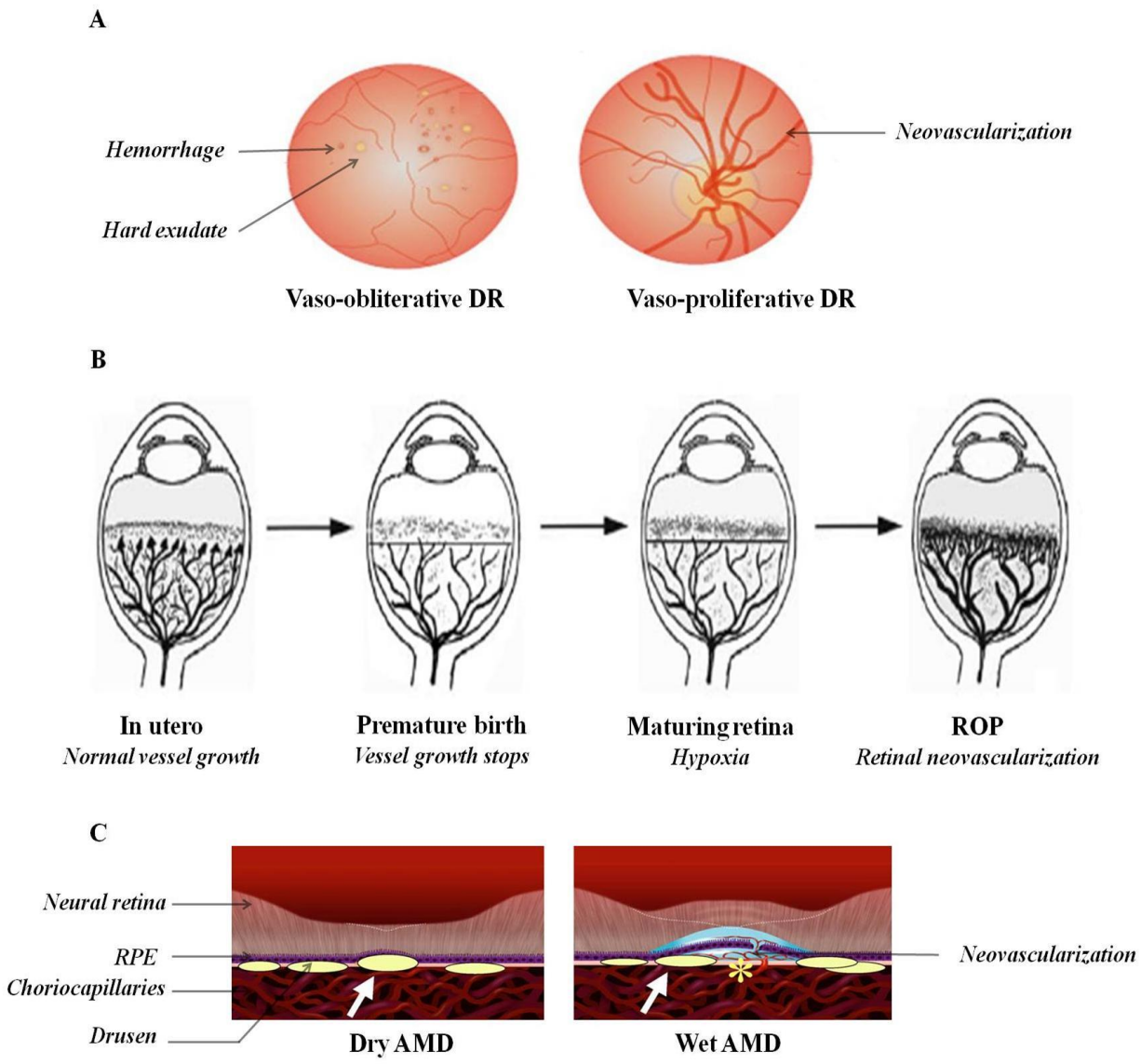

Figure 43. (A) Stages of diabetic retinopathy (DR). (B) Development of retinopathy of prematurity (ROP). (C) Schematic representation of dry and wet age-related macular degeneration (AMD). Reproduced with permission from references [402, 426].

Pegaptanib (50 kDa) is the first anti-VEGF agent approved by US Food and Drug Administration (FDA) in 2001 for the treatment of exudative AMD. It is a 28-base ribonucleic acid aptamer, covalently linked to two branched polyethylene glycol (PEG- 20 kDa) moieties. It binds to extracellular $VEGF_{165}$ with high affinity and prevents the interaction of VEGF with its receptor. Since pegaptanib binds specifically to only one isoform, it exhibits limited efficacy. Approval of this drug molecule began a new era in anti-VEGF therapy. Bevacizumab (149 kDa) is a humanized recombinant full-length monoclonal antibody. It binds to all VEGF isoforms. Although not approved for specific intraocular use, bevacizumab has been indicated as an off-label therapeutic in the treatment of ocular diseases. Ranibizumab (48 kDa) is the Fab fragment of the former, approved by US FDA in 2010 for treatment of macular edema and vein occlusion. Both these molecules bind all forms of VEGF. Compared to bevacizumab, ranibizumab demonstrates 5-20 fold greater potency due to higher affinity and lack of immunogenicity. Aflibercept (115 kDa) was approved by US FDA in 2011 for treatment of AMD. It is a recombinant fusion protein consisting of the VEGF binding domains of human VEGFR1 and VEGFR2 fused to the Fc domain of human immunoglobulin-G1. It acts as a decoy receptor binding free VEGF [450-453].

Although large molecule therapeutics appear to be promising, their long term usage must be considered with caution. A recent investigation by Kurihara et al., reported the deleterious effects following deletion of *Vegfa* gene in adult mice. Choriocapillaris are completely attenuated following three days of RPE-specific *Vegf* inactivation. Further, cone photoreceptors were damaged and cone dysfunction was noticed. These dramatic secondary “off-target” effects of *Vegf* antagonism were not observed when *Hif1a*, *Epas1*, and *Hif1a/Epas1* were genetically ablated. The transcriptional mutants did not exhibit any morphological, functional, or

transcriptional differences relative to control adult mice. Further, deletion of transcription factors reduced pathological angiogenesis in laser photocoagulation model of CNV. These studies clearly reinforce the strategy that molecules aiming at HIF pathway may be an alternative, safer and effective mode of treatment than attenuating VEGF levels alone [454, 455].

Development of HIF-1 Inhibitors for Ocular Diseases

Significant research has been conducted in recent years to identify inhibitors of HIF-1 pathway. Based on their putative mechanism of action, HIF inhibitors may be classified as modulating i) HIF-1α mRNA expression, ii) HIF-1α protein translation, iii) HIF-1α protein degradation, iv) HIF-1α DNA binding activity and v) HIF-1α transcriptional activity. Examples of HIF-1 inhibitors are summarized in **Table 9** while few of them are described below.

HIF-1α mRNA Expression

It has been hypothesized that transcriptional level of HIF-1α is the rate limiting factor of HIF-1 activity under hypoxic conditions [456]. Hence, inhibitors that effect HIF-1α mRNA expression can lower the rate of HIF-1 translation. Chen et al., have studied the role of HIF-1α inhibition by RNA interference (RNAi) with shRNA in BALB/C mouse model of corneal neovascularization. The effect of shRNA treatment was assessed by measuring mean neovascularization score. The mean score values were as follows: normal (0), control neovascular eyes (3.59 ± 1.1), saline treated (4.05 ± 0.75), vehicle-treated (3.64 ± 1.02) and RNAi- treated (1.13 ± 0.96). HIF-1α shRNA reduced neovascularization by more than 3 fold compared to control eyes. Further, the expression of angiogenic factors (VEGF and MMPs) and inflammatory mediator (IL-1β) was also diminished. In summary, this study confirmed the role of HIF-1α transcriptional inhibition in reducing corneal neovascularization and associated inflammation [457].

Table 9. Inhibitors of HIF-1 pathway.

Target Pathway / Mechanism of Action	Small Molecules
HIF-1α mRNA expression	
Transcription	Aminoflavone
	EZN-2968
	RNA interference
HIF-1α protein expression	
Translation	Digoxin
	PX-478
	Topotecan
Receptor tyrosine kinases	Erotinib
	Gefitinib
	Genistein
PI3K-AKT pathway	LY294002
	Nelfinavir
	Wortmannin
ERK-AKT pathway	Resveratrol
mTOR pathway	Everolimus
	Rapamycin
	Silibinin
	Temsirolimus
Ras-MAPK pathway	PD98059
	Sorafenib
HIF-1α protein degradation	
Hsp90 inhibitor	Apigenin
	Deguelin
	Geldanamycin
HDAC inhibitor	FK228
	SAHA
	Trichostatin A
Others	
Cyclin-dependent kinase	Flavopiridol
DNA binding	Cisplatin
	Doxorubicin
	Echinomycin
	Pyrrole - imidazole polyamide
Microtubules	2-methoxyestradiol (2ME2)

A similar study with RNAi of HIF-1α was performed by Jiang et al., in C57BL/6J mice of ischemic retinopathy. The researchers have counted the number of neovascular nuclei on the vitreal side of inner limiting membrane. The number of nuclei per cross section were as follows: normoxia (0.05 ± 0.29), hypoxia (41 ± 2.8), vehicle-treated (41 ± 2.6) and siRNA-treated (28 ± 2.8). The number of neovessels significantly decreased in transfected group relative to hypoxic group ($p<0.01$). This report clearly demonstrates the application of HIF-1α RNAi as a novel therapeutic for the treatment of neovascular eye diseases [412].

HIF-1α Protein Translation

Although the precise mechanism of HIF-1α protein translation in hypoxic conditions is not clear, several translational inhibitors have been identified. These molecules can directly inhibit translation or inhibit various signaling pathways (receptor tyrosine kinases, PI3K/AKT/mTOR and Ras-MAPK pathway). These signaling pathways play a predominant role in upregulating HIF-1α translation and thus inhibition of these growth factors can alter hypoxic regulation. Cardiac glycosides can inhibit HIF-1α protein translation and digoxin has been identified as a potent inhibitor in a cell-based reporter assay [458]. The effect of digoxin on ocular neovascularization was demonstrated by Yoshida et al., in C57BL/6 mice with ischemic retinopathy. Intraocular injection of digoxin lowered retinal neovascularization by almost 75% compared to saline group. Also, the area of CNV at Bruch's membrane was significantly lowered in presence of digoxin. Apart from inhibiting HIF-1α protein expression, digoxin also inhibited the expression of several angiogenic factors including VEGF, PDGF-B, SDF-1, VEGFR2, chemokine receptor (CXCR4) and Tie2 receptor in ischemic retina. This observation suggests that digoxin may offer advantages over VEGF antagonists in the treatment of neovascular diseases due to inhibition of several proangiogenic pathways. This study signifies that digoxin, a

potent HIF-1α inhibitor, can possibly provide a better therapeutic intervention [459].

Genistein, a naturally occurring isoflavonoid, exhibits strong antiangiogenic activity. The underlying mechanism is hypothesized as inhibition of HIF-1α translation caused by inhibition of tyrosine kinases [460]. Wang et al., examined the effects of genistein on retinal neovascularization in C57BL/6 OIR mouse model. Number of vascular nuclei anterior to inner limiting membrane was quantified and data obtained was represented as: normoxia (0.76 ± 0.81), hypoxia (23.9 ± 4.4), genistein (50 mg/kg) (20.9 ± 4.7), genistein (100 mg/kg) (17.2 ± 4.0) and genistein (200 mg/kg) (14.2 ± 3.2). The nuclei numbers were diminished by 87, 72 and 59% respectively, as the dose of genistein was raised. Further, dose-dependent reduction in HIF-1α and VEGF levels were also observed. This report suggests possible pharmacological application of genistein in ocular neovascularization [461].

HIF-1α Degradation

The molecular chaperone, heat shock protein 90 (Hsp90) is required for activity of various signaling proteins [462]. The interaction of Hsp90 with HIF-1α is required for proper conformational stability. Inhibitors of Hsp90 can promote degradation of HIF-1α *via* oxygen-independent proteasomal degradation [463]. Geldanamycin and its analogs (17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG/Deguelin)) may interfere with Hsp90/HIF-1α interaction by competing with the ATP binding site [464]. Kim et al. evaluated the potential of deguelin in the treatment of vaso-proliferative retinopathies. In an OIR mouse model, deguelin treated mice exhibited lower neovascularization as assessed by fluorescein angiography (**Figure 44**). Further, the number of vascular lumens between posterior lens and anterior inner limiting membrane were estimated. Compared to control group the deguelin injected group showed lower number of

vascular lumens (19 ± 3.4 vs 4 ± 2.1). Moreover, deguelin treatment did not alter normal retinal morphology as evident by normal retinal thickness and lack of any inflammation in vitreous, retina or choroid. This data clearly implies that modulation of HIF pathway reduces retinal neovascularization without any retinal toxicity [465].

HIF-1α DNA Binding and Transcriptional Activity

Binding of active heterodimeric HIF-1 to the consensus -RCGTG- enhancer element of target genes is another crucial step necessary for transcription of hypoxia-inducible target genes. Also, the interaction of coactivator p300 with HIF-1α is another potential mechanism required for transcriptional activity of HIF-1. These molecular pathways can also be targeted in the treatment of various neovascular diseases [466-468].

Conclusions and Future Perspectives

In summary, high levels of energy is required for proper retinal function. Any perturbations in oxygenation may lead to progression of several retinal degenerative diseases including DR, ROP and AMD. Mechanistic studies of cellular and molecular components of hypoxia signaling have opened a new era in the treatment of retinopathies. Given the role of HIF-1 in the etiology of these diseases, it is evident that manipulation of this pathway at various stages can lead to more effective treatment of oxygen-dependent ocular diseases. As discussed in this chapter, many inhibitors have been identified and evaluated both in *in vitro* cell culture models and animal models. However, further work validating the pharmacological intervention of these inhibitors in retinal diseases and their translation from bench to bedside is necessary. Nonetheless, there is significant optimism that modulation of HIF pathway can provide new treatments for ocular neovascular diseases.

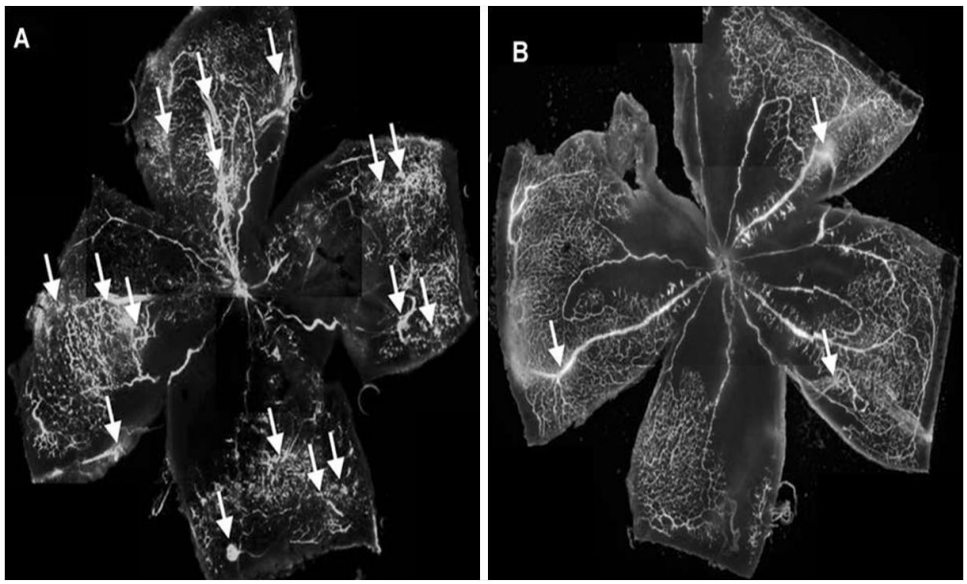

Figure 44. Estimation of retinal neovascularization in (A) control and (B) deguelin treated oxygen-induced retinopathy mouse model. Reproduced with permission from reference [465].

CHAPTER 6

MOLECULAR EXPRESSION AND FUNCTIONAL ACTIVITY OF EFFLUX AND INFLUX TRANSPORTERS IN HYPOXIA INDUCED RETINAL PIGMENT EPITHELIAL CELLS

Rationale

Retina, a light sensitive tissue, forms the inner lining of posterior segment of the eye and is metabolically one of the most active tissues. It is highly sensitive to oxygen tension. Mammalian cells require oxygen as an electron acceptor during oxidative phosphorylation for ATP production to maintain normal metabolic functions. Oxygen serves as a major contributor for regulating membrane transport, intracellular signaling, expression of many genes, and initiation of apoptosis [286, 287, 469, 470]. Exposure to low oxygen tension even for short time periods may bring about alterations in the maintenance of oxygen homeostasis [471]. Hypoxia is a condition in which oxygen is found to be below the normal level. Many regulatory systems in the human body are activated by hypoxia and consequently cells under this hypoxic stress elicit various adaptive responses.

A master regulator of hypoxic response is hypoxia-inducible factor (HIF). It is a heterodimer consisting of oxygen-dependent HIF-α subunit and independent HIF-β subunit [291, 472]. Under normal oxygen levels, HIF-α subunit is hydroxylated by prolyl hydroxylases (PHD1-3) and rapidly degraded by E3 ubiquitin-proteasome system [312, 321, 473]. Under hypoxic conditions, hydroxylation is inhibited due to inactive PHDs leading to translocation of HIF-α to the nucleus. HIF-α heterodimerizes with HIF-β, recruits various co-activators and binds to the pentanucleotide hypoxic response element sequence (RCGTG) to modulate the expression of various genes. To date several HIF responsive genes have been identified, which play a key role in regulating many cellular processes. Few important genes include vascular endothelial

growth factor (VEGF), erythropoietin (EPO), glucose transporter-1 (GLUT-1), angiopoietin 2, endothelin-1 (ET1), heme oxygenease-1 (HO-1), nitric oxide synthase, placental growth factor (PGF), platelet derived growth factor-B (PDGF-B) and stromal derived growth factor-1 (SDF-1) [294, 474-476].

There is a general consensus that hypoxic conditions contribute to many retinal diseases such as von Hippel-Lindau (VHL) disease, diabetic retinopathy (DR), retinopathy of prematurity (ROP), macular edema, glaucoma, age-related macular degeneration (AMD) and retinitis pigmentosa [388, 402, 440, 477-480]. In response to hypoxia, retinal cells release various growth factors which produce paracrine effects, resulting in angiogenesis, fibro vascular tissue formation, retinal ablation and ultimately loss of vision. Inspite of recent advances in therapeutic intervention for these diseases, currently available agents demonstrate only modest effects. It could be attributed to cascade of reactions underlying hypoxia signaling. Since drug disposition is predominantly regulated by membrane transporters, evaluating the molecular response of efflux and influx transporters to reduced oxygen levels is of considerable interest. Efflux transporters such as P-glycoprotein (P-gp/MDR1), multidrug resistance protein 2 (MRP2), breast cancer resistant protein (BCRP) are the leading membrane bound protein families in both prokaryotes and eukaryotes. These proteins are also referred as ATP binding cassette (ABC) transporters since these efflux pumps derive energy from ATP hydrolysis. Expression and functional activity of various efflux transporters was characterized on human and rabbit retinal cells [481, 482]. These efflux pumps are mainly responsible for extruding molecules out of cytoplasm. In order to facilitate enhanced cellular absorption and evasion of efflux transporters, several strategies were examined [483-485]. One of such approaches include utilization of influx transporters such as neutral and cationic amino acid transporter ($B^{(0,+)}$), folate receptor-alpha

(FR-α) and sodium dependent multivitamin transporter (SMVT). $B^{(0,+)}$ is an amino acid transporter which recognizes neutral as well as cationic amino acids for membrane translocation. FR-α is a membrane receptor anchored to the plasma membrane by glycosylphosphatidylinositol (GPI), with greater affinity for folic acid and reduced forms such as methyltetrahydrofolate [486, 487]. SMVT is an important transmembrane protein responsible for translocation of vitamins and other essential cofactors such as biotin, pantothenic acid and lipoic acid [488]. The role of these influx transporters in ocular drug delivery has also been explored. Therefore, in this study, we have examined the differential expressions of efflux transporters (MDR1, MRP2 and BCRP) and influx transporters ($B^{(0,+)}$, FR-α and SMVT) in human retinal pigment epithelial (RPE) (D407 and ARPE-19) cells under hypoxic and normoxic conditions.

Materials and Methods

Materials

Human retinal pigment epithelial (D407) cells were obtained as a generous gift from Dr. Richard Hunt (University of South Carolina, Columbia, SC). ARPE-19 cells were procured from American Type Culture Collection (Manassas, VA). Dulbecco's modified eagle's medium (DMEM), DMEM: Nutrient Mixture F-12 (DMEM/F12), trypsin replacement (TrypLE™ Express), non-essential amino acids, and TRIzol® were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 and 25 cm^2growth area), 12-well plates (3.8 cm^2 growth area per well) and 96-well plates (0.32 cm^2 growth area per well) were procured from Corning Costar Corp. (Cambridge, MA). [^{14}C] Arginine (specific activity: 346 mCi/mMol), [^{3}H] abacavir (specific activity: 40 Ci/mMol) and [^{3}H] biotin (specific activity: 53.4 Ci/mMol) were procured from PerkinElmer (Waltham, MA). [^{3}H] Digoxin (specific activity: 0.1 Ci/mMol),

[^{3}H] folic acid (specific activity: 25.6 Ci/mMol) and [^{3}H] lopinavir (specific activity: 0.5 Ci/mMol) were purchased from Moravek Biochemicals (Brea, CA). OligodT, dNTP, M-MLV reverse transcriptase and CellTiter 96® aqueous non-radioactive cell proliferation assay were obtained from Promega Corporation (Madison, WI). Light Cycler 480® SYBR I green master mix was obtained from Roche Applied Science (Indianapolis, IN). Human VEGF ELISA kit was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Unless otherwise mentioned, all other chemicals were purchased from Sigma Chemicals (St. Louis, MO) and used without further purification.

Cell Culture

D407 cells (passage numbers 65-70) were employed in this study [489]. Cells were maintained in DMEM medium supplemented with 10% FBS (heat inactivated), 1% non-essential amino acids, 20 mM HEPES, 29 mM sodium bicarbonate and 100 U/mL of penicillin and 100 µg/mL of streptomycin. ARPE-19 cells (passage numbers 20-25) were cultured in D-MEM/F-12 supplemented with 10% FBS (heat inactivated), 15 mM HEPES, 29 mM sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin [490]. Cells were cultured in T-75 flasks at 37°C in a humidified atmosphere of 5% CO_2 and 90% relative humidity. The medium was replaced every alternate day and passaged using TrypLE™ Express after reaching 80% confluency. Cells were seeded at a density of 1 million cells in T-25 flasks and 250,000 cells/well in 12-well plates. After reaching 80% confluency, cells were subjected to hypoxic conditions for a specific period of time in a bactron anaerobic chamber with a gas mix consisting of 1% O_2, 5% CO_2 and 94% N_2, following a previously published method [489, 491].

Extraction of RNA

Cells cultured under hypoxic conditions were processed in the anaerobic chamber itself to avoid any exposure to normoxic conditions. Cells were lysed in TRIzol® reagent and the lysate was transferred into an eppendorf tube. To this lysate, chloroform was added to facilitate phase separation. The aqueous layer was separated and isopropanol was added to precipitate RNA. The RNA pellet obtained was washed twice with 75% ethanol and dissolved in DNase/RNase free water. The concentration of RNA was determined by detecting UV absorbance at 260 nm with Nanodrop (Thermo Fisher Scientific, Wilmington, DE). RNA (4 µg) was reverse transcribed into complementary DNA (cDNA) with OligodT as a template and M-MLV reverse transcriptase. The conditions for reverse transcription were denaturation for 5 min at 70°C, reverse transcription for 1 hour at 42°C and final extension for 5 min at 72°C.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) Studies

Primers were custom designed with Primer-Blast tool from Pubmed and listed in **Table 10**. All the primers were designed such that 100-150 bp long amplicons could be generated to increase the efficiency of amplification. For quantitative gene expression analysis, 80 µg of cDNA was amplified using 10 µM of forward and reverse primers and Light Cycler 480® SYBR I green master mix using ABI Prism 5700 Sequence Detection System (Applied Biosystems, CA). The reactions were carried out with initial denaturation at 70°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 10 sec. The specificity of PCR products was confirmed by melting curve analysis. Relative amount of RNA levels was normalized to the internal standard GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in each sample. GAPDH was selected as an internal control from previously published results [492]. Initial experiments were carried out to ensure that the target and control genes were amplified with equal efficiencies.

Table 10. Primers used in quantitative gene expression analysis. Sequence is given from 5'->3'.

Gene	Forward primer	Reverse primer
GAPDH	ATCCCTCCAAAATCAAGTGG	GTTGTCATGGATGACCTTGG
MDR1	CTTATGCTCTGGCCTTCTGG	TGCTTCAATGCTTGGAGATG
MRP2	AAATATTTTGCCTGGGAACC	TGTGACCACAGATACCAGGA
BCRP	AGCTGCAAGGAAAGATCCAA	TGCCCATCACAACATCATCT
$B^{(0,+)}$	AGCCGAGGGAGTGAACCATG	GGACCAGTTACCACGGTCCT
FR-α	AGGACAGACATGGCTCAGCG	TGTGGTGCTTGGCGTTCATG
SMVT	TACCAGTTCTGCCAGCCACAGTG	CAGGGACACCAAAACCTCCCTCT
HIF-1α	GTACCCTAACTAGCCGAGGAAGAA	GTGAATGTGGCCTGTGCAGT
VEGF	CTTGCCTTGCTGCTCTAC	TGGCTTGAAGATGTACTCG
GLUT-1	CGGGCCAAGAGTGTGCTAAA	TGACGATACCGGAGCCAATG
PEPT1	CAAGAAGTTCAAGCCACAGG	GAAATGCCTTACTCCGATGC
PEPT2	CCTTCAGCAGCCTCTGTTAT	AGGACTGTGTGTACCACTTG
OCT1	GTGGGATAATCACCCCCTTC	TCTGGAAGAAGTAGCGTCAC
OCT2	TTGGCTCTATGAGTATCGGC	AGGACTGTAGTTAGGAGGCA

Enzyme-Linked Immunosorbent Assay (ELISA)

A sandwich ELISA was performed to determine the amount of VEGF secreted in cell culture medium post exposure to normoxic and hypoxic conditions, following manufacturer's instructions. Briefly, an anti-human $VEGF_{165}$ antibody was precoated to the 96-well microplate. The samples/standards (50 µL) were added to the wells and incubated for 2 hours. After removing unbound antigen, biotinylated secondary detecting antibody (100 µL) was added and further incubated for 1 hour. Excess detecting antibody was removed and streptavidin - horseradish peroxidase (HRP) was added which subsequently reacts with TMB (3,3′,5,5′-tetramethylbenzidine) substrate to produce a colorimetric signal. This signal was estimated by detecting the absorbance at 450 nm using a plate spectrophotometer (SpectraFluor Plus, Maennedorf, Switzerland).

Immunoblot Analysis

Following growth under normoxic and hypoxic conditions, cells were washed with PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO4, 1.3 mM KCl and 135 mM NaCl) and lysed in PBS with 1% Triton X-100 and protease inhibitor cocktail maintained at pH 7.4, for 15-20 min on ice. Upon homogenization, cells were centrifuged at 12,000 rpm for 10 min and the protein lysate was collected. The amount of protein in the lysate was determined using Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as the standard. An equal amount of protein (20 µg) in all samples was separated using poly acrylamide gel electrophoresis (PAGE). The separated protein bands were transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked overnight at $4^{\circ}C$ with 2% non-fat dry milk and 0.25% BSA prepared in 0.05 M tris buffered saline. The blot was exposed to primary antibody at $25^{\circ}C$ for 4 hours. After subsequent washes the blot was again exposed to secondary antibody-HRP

conjugate for 1 hour. The blot was visualized for the protein of interest using chemiluminescent substrate in chemiImager 8900 (AlphaInnotech, San Leandro, CA).

Uptake Studies

Uptake studies were performed on confluent cell monolayers cultured in 12-well plates. Uptake experiments for hypoxic conditions were performed in the anaerobic chamber itself to prevent cell exposure to normal oxygen levels during the study. After culturing in normoxic or hypoxic conditions, the medium was removed and cells were washed three times with 1 mL of dulbecco's phosphate buffered saline (DPBS) (130 mM NaCl, 0.03 mM KCl, 7.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 20 mM HEPES and 5 mM glucose) maintained at pH 7.4. Test solution (500 µL) was added and cells were incubated for 30 min at 37°C. Uptake was arrested with ice cold PBS and 1 mL of lysis solution (0.1% (v/v) Triton-X in 0.3 N NaOH) was added. Cells were lysed overnight and the lysate was then quantified for radioactivity with scintillation cocktail (Fisher Scientific, Fair Lawn, NJ) in a scintillation counter BeckmanCounter, Fullerton, CA). Uptake in each well was normalized to the respective protein content, as determined by Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA). [^{3}H] Digoxin, [^{3}H] lopinavir and [^{3}H] abacavir were used as model substrates for MDR1, MRP2 and BCRP mediated transport respectively. [^{14}C] Arginine, [^{3}H] folic acid and [^{3}H] biotin were used as model influx transporter substrates for $B^{(0,+)}$, FR-α and SMVT respectively.

Cell Proliferative Assay

A cell proliferative assay (MTS assay) was performed to determine the effect of hypoxia on RPE cells. Post exposure to normoxic or hypoxic conditions, medium was aspirated and 100 µL of serum free media and 20 µL of MTS and PMS reagent were added. Following incubation for 3 hours, the quantity of formazan formed from MTS is measured at 490 nm using a plate

spectrophotometer. The amount of formazan formed is directly proportional to the number of viable cells.

Data and Statistical Analysis

Relative expression of each gene was quantified following delta-delta ($\Delta\Delta C_t$) method according to Eq.s 1, 2 and 3

$$\Delta C_t = C_{t\,(treated)} - C_{t\,(control)} \quad \text{Eq.1}$$

Treated represents hypoxic samples and control represents normoxic samples

$$\Delta\Delta C_t = \Delta C_{t\,(target\,gene)} - \Delta C_{t\,(reference\,gene)} \quad \text{Eq.2}$$

Target gene represents either MDR1, MRP2, BCRP, $B^{(0,+)}$, FR-α, SMVT, HIF-1α, VEGF or GLUT-1 and reference gene represents GAPDH. Relative fold expression is calculated according to Eq.3

$$\text{Relative fold expression} = 2^{\wedge\,\Delta\Delta Ct} \quad \text{Eq.3}$$

Cellular accumulation of radioactive substrates was calculated with Eq.4

$$C_{sample} = (DPM_{sample} \times C_{donor}) / DPM_{donor} \quad \text{Eq.4}$$

DPM represents disintegrations per min and C represents concentration.

All the experiments were performed in quadruplicates. The results are expressed as mean ± standard deviation (SD). One way analysis of variance (ANOVA) or student's t test (Graph Pad InStat Version 4.0) was performed to confirm statistical significance of the experimental values. A P-value of less than 0.05 was considered to be statistically significant.

Results

Quantitative Gene Expression Analysis

Among the different cell culture models of retinal pigment epithelial cells, D407 and ARPE-19 cells were selected for their expression of efflux and influx transporters [481, 493-495]. Further, both these cells induce VEGF expression under hypoxic conditions [496, 497]. Both D407 and ARPE-19 cells represent RPE with respect to epithelial morphology, transporter expression and polarization [490, 498]. D407 cells were cultured for 3, 6, 12, 24, 48 and 72 hours under both normoxic and hypoxic conditions to determine the optimum time for induction of membrane transporters. RNA was extracted and cDNA was synthesized. Real-time PCR analysis was performed to determine the quantitative gene expression levels of efflux transporters (MDR1, MRP2 and BCRP), influx transporters ($B^{(0,+)}$, FR-α, and SMVT), hypoxic regulator (HIF-1α) and hypoxic markers (VEGF and GLUT-1).

Expression of Efflux Transporters: MDR1 expression did not change significantly following exposure to hypoxic conditions for 3 and 6 hours. The expression was elevated to almost 2.5 fold within 12 hours of hypoxic exposure which was maintained for over 72 hours. The expression of MRP2 remained the same within 3 hours of hypoxic exposure. Once again expression level was significantly induced from 6 to 24 hours, with highest induction noticed at 24 hours and then an attenuation of MRP2 mRNA levels was observed within 48 and 72 hours. Exposure to hypoxia for 3 and 6 hours did not alter the expression levels of BCRP. A significant rise in BCRP expression was evident when cells were exposed to hypoxia for 12 (2.5 fold) and 24 hours (3.1 fold). A sudden drop in RNA expression compared to control levels was observed as time progressed from 48 to 72 hours (**Figure 45**).

Expression of Influx Transporters: The expression levels of influx transporters- $B^{(0,+)}$, FR-α, and SMVT did not rise significantly following hypoxic exposure over 3 and 6 hours. The expression level of $B^{(0,+)}$ was induced by 2 fold upon hypoxic exposure for 12 and 24 hours and

then dropped to basal levels upon prolonged exposure to 72 hours. FR-α showed incremental induction as hypoxic exposure was prolonged. A maximum of 3 fold induction was observed in 48 to 72 hours. The expression level of SMVT elevated to 2.5 fold after 12 hours and reached to a peak of 4 fold with 24 hours of hypoxic exposure. Interestingly, prolonged exposure to hypoxia dramatically reduced SMVT expression to basal levels (**Figure 46**). Among all the time points studied, hypoxic exposure to 24 hours demonstrated elevated RNA expression levels of efflux and influx transporters, HIF-1α, VEGF and GLUT-1 in D407 cells. Therefore, 24 hour exposure duration was selected for all subsequent studies.

With these promising results, ARPE-19 cells were exposed to hypoxia for 24 hours. A similar trend in RNA expression levels of efflux and influx transporters, HIF-1α, VEGF and GLUT-1 was also noticed in ARPE-19 (**Figure 47**).

ELISA

The exposed cell culture medium was collected and assayed to determine the amount of VEGF secreted, using sandwich ELISA method. This assay can detect different concentrations of VEGF varying from 31.25 - 2000 pg/mL (R^2 = 0.99). From this standard graph, the concentration of VEGF in D407 normoxic conditions was calculated to be 272.83 ± 25.69 pg/mL. However, exposure to hypoxia for 24 hours increased the concentration of VEGF to 583.83 ± 14.84 pg/mL (2 fold increase). Similarly, the concentration of VEGF in ARPE-19 cells increased to 329.33 ± 14.14 pg/mL (3.5 fold increase) in hypoxic conditions compared to 93.66 ± 11.83 pg/mL in normoxic conditions (**Figure 48**). The induction observed at the RNA level for VEGF was well correlated with the values obtained from ELISA.

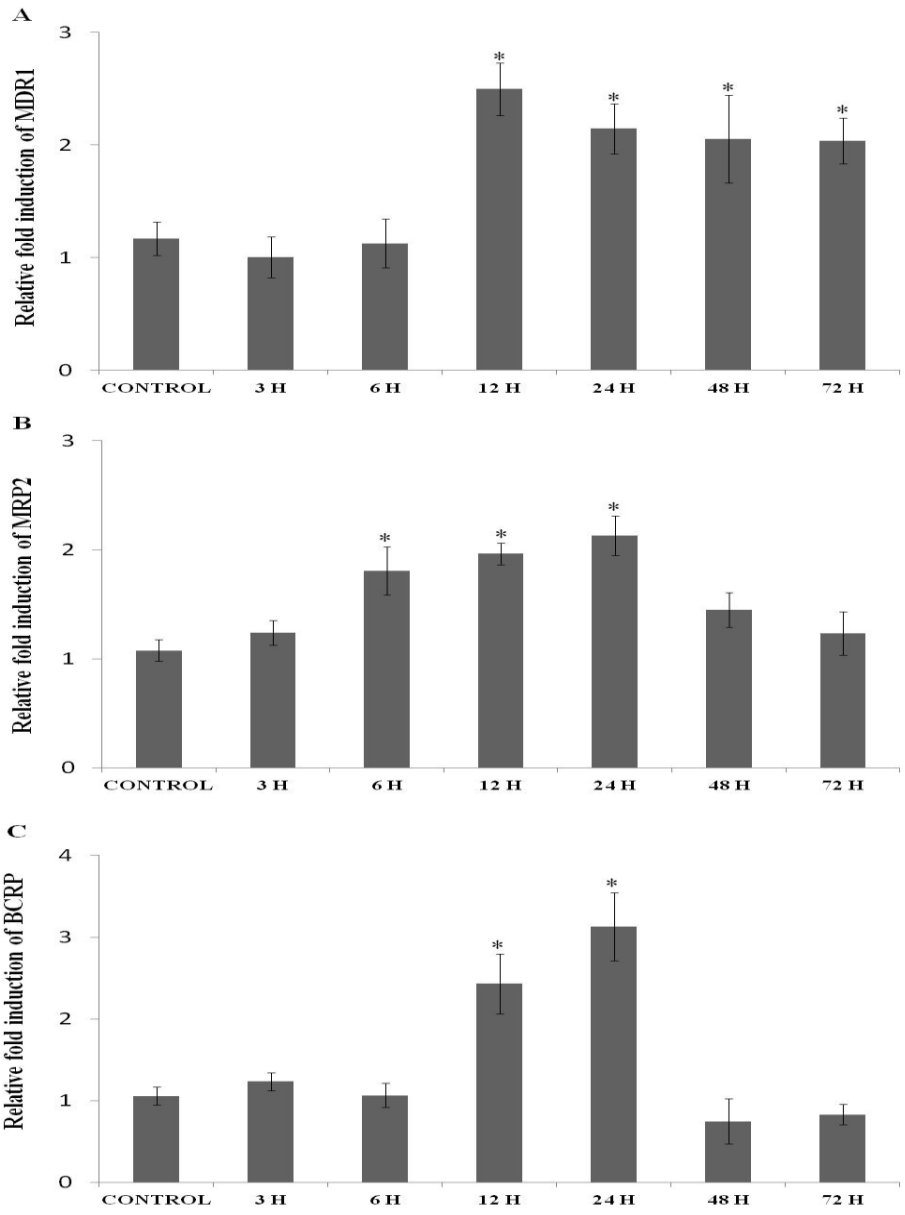

Figure 45. Relative fold RNA expression of various efflux transporters (A) MDR1, (B) MRP2 and (C) BCRP in D407 cells exposed to hypoxic conditions for various time points (3, 6, 12, 24, 48 and 72 hours). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

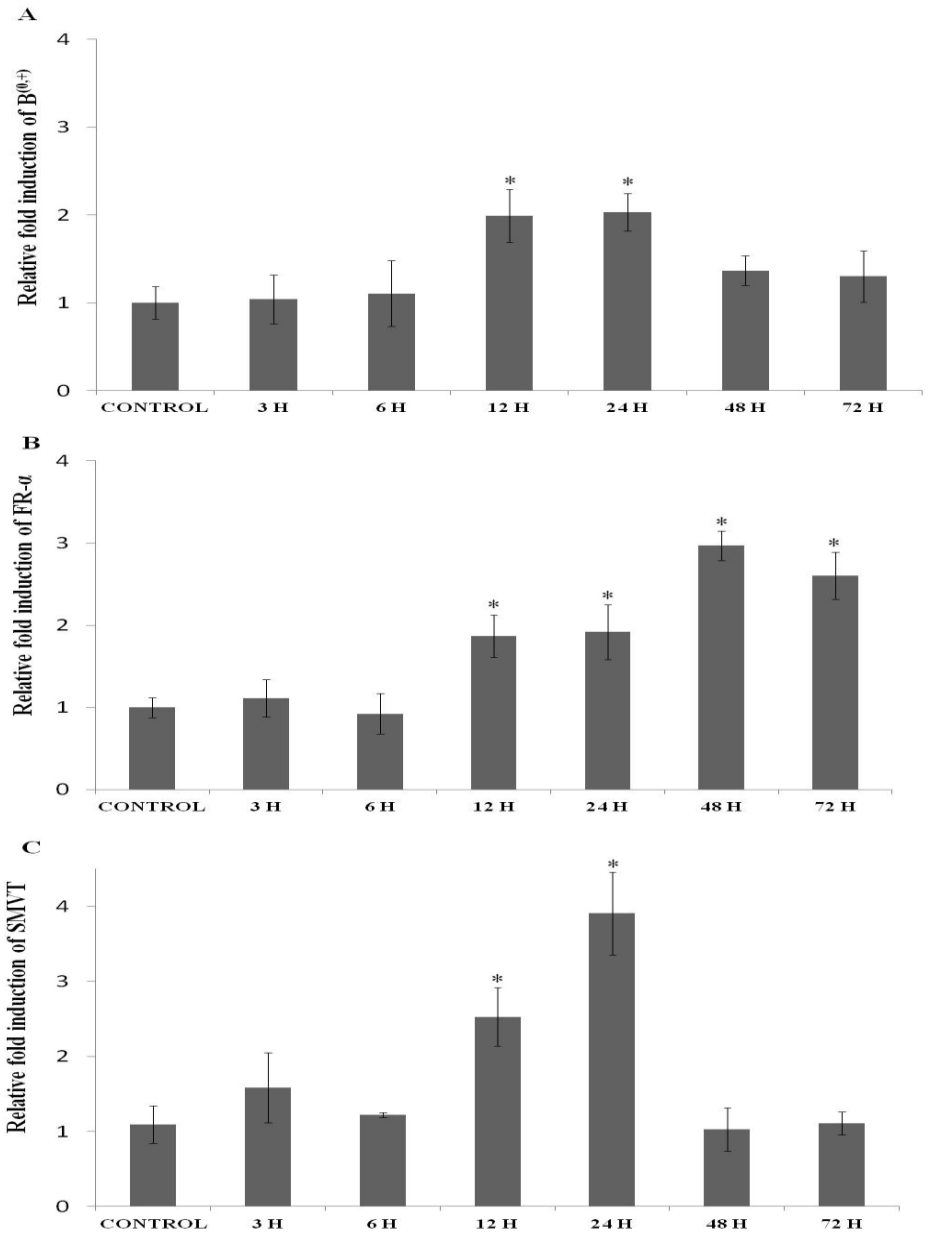

Figure 46. Relative fold RNA expression of various influx transporters (A) $B^{(0,+)}$, (B) FR-α and (C) SMVT in D407 cells exposed to hypoxic conditions for various time points (3, 6, 12, 24, 48 and 72 hours). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

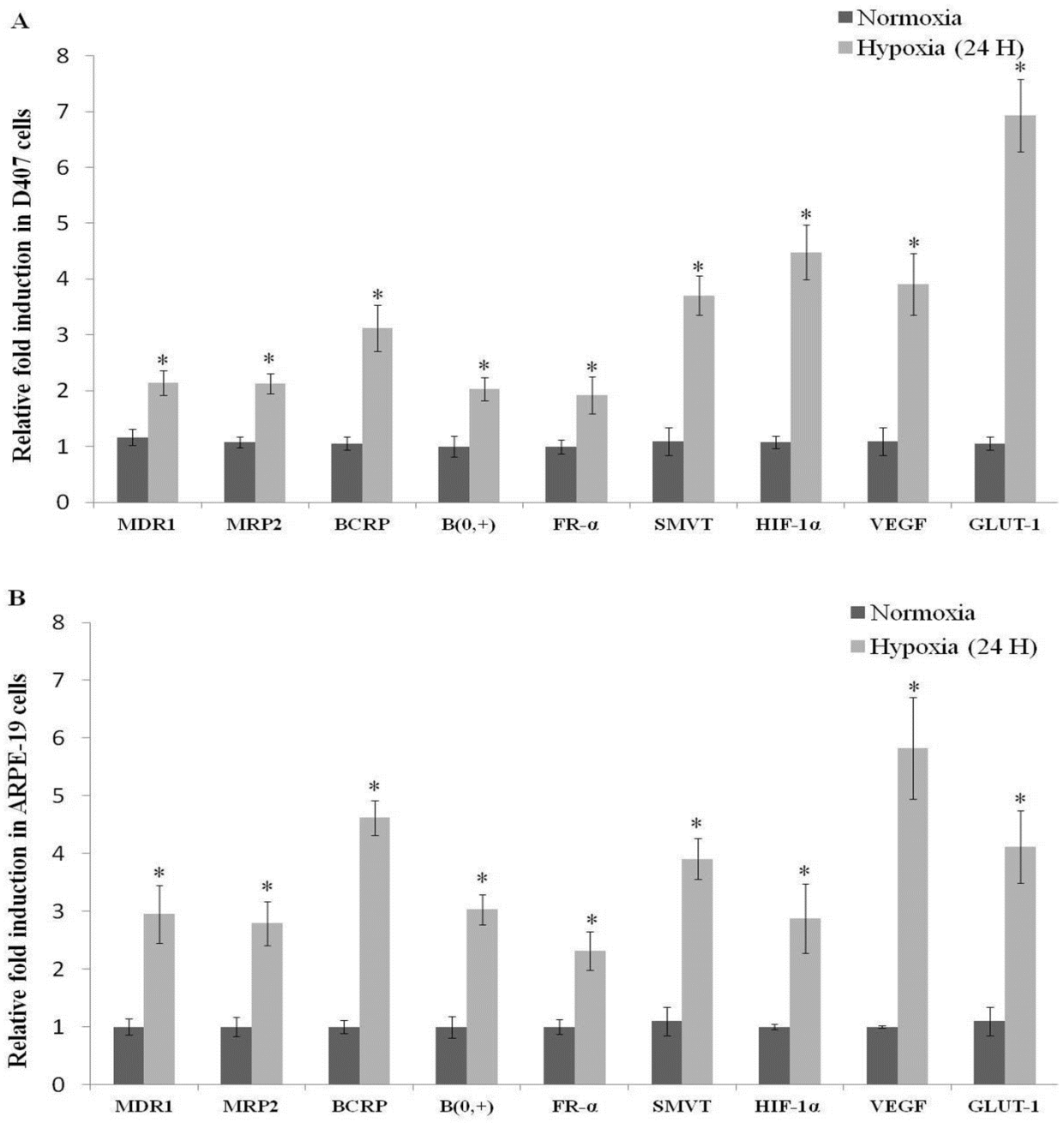

Figure 47. Relative fold RNA expression of efflux transporters (MDR1, MRP2 and BCRP), influx transporters ($B^{(0,+)}$, FR-α and SMVT) and hypoxic markers (HIF-1α, VEGF and GLUT-1) post hypoxic exposure for 24 hours in (A) D407 and (B) ARPE-19 cells. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

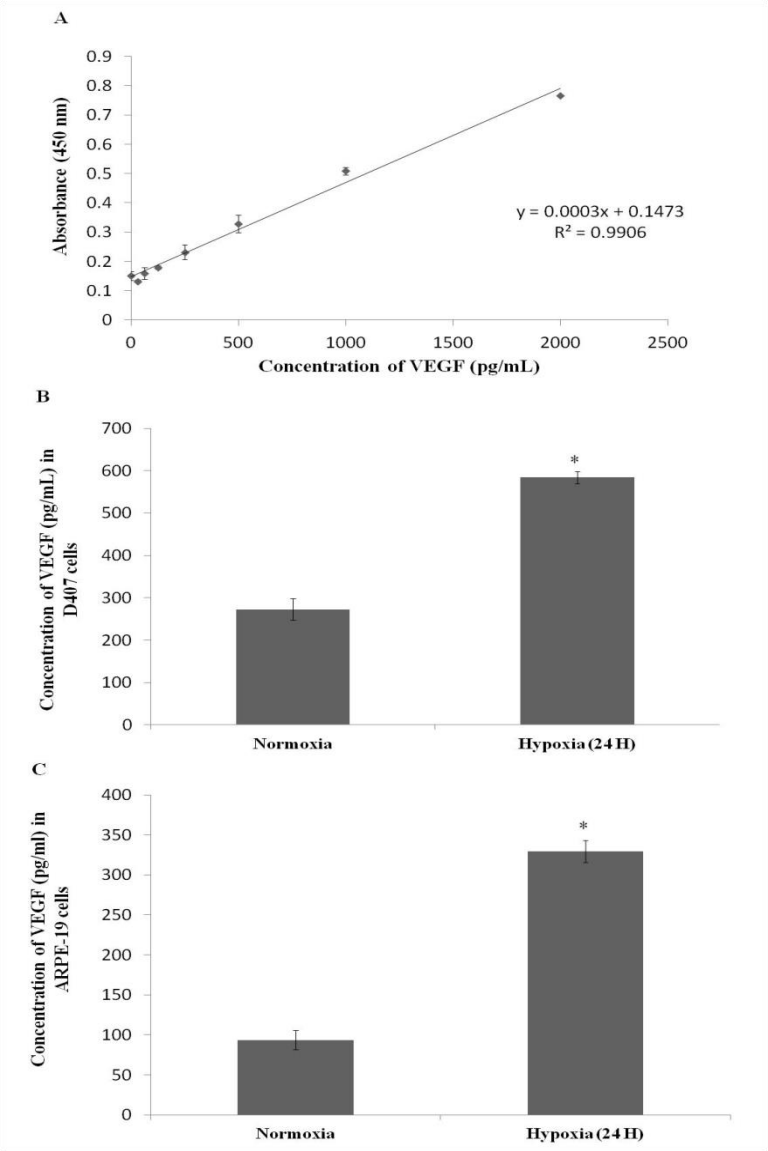

Figure 48. Sandwich ELISA measuring (A) absorbance at 450 nm post exposure to different concentrations of VEGF standards (31.25 - 2000 pg/mL), (B) concentration of VEGF in D407 cell culture supernatant post exposure to normoxic and hypoxic conditions (24 hours) and (C) concentration of VEGF in ARPE-19 cell culture supernatant post exposure to normoxic and hypoxic conditions (24 hours). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Immunoblot Analysis

Immunoblot analysis was performed to evaluate if the induction in gene expression levels correlate with protein expression on exposure to hypoxia. Since BCRP and SMVT were highly upregulated as evident from qPCR analysis, these two transporters were examined for their protein expression. RPE cells were cultured for 24 hours in both normoxic and hypoxic conditions. Total protein was extracted, separated using PAGE and amount of specific protein was determined using antibody staining. The protein level of β-actin was measured as a loading control during the experiment. Both the proteins BCRP and SMVT demonstrated significant induction in hypoxic relative to normoxic conditions in both D407 and ARPE-19 cells (**Figure 49**). The induction observed at the protein level for representative efflux and influx transporters correlated well with the mRNA change observed in qPCR analysis.

Uptake Studies

Uptake of Efflux Transporter Substrates: [^{3}H] Digoxin, [^{3}H] lopinavir and [^{3}H] abacavir were used as model substrates to study MDR1, MRP2 and BCRP mediated efflux, respectively. A significant decrease in cellular accumulation of these substrates in hypoxic conditions was evident in D407 cells. Uptake of [^{3}H] digoxin and [^{3}H] lopinavir was lower by 30% and 25%, respectively. Notably, a 65% reduction in uptake of [^{3}H] abacavir was noticed. In ARPE-19 cells, uptake of [^{3}H] digoxin, [^{3}H] lopinavir and [^{3}H] abacavir was reduced by 25%, 30% and 55%, respectively. This data confirms increased activity of efflux transporters under hypoxic conditions in both the cells (**Figure 50**).

Uptake of Influx Transporter Substrates: [^{14}C] Arginine, [^{3}H] folic acid and [^{3}H] biotin were employed as model influx substrates to study the functional activity of $B^{(0,+)}$, FR-α, and SMVT respectively. [^{14}C] Arginine demonstrated 85% rise in cellular accumulation under

hypoxic conditions in D407 cells. [^{3}H] Folic acid and [^{3}H] biotin also exhibited an elevation in cellular uptake in hypoxic conditions by 40% and 50%, respectively. Uptake of all the three influx transporter substrates raised by 65%, 60% and 40%, respectively in ARPE-19 cells exposed to hypoxia for 24 hours. This study demonstrates the enhanced activity of influx transporters under hypoxic conditions (**Figure 51**).

Cell Proliferation Assay

MTS assay was performed to examine the cytotoxicity of cells post exposure to hypoxic conditions (24 hours). RPE cells (D407 and ARPE-19) did not exhibit any significant cell death (**Figure 52**).

Discussion

Deficiencies in oxygenation (hypoxia) stimulate the expression of proangiogenic factors leading to the development of neovascularization in retinal diseases [375]. In the past decade, significant research has been conducted to comprehend the pathogenesis and genetics of oxygen-dependent retinal diseases. Most of the therapies aimed at inhibiting this pathway demonstrated partial success owing to the underlying complex multifactorial signaling process. Significant attention has not been paid to transporter expression during retinal hypoxic complications, regardless of their potential value in drug absorption. Therefore, the aim of this study was to examine the changes in molecular expression and functional activity of efflux and influx transporters under hypoxic conditions in retinal pigment epithelial cells (D407 and ARPE-19 cells). These observations would be helpful for predicting drug accumulation in ocular tissues under hypoxic conditions.

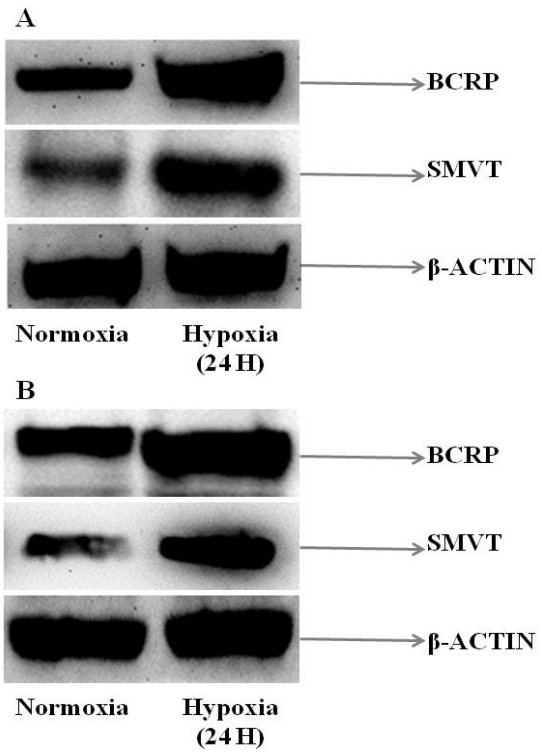

Figure 49. Immunoblot analysis showing the protein expression of BCRP and SMVT in (A) D407 and (B) ARPE-19 cells post exposure to normoxic and hypoxic conditions (24 hours). β-actin was used as an internal control.

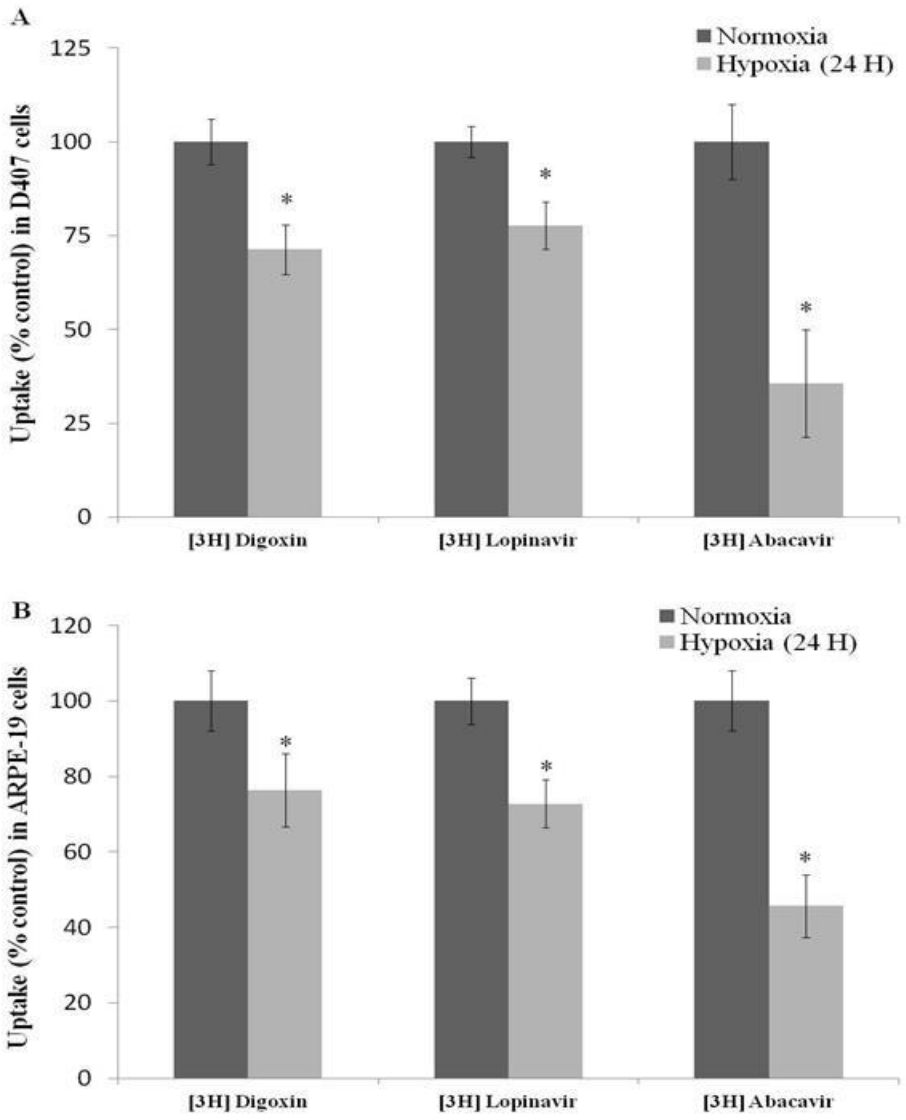

Figure 50. Uptake of efflux transporter substrates [^{3}H] digoxin, [^{3}H] lopinavir and [^{3}H] abacavir in (A) D407 and (B) ARPE-19 cells post exposure to normoxic and hypoxic conditions (24 hours). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

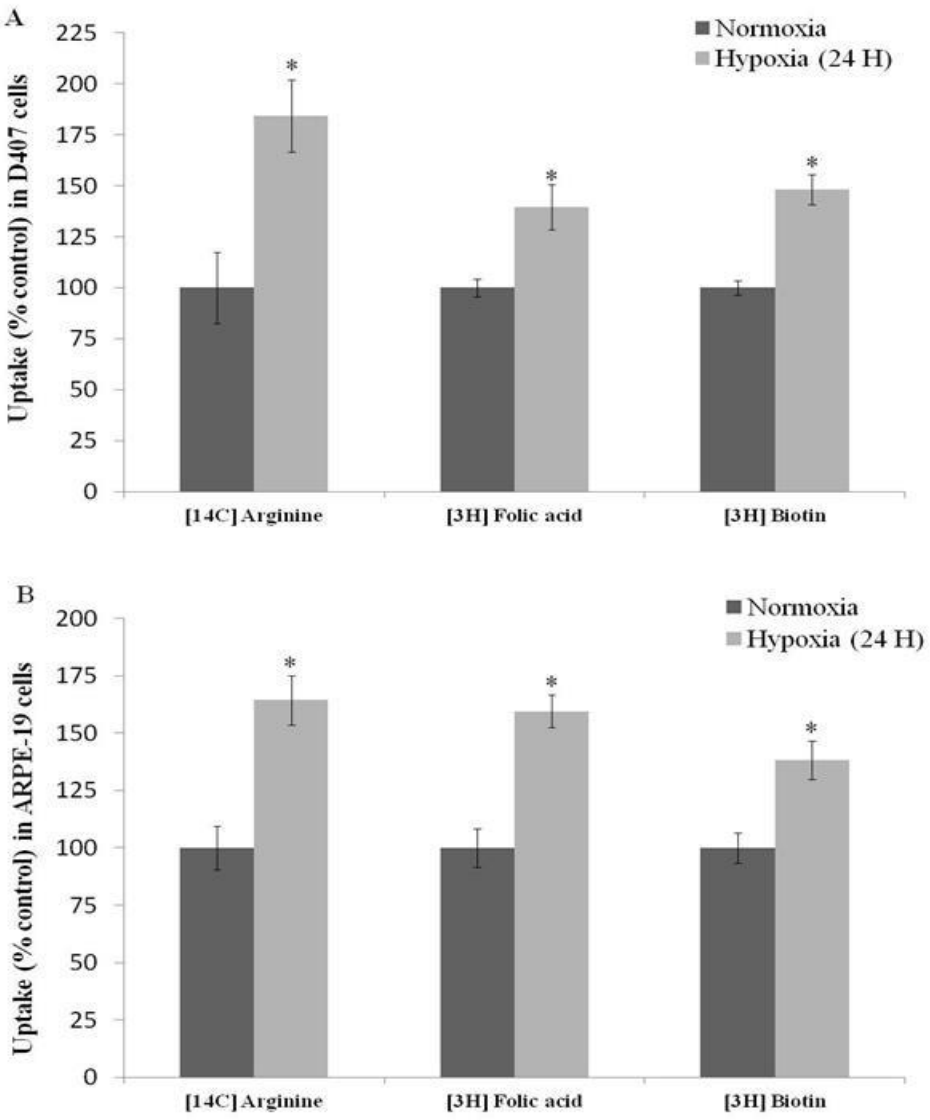

Figure 51. Uptake of influx transporter substrates [^{14}C] arginine, [^{3}H] folic acid and [^{3}H] biotin in (A) D407 and (B) ARPE-19 cells post exposure to normoxic and hypoxic conditions (24 hours). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

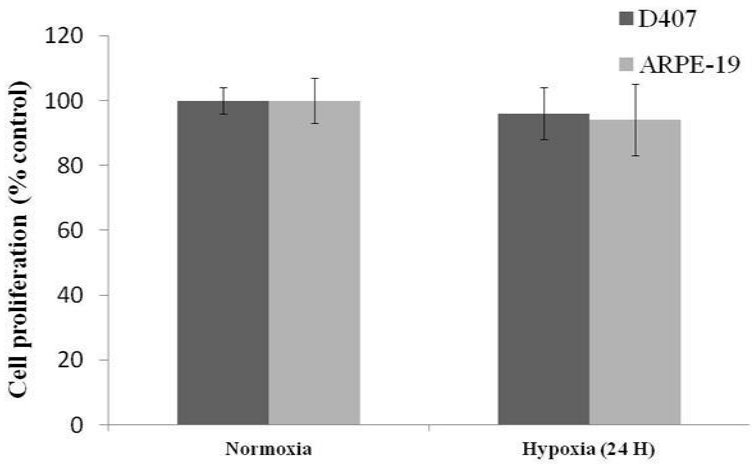

Figure 52. Cell proliferation assay of D407 and ARPE-19 cells post exposure to normoxic and hypoxic conditions (24 hours). Values represent mean ± SD.

Real time-PCR studies revealed that RNA levels of efflux transporters were strongly induced in hypoxic cells. Exposure of D407 cells induced the expression of MDR1 gene levels after 12 hours and continued induction upto 72 hours. These results are consistent with earlier studies which demonstrated that the basal expression of MDR1 is indeed regulated by HIF-1α [67]. The expression of MRP2 in hypoxic cells was upregulated after incubation from 6 to 24 hours, with the differences being statistically significant. However, such induction of MRP2 expression was diminished after continuous exposure to hypoxia for 48 and 72 hours. Also, a similar trend was observed for BCRP expression in D407 cells. These results suggest that the induction of both MRP2 and BCRP genes at hypoxic state was transient. Elevation in BCRP levels was also suggested to be upregulated by HIF- α, thus conferring a survival advantage under hypoxic conditions [66, 499, 500]. Cheng et al. have identified a region within BCRP promoter site in close proximity to the reported HIF-1α response element, which is responsible for regulation of BCRP *via* HIF-1α [501]. BCRP has also been reported to play a major role in heme and folate homeostasis, which may help in cell survival under hypoxic conditions. Thus, malignant or diseased cells may utilize the over expressing efflux transporters to survive against hypoxic stress [132, 133].

Besides efflux transporters, the expression level of influx transporters including $B^{(0,+)}$, FR-α, and SMVT were also examined in these studies. Elevated expression of $B^{(0,+)}$, FR-α, and SMVT after hypoxic exposure suggest that these transporters are also susceptible to oxygen tension. It appears that cells exposed to hypoxic stress may require high intake of amino acids, vitamins and other nutrients as compared to normal cells. This requirement is generally met by acquisition of genetic mutations that functionally alter receptor-initiated signaling pathways in malignant or diseased cells. Consequently such pathways aid in activating the uptake and

metabolism of amino acids, vitamins and other nutrients necessary for promotion of cell survival and growth. Enhanced expression of nutrient transporters occurs as a result of adaptive response to hypoxic insult [502, 503]. Apart from $B^{(0,+)}$, FR-α, and SMVT, other influx transporters including peptide transporters (PEPT1 and PEPT2) and organic cation transporters (OCT1 and OCT2) are also identified on RPE cells and their importance in ocular drug disposition has been documented in literature [504]. Hence, a preliminary study was conducted to evaluate the differential expression of PEPT1, PEPT2, OCT1 and OCT2 in hypoxic conditions. Surprisingly, RNA expression of PEPT1 and PEPT2 did not alter significantly while the expression of OCT1 and OCT2 was downregulated in RPE cells following 24 hours of hypoxic exposure (**Figure 53**).

OCTs play an important role in the transport of ionic drug molecules across ocular barriers. Consistent with our results, Kadam RS and colleagues have demonstrated that hypoxia downregulates OCT mRNA expression in rat choroid-retina. Also, the apparent permeability of MPP^+ (OCT substrate) across hypoxic calf's sclera-chorid-RPE and cornea was diminished by 61% and 49%, respectively. This study suggests that both the expression and functional activity of OCTs were significantly reduced in ocular tissues under chronic hypoxic conditions [505]. Literature also reports that the expression of organic cation/carnitine transporter 1 (OCTN1) is reduced under hypoxic conditions. Also, decreased carnitine (OCTN1 substrate) uptake is evident in placental explants and BeWo cells under hypoxia. The study suggests that hypoxia plays an important role in the regulation of OCTN expression, probably *via* regulation of transcriptional factor - PPARα and its DNA-binding partner - RXRα [506].

A sandwich ELISA assay confirmed the increased secretion of VEGF during hypoxic conditions. This assay further validates the hypoxic conditions used in our studies. Results from immunoblot analysis further indicated that the RNA expression of these transporters is being

well translated to protein expression. Results from gene expression studies were further confirmed by performing uptake studies to delineate the functional activity of various transporters in hypoxic conditions. Lower uptake of [^{3}H] digoxin, [^{3}H] lopinavir and [^{3}H] abacavir was observed as a result of elevated efflux by MDR1, MRP2 and BCRP, respectively in RPE cells. This decrease in intracellular accumulation of radiolabeled substrates suggested that hypoxia may inhibit the uptake of various active molecules that are substrates of efflux transporters. An interesting observation was that uptake of [^{3}H] abacavir was reduced to a greater degree in cells exposed to hypoxia for 24 hours suggesting that BCRP is more susceptible to hypoxic stress compared to MDR1 and MRP2.

We also examined the functional activity of influx transport systems-B $^{(0, +)}$, FR-α and SMVT by measuring uptake of [^{14}C] arginine, [^{3}H] folic acid and [^{3}H] biotin, respectively. Enhanced uptake of these substrates suggest that rise in cellular accumulation may be due to induction of B $^{(0, +)}$, FR-α and SMVT respectively, further confirming the results from gene expression studies. [^{14}C] Arginine accumulation was higher compared to [^{3}H] folic acid and [^{3}H] biotin in both the RPE cells, suggesting that B $^{(0, +)}$ transporter is functionally more active under hypoxic conditions relative to FR-α and SMVT. However, data from gene expression analysis revealed that SMVT is highly induced compared to B $^{(0, +)}$ and FR-α. The discrepancy can be attributed to posttranscriptional modifications of the respective protein. MTS assay was carried out to check cell proliferation under normoxic and hypoxic conditions. Results from this assay revealed that hypoxic insult for 24 hours did not induce RPE cell death. This result is consistent with earlier report in which another human RPE cell line (R-50) did not exhibit any cytotoxic effects even after 48 hours of hypoxic exposure [507].

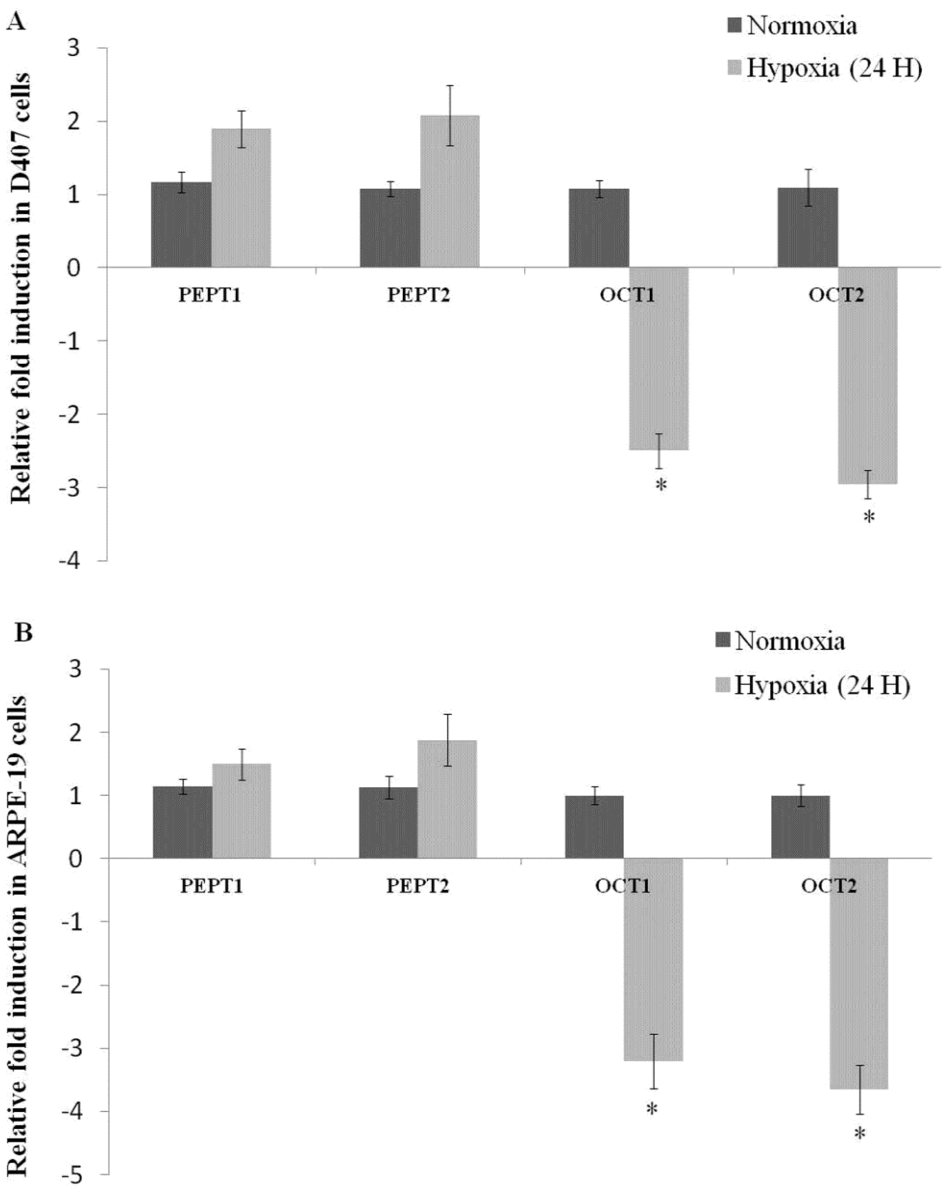

Figure 53. Relative fold RNA expression of peptide transporters (PEPT1 and PEPT2) and organic cation transporters (OCT1 and OCT2) post hypoxic exposure for 24 hours in (A) D407 and (B) ARPE-19 cells. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Conclusions

In conclusion, this study demonstrates for the first time that hypoxic conditions alter expression levels of efflux and influx transporters in human RPE cells. These molecular and functional alterations in the expression of membrane transporters/receptors may facilitate cell survival and growth under hypoxic conditions. Also, hypoxic conditions in retinal diseases may alter cellular accumulation of various molecules targeted to ocular tissues. Better knowledge of physiological and pathological conditions of hypoxia induced disorders offers a promising approach for effective ocular drug delivery.

CHAPTER 7

RITONAVIR INHIBITS HIF-1α MEDIATED VEGF EXPRESSION IN RETINAL PIGMENT EPITHELIAL CELLS

Rationale

Retina is the most metabolically active tissue and consumes more oxygen than any other tissue in the human body [508]. Retinal hypoxia (oxygen deficiency) leading to angiogenesis is a major pathological condition underlying a number of sight threatening diseases including diabetic retinopathy (DR), retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and neovascular glaucoma [375]. In response to hypoxia, retinal pigment epithelial (RPE) cells release various growth factors resulting in angiogenesis, fibro vascular tissue formation, retinal ablation and ultimately loss of vision. This response is mediated *via* the transcriptional regulator hypoxia-inducible factor (HIF). It is a heterodimeric complex consisting of hypoxically stabilized subunit (HIF-1α) and stable nuclear subunit (HIF-1β/ARNT). In normoxic conditions, HIF-1α protein is rapidly hydroxylated on proline residues by 2-oxoglutarate dependent oxygenases. Post hydroxylation, von Hippel-Lindau tumor suppressor protein (pVHL) marks HIF-1α for E3 ubiquitin ligase mediated proteosomal degradation. Under hypoxic conditions, hydroxylation is inhibited leading to nuclear translocation of HIF-1α and subsequent dimerization with HIF-1β. This transcriptionally active complex recruits several coactivators and modulates the gene expression of various target genes by binding to hypoxia response element (HRE) [474, 475, 509].

To date, more than hundred HIF target genes have been identified, many of which play a critical role in mediating angiogenesis, vascular tone, glucose and iron metabolism, cellular differentiation, invasion/metastasis, apoptosis and maintenance of stem cells [510-512]. Few

important target genes include vascular endothelial growth factor (VEGF), angiopoietin 2, platelet-derived growth factor-B (PDGF-B), placental growth factor (PGF) and erythropoietin (EPO) [374]. Many investigations have demonstrated that VEGF is a common denominator promoting ocular angiogenesis. It is a predominant mitogen promoting vascular endothelial cell proliferation and migration [352, 374]. Currently available anti-VEGF therapeutics for the treatment of ocular neovascular diseases includes pegaptanib (Macugen), bevacizumab (Avastin®) and ranibizumab (Lucentis®). Pegaptanib is a 28-base RNA aptamer, bevacizumab is a humanized monoclonal antibody while ranibizumab is an antibody binding fragment of the former. All these molecules bind to secreted VEGF and prevent its interaction with VEGF receptors [280, 513, 514]. These molecules are administered repeatedly at very high doses. Intravitreal injections of these antagonists resulted in serious complications such as endophthalmitis, vitreous hemorrhage and retinal detachment. Further, systemic side effects also warrant their utility in the treatment of ocular diseases [515, 516]. Hence, therapeutic molecules that aim at inhibiting the signaling mechanisms underlying neovascularization (HIF-pathway) appear to have promising antiangiogenic roles with fewer side effects.

Resurgent interest is directed towards identifying new implications of existing drugs, rather than discovering new molecules. This process of drug repositioning can limit the associated cost and expedite the time required from bench to bedside transition of therapeutic molecules. Ritonavir, a HIV protease inhibitor exhibits antineoplastic effects independent from its ability to inhibit HIV protease [279-281]. Also, research has demonstrated that ritonavir can inhibit angiogenesis in Kaposi sarcoma, head and neck carcinoma [282, 517]. However, the inhibitory potency of ritonavir on HIF pathway and *de novo* synthesized VEGF in retinal tissues have not been so far investigated. Hence, the primary objective of this study was to investigate

the expression of HIF-1α and VEGF in human RPE cells (ARPE-19 and D407) treated with ritonavir under hypoxic and normoxic conditions. These results may assist in understanding the molecular mechanism of VEGF inhibition by ritonavir. Further, inhibition of VEGF *via* HIF pathway may serve the possibility of using ritonavir in the treatment of various ocular angiogenic diseases.

Materials and Methods

Materials

ARPE-19 and RF/6A cells were procured from American Type Culture Collection (Manassas, VA). D407 cells were obtained as a generous gift from Dr. Richard Hunt (University of South Carolina). Ritonavir was obtained from Sigma Chemicals (St. Louis, MO). Dulbecco's modified eagle's medium (DMEM), DMEM: Nutrient Mixture F-12 (DMEM/F12), RPMI1640, trypsin replacement (TrypLE™ Express), non-essential amino acids, and TRIzol®, and custom designed primers were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75 cm^2 and 25 cm^2 growth area) and 96-well plates (0.3 cm^2 growth area per well) were procured from Corning Incorporated (Cambridge, MA). The reagents for cDNA synthesis (OligodT, dNTP, M-MLV reverse transcriptase) and CellTiter 96® aqueous non-radioactive cell proliferation assay were supplied by Promega Corporation (Madison, WI). Light Cycler 480® SYBR I green master mix was obtained from Roche Applied Science (Indianapolis, IN). BCA protein assay reagent and human VEGF ELISA kit were purchased from Thermo Fisher Scientific Inc (Rockford, IL). Unless otherwise mentioned, all other chemicals were purchased from Sigma Chemicals and used without further purification.

Cell Culture

ARPE-19 cells (passage numbers 25-30) were cultured in D-MEM/F-12 supplemented with 10% FBS (heat inactivated), 15 mM HEPES, 29 mM sodium bicarbonate, 100 U/mL penicillin and 100 µg/mL streptomycin. D407 cells (passage numbers 65-70) were maintained in DMEM medium supplemented with 10% FBS (heat inactivated), 1% non-essential amino acids, 20 mM HEPES, 29 mM sodium bicarbonate and 100 U/mL of penicillin and 100 µg/ml of streptomycin. RF/6A cells (passage numbers 5-10) were cultured using RPMI1640 supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. All the cells were maintained in a humidified atmosphere of 5% CO_2 and 90% relative humidity at 37°C in T-75 flasks. The medium was replaced every alternate day and cells were passaged using TrypLE™ Express after reaching ~80% confluency. Cells were seeded at a density of 1 million cells in T-25 flasks. After reaching ~80% confluency, cells were subjected to hypoxic conditions in a bactron anaerobic chamber with a special gas mix (1% O_2, 5% CO_2 and 94% N_2) for specified time periods following a previously published method [489]. Cells cultured under hypoxic conditions were processed in the chamber itself to avoid any exposure to normoxic conditions.

Extraction of RNA

RNA was extracted using the standard phenol-chloroform-isopropanol method. Briefly, cells were lysed in TRIzol® reagent and the lysate was transferred into an eppendorf tube. To facilitate phase separation, chloroform was added to lysate. The aqueous layer containing RNA was separated and isopropanol was added to precipitate RNA. RNA pellet was washed twice with 75% ethanol and dissolved in DNase/RNase free water. The concentration of RNA was estimated by detecting UV absorbance at 260 nm with Nanodrop (Thermo Fisher Scientific, Wilmington, DE). Reverse transcription of RNA (4 µg) into complementary DNA (cDNA) was carried out with OligodT as a template and M-MLV reverse transcriptase. The conditions for

reverse transcription include denaturation for 5 min at 70°C, reverse transcription for 1 hour at 42°C and final extension for 5 min at 72°C.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) Studies

For quantitative gene expression analysis, 80 µg of cDNA was amplified for specific genes with corresponding forward and reverse primers (10 µM) and SYBR green master mix using ABI Prism 5700 Sequence Detection System (Applied Biosystems, CA). Primers were designed with Primer-Blast tool from Pubmed. β-actin was selected as an internal standard in all the samples The sequences of the primers are as follows: β-actin forward 5'-GCATTGCTTTCGTGTAAATTATGT-3' reverse 5'- ACCAAAAGCCTTCATACATCTCA-3'; *HIF-1α* forward 5'-GTACCCTAACTAGCCGAGGAAGAA -3' reverse 5'-GTGAATGTGGCCTGTGCAGT-3'; *VEGFA* forward 5'- CTTGCCTTGCTGCTCTAC-3' reverse 5'-TGGCTTGAAGATGTACTCG-3'. The reactions were carried out with initial denaturation at 70°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec and extension at 72°C for 10 sec. Melting curve analysis was used to check the specificity of PCR products. The relative fold mRNA expression in treated vs control samples was quantified by comparative threshold method as mentioned in data analysis section.

Immunoblot Analysis

Following exposure to normoxic and hypoxic conditions, cells were washed with PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl and 135 mM NaCl). Protein was extracted using radio immunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail at pH 7.4. The amount of protein in the lysate was quantified using BCA protein assay kit. An equal amount of protein (20 µg) in all samples was separated using poly acrylamide gel electrophoresis (PAGE). The separated protein bands were transferred onto a polyvinylidene difluoride (PVDF)

membrane and blocked for 2 hours with 5% non-fat dry milk prepared in 0.05 M tris buffered saline. The blot was exposed overnight at 4°C to primary antibody. After subsequent washes, the blot was exposed to secondary antibody conjugated with horseradish peroxidase (HRP) for 2 hours. Finally, the blot was visualized for the protein of interest using chemiluminescent substrate in ChemiImager 8900 (Alpha Innotech, San Leandro, CA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The amount of VEGF secreted into the culture medium post exposure to normoxic or hypoxic conditions was estimated by performing a sandwich ELISA, following manufacturer's instructions. An anti-human $VEGF_{165}$ antibody was precoated to the 96-well microplate. The samples/standards (50 µL) were added to the antibody coated wells and incubated for 2 hours. Unbound antigen was washed, followed by addition of biotinylated secondary detecting antibody (100 µL) and subsequent incubation for 1 hour. Excess detecting antibody was removed and streptavidin-HRP was added. It reacts with TMB (3,3′,5,5′-tetramethylbenzidine) substrate to produce a colorimetric signal. This signal was detected by measuring the absorbance at 450 nm using a plate spectrophotometer (SpectraFluor Plus, Maennedorf, Switzerland).

Cell Proliferation Assay

Relative proliferation of choroid-retinal endothelial cells (RF/6A) was determined using spent culture medium of ARPE-19 and D407 cells exposed to hypoxic or normoxic conditions, following a previously published method [518]. Briefly, RF/6A cells were seeded in a 96-well plate at a density of 5000 cells/well. Following 24 hours of seeding, cells were serum-starved overnight. Later, cells were treated for 24 hours with medium collected from either normoxic or hypoxic conditions. Subsequently, solutions were aspirated and 100 µL of serum free medium was added. Twenty microliters of MTS and PMS reagent was then added to wells. After

incubating for 4 hours, the quantity of formazan formed from MTS was measured at 490 nm using a plate reader.

Cytotoxicity Assay

A cytotoxicity assay was performed to determine the possible toxic effect of ritonavir on ARPE-19 and D407 cells. Briefly, RPE cells were seeded in a 96-well plate at a density of 10000 cells/well. Following 24 hours of seeding, cells were exposed to either normoxic or hypoxic conditions in the absence or presence of three different concentrations of ritonavir for 12 hours. The solutions were aspirated and cytotoxicity was determined using MTS and PMS reagents as described above.

Data and Statistical Analysis

Relative expression of each gene was quantified by delta-delta ($\Delta\Delta C_t$) analysis following previously published method [277]. All the experiments were performed in biological duplicates and experimental quadruplicates. Results were expressed as mean ± standard deviation (SD). Statistical significance between experimental values was checked by performing one way analysis of variance (ANOVA) or student's t test (Graph Pad InStat Version 4.0). A P-value of less than 0.05 was considered to be statistically significant and represented by *.

Results

Quantitative Gene Expression Analysis

Two RPE cell culture models (ARPE-19 and D407) were selected. Cells were exposed to normoxic and hypoxic conditions for 3, 6 and 12 hours. The expression of HIF-1α did not change significantly following exposure of ARPE-19 cells for 3 hours of hypoxic conditions. However, 6 and 12 hours of hypoxic exposure induced the expression of HIF-1α to almost 2 and 3 fold respectively. The expression of VEGF was significantly elevated by 2.5 to 6 fold upon

hypoxic exposure from 3 to 12 hours. Three hours of hypoxic exposure did not alter the expression levels of both HIF-1α and VEGF in D407 cells. A significant rise in their expression was evident when cells were exposed to 6 and 12 hours of hypoxia. Among the three time points studied, hypoxic exposure to 12 hours demonstrated significant elevation of HIF-1α and VEGF mRNA expression levels in both cell lines (**Figure 54**). Hence, this time point was selected for all subsequent studies.

To evaluate if ritonavir can inhibit HIF-1α and subsequent VEGF expression, cells were exposed to normoxic and hypoxic conditions in the presence or absence of ritonavir for 12 hours and expression levels were monitored. Three different concentrations of ritonavir (5, 10 and 20 μM) were selected. Ritonavir did not alter mRNA expression of HIF-1α in both ARPE-19 and D407 cells under hypoxic conditions. Surprisingly, the presence of ritonavir strongly inhibited hypoxia-induced expression of VEGF in a concentration-dependent manner in ARPE-19 cells. A similar trend was noticed even in D407 cells; ritonavir dramatically reduced the expression of VEGF to basal levels (**Figure 55**).

Protein Expression Analysis

Immunoblot analysis was performed to determine HIF-1α protein expression levels. ARPE-19 and D407 cells were cultured for 12 hours in normoxic or hypoxic conditions in the absence or presence of ritonavir. In ARPE-19 cells, the normoxic expression of HIF-1α was negligible. The expression of HIF-1α was stabilized following 12 hours of hypoxic conditions. However, the presence of ritonavir (10 μM) in hypoxic conditions significantly inhibited HIF-1α protein expression. Similar results were also obtained in D407 cells. Exposure to hypoxia alone stabilized the expression of HIF-1α, while presence of ritonavir in hypoxic conditions inhibited HIF-1α protein translation (**Figure 56**).

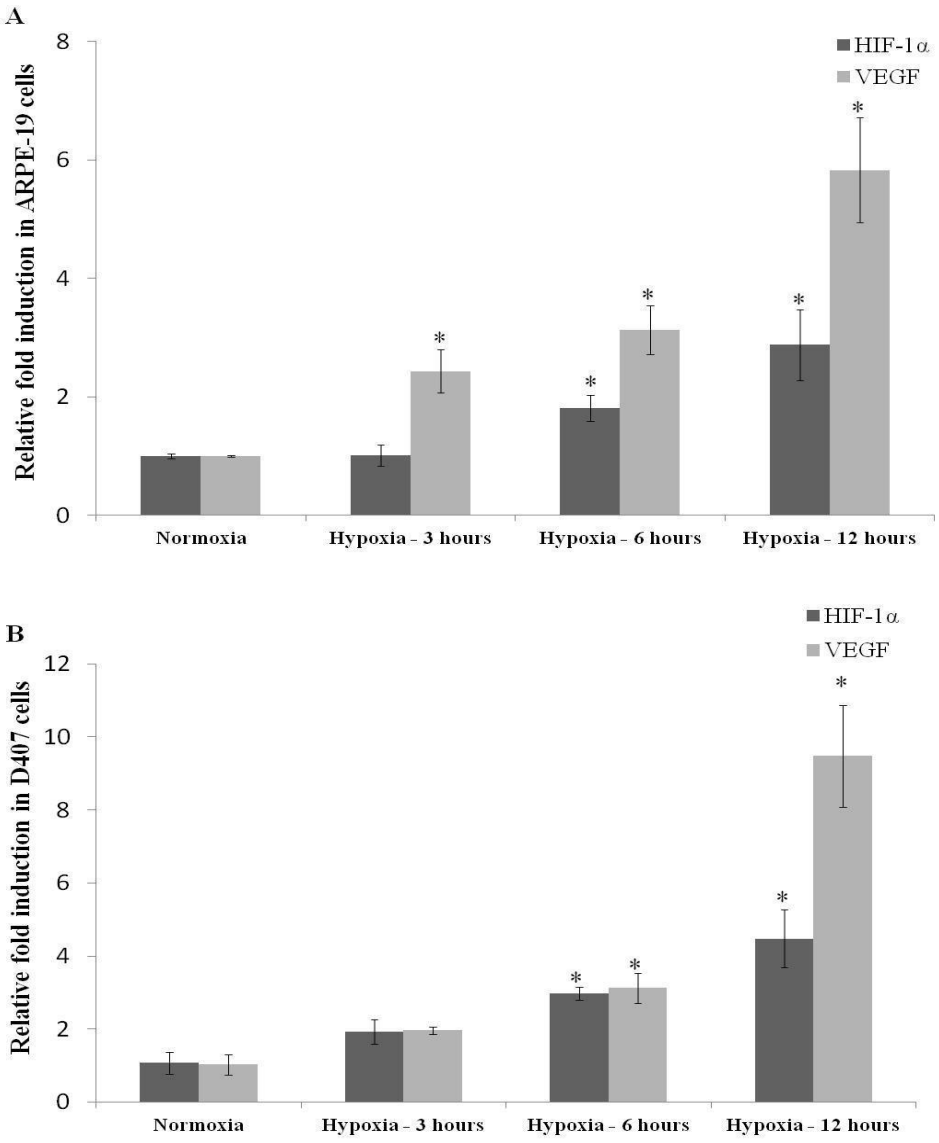

Figure 54. Relative fold RNA expression of HIF-1α and VEGF in (A) ARPE-19 and (B) D407 cells post exposure to 3, 6 and 12 hours of hypoxia. Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

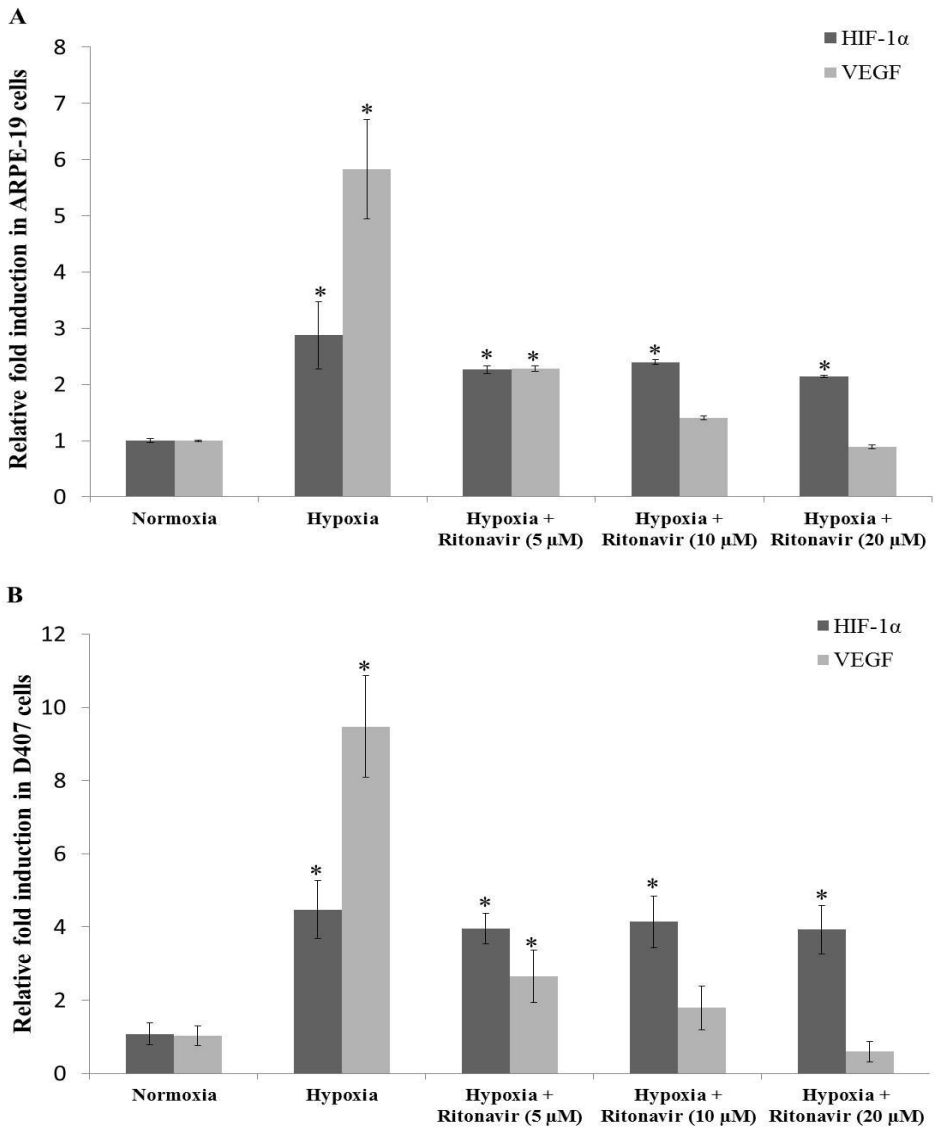

Figure 55. Relative fold mRNA expression of HIF-1α and VEGF in (A) ARPE-19 and (B) D407 cells post exposure to hypoxia (12 hours) in the absence or presence of three different concentrations of ritonavir (5, 10 and 20 µM). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

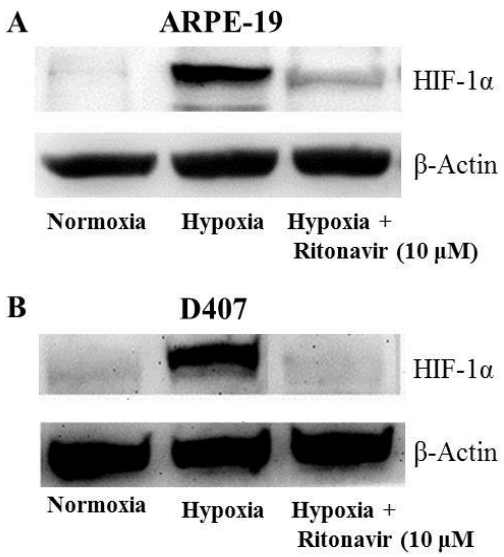

Figure 56. Immunoblot analysis showing the protein expression of HIF-1α in (A) ARPE-19 and (B) D407 cells post exposure to hypoxia (12 hours) in the absence or presence of ritonavir (10 µM). β-actin was used as an internal control.

Although ritonavir did not alter HIF-1α mRNA expression, the protein expression was significantly diminished. This study suggests that ritonavir inhibits HIF-1α synthesis only at the translational level. Many signaling mechanisms including receptor tyrosine kinases, PI3K/AKT/mTOR and Ras-MAPK pathways play a predominant role in HIF-1α translation. Hence, an immunoblot analysis was performed to delineate the mechanism of HIF-1α inhibition by ritonavir. Our study demonstrates that ritonavir decreased the level of AKT phosphorylation (at Ser^{473}) in both ARPE-19 and D407 cells (**Figure 57**). Although the levels of total AKT remained the same, a decrease in phosphorylation resulted in inhibition of HIF-1α protein translation.

Estimation of VEGF by ELISA

ARPE-19 and D407 cells were exposed to 12 hours in normoxic or hypoxic conditions, either in the absence or presence of ritonavir (5, 10 and 20 μM). The exposed culture media were collected and analyzed for concentration of VEGF secreted, using sandwich ELISA method. This method was able to detect different VEGF concentrations varying from 31.25 - 2000 pg/mL with an estimated R^2 value (Pearson coefficient) of 0.99. Concentration of VEGF in ARPE-19 cells exposed to normoxic conditions was estimated to be ~94 pg/mL. Three fold rise in VEGF concentration (~330 pg/mL) was noticed in cells exposed to hypoxia. Interestingly, when cells were exposed to hypoxia in the presence of ritonavir, there was a concentration-dependent decrease in the amount VEGF secreted. A similar trend was also observed in D407 cells. Post exposure to hypoxic conditions, concentration of VEGF increased from ~273 pg/mL to ~584 pg/mL. However, hypoxia induced VEGF secretion was inhibited by the presence of ritonavir in culture medium (**Figure 58**). Two concentrations of ritonavir (10 and 20 μM) completely diminished overexpression of VEGF in hypoxic conditions.

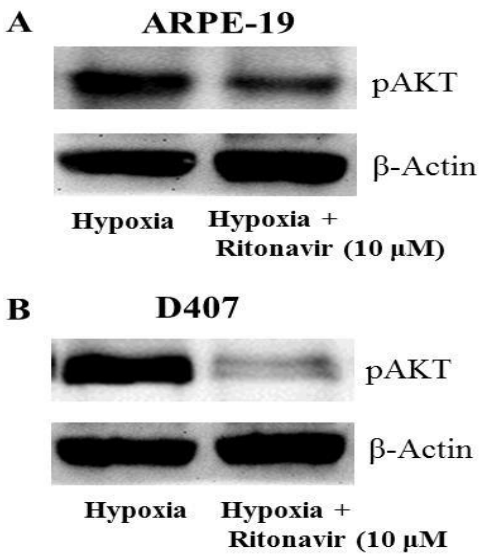

Figure 57. Immunoblot analysis showing the protein expression of pAKT in (A) ARPE-19 and (B) D407 cells post exposure to hypoxia (12 hours) in the absence or presence of ritonavir (10 μM). β-actin was used as an internal control.

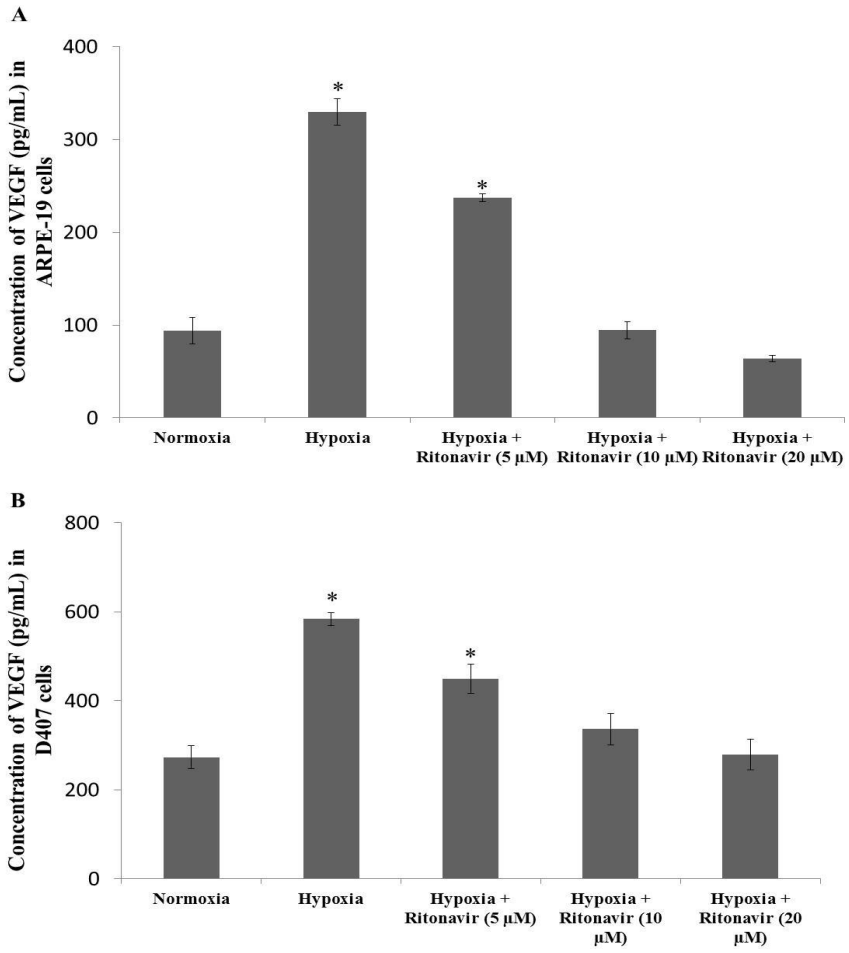

Figure 58. Sandwich ELISA assay measuring concentration of VEGF in (A) ARPE-19 and (B) D407 cell culture supernatant post exposure to hypoxia (12 hours) in the absence or presence of ritonavir (5, 10 and 20 µM). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

Estimation of RF/6A Cell Proliferation

RPE cells were cultured for 12 hours in normoxic or hypoxic conditions, with or without ritonavir (5, 10 and 20 μM). The exposed culture media were collected, sterile filtered using 0.22 μm syringe filters and tested for their ability to induce endothelial (RF/6A) cell proliferation. Following serum starvation, RF/6A cells were incubated with media from ARPE-19 and D407 cultures for 24 hours. Proliferation of endothelial cells was enhanced by 2.2 fold when treated with ARPE-19 hypoxic medium. However, ritonavir (10 and 20 μM) significantly inhibited this VEGF-induced proliferation. Similarly, hypoxic culture medium of D407 cells also induced the proliferation of RF/6A cells by 2 fold. Compared to the hypoxic group, cell proliferation was reduced in the presence of two concentrations of ritonavir tested (10 and 20 μM) (**Figure 59**).

Cytotoxicity assay

MTS assay was performed to examine the possible cytotoxicity of various concentrations of ritonavir. All the three concentrations used were found to be non-cytotoxic to the RPE cells either under normoxia or hypoxia (**Figure 60**). This study suggests that any alterations in mRNA or protein expression levels are not attributed to drug toxicity.

Discussion

Ocular angiogenesis or formation of new blood vessels is a leading cause of ischemic retinopathies. It results from complex interplay between multiple gene products. Hypoxia or reduced oxygenation resulting in activation of HIF-1 was identified as a key transcriptional regulator of angiogenesis [519]. In response to hypoxic conditions, HIF-1 induces transcription of many angiogenic stimulators in RPE cells [520]. Current therapies aimed at treating ocular neovascular diseases include the utilization of large molecule therapeutics targeting VEGF. Although promising, long term usage of such therapeutics is not free of side effects.

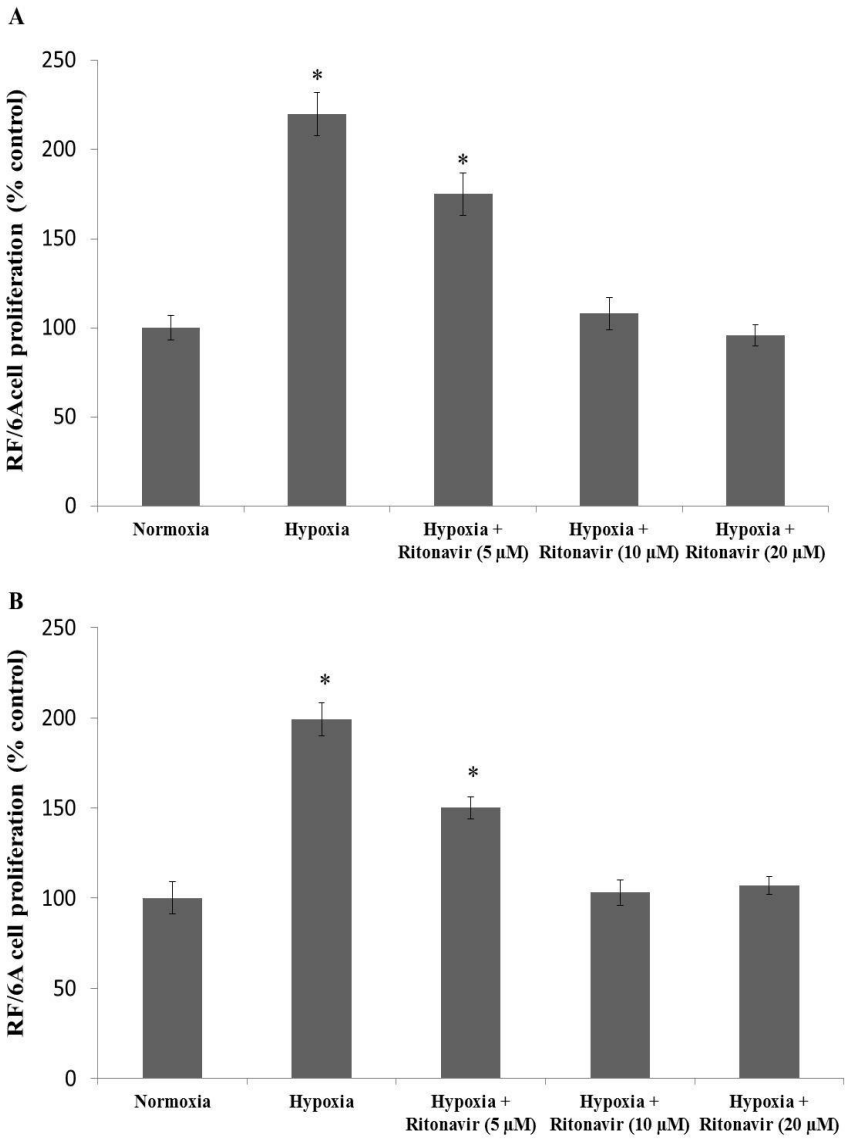

Figure 59. RF/6A cell proliferation with (A) ARPE-19 and (B) D407 spent cell culture media post exposure to hypoxia (12 hours) in the absence or presence of ritonavir (5, 10 and 20 µM). Values represent mean ± SD. * represents statistical significance from control at P-value of <0.05.

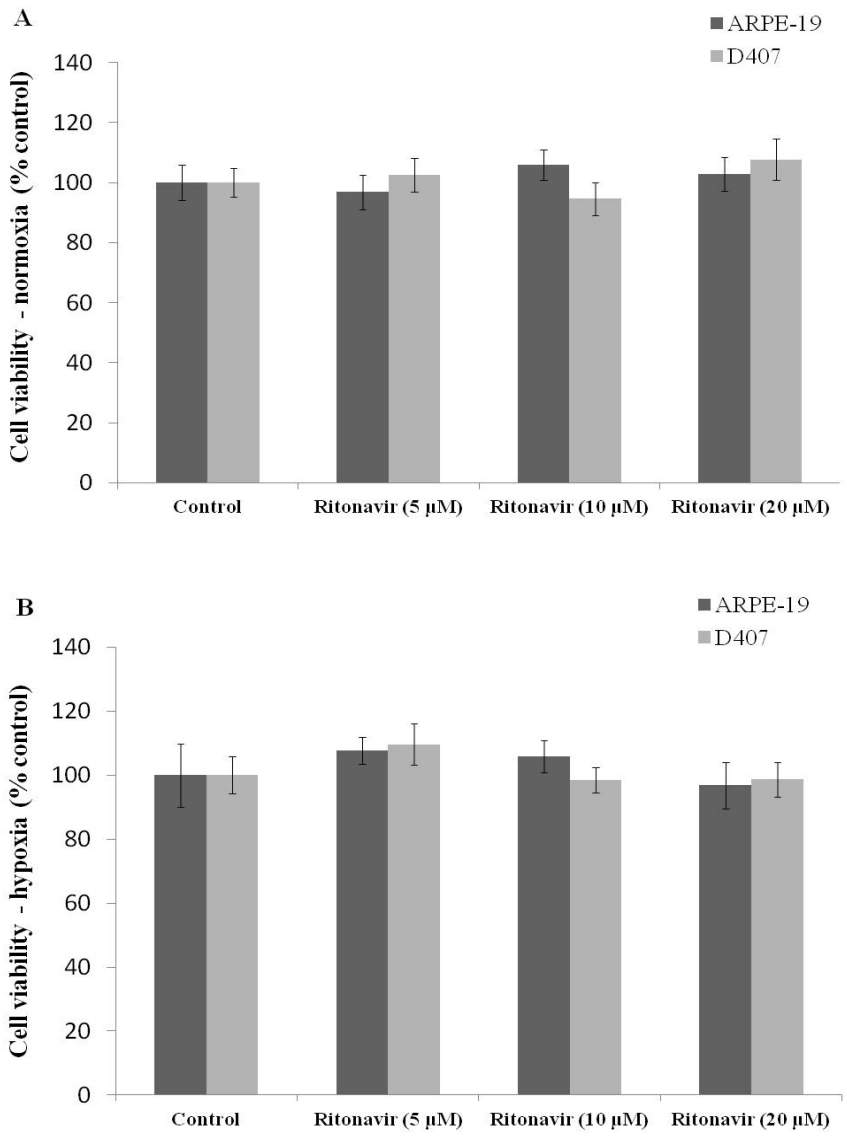

Figure 60. Cytotoxicity assay on ARPE-19 and D407 cells post exposure to (A) normoxia or (B) hypoxia (12 hours) in the absence or presence of ritonavir (5, 10 and 20 µM).

Kurihara et al., have demonstrated the off-target effects resulting from specific knock-out of *Vegfa* gene in adult mice. The knock-out mice exhibited degeneration of choriocapillaris, dysfunction of cone photoreceptors and ultimately vision loss. However, the transcriptional (HIF) mutants did not elicit any dramatic secondary off-target effects [454, 455]. These studies clearly reinforce that therapeutics aiming at HIF pathway may be an alternative, safer and effective mode of treatment than attenuating VEGF levels alone in the treatment of ocular neovascular diseases. Hence, there is an immediate necessity to identify modulators of HIF pathway for better treatment of angiogenic diseases.

Significant research has been conducted in the past few years to identify inhibitors of the HIF pathway in RPE cells, which may provide better treatment for ischemic retinopathies [457, 521-525]. Although RNAi and many other small molecule inhibitors have been identified, their clinical translation is slow. Hence, interest is directed towards identifying new implications of existing drugs, rather than discovering new molecules. This process of drug repositioning may expedite the clinical transition of therapeutic molecules. Ritonavir, a HIV protease inhibitor exhibits antiangiogenic, antiproliferative and antitumorigenic effects. Hence, ritonavir was evaluated for inhibition of HIF-1α and VEGF in RPE cells, since this inhibition can provide therapeutic interventions for diseases caused by pathological neovascularization.

Since RPE cells are an important source of angiogenic factors in retina, we selected two *in vitro* RPE models (ARPE-19 and D407) [526]. These cells have structural and functional characteristics similar to RPE cells and have been selected for studying the effects of hypoxia in RPE physiology. Hypoxic exposure for 12 hours exhibited a significant rise in expression levels of HIF-1α and VEGF in both ARPE-19 and D407 cells. When RPE cells were cultured in presence of three different concentrations of ritonavir, the mRNA expression of HIF-1α

remained unaltered. Interestingly, the expression of VEGF was inhibited in a concentration-dependent manner in the presence of ritonavir. Based on their putative mechanism of action, HIF inhibitors may be classified as modulating RNA expression, protein translation, protein degradation, DNA binding activity or transcriptional activity [527]. Although ritonavir did not alter HIF-1α mRNA expression, immunohistochemical results suggest that it significantly inhibited HIF-1α protein translation. Our results corroborated with Pore et al., who demonstrated that nelfinavir, another HIV protease inhibitor, interfered with HIF-1α protein translation in carcinoma cells [528]. An active PI3K/AKT pathway is required for hypoxia induced expression of HIF-1α protein translation [529, 530]. Hence, a preliminary study was performed to analyze the levels of AKT in RPE cells post treatment with ritonavir. Our study indicates that ritonavir inhibits HIF-1α protein translation *via* inhibition of AKT phosphorylation.

A major angiogenic stimulator in both physiological and pathological ocular conditions is VEGF. Although it exists as multiple isoforms due to alternative splicing, $VEGF_{165}$ plays a vital role in augmenting vascular leakage and breakdown of blood retinal barrier [531]. Our study has demonstrated that hypoxic RPE cells exhibited high levels of VEGF secretion. However this hypoxia induced expression diminished significantly following treatment with ritonavir. This could possibly result from decreased HIF-1α translation and subsequent VEGF transcription. The secreted VEGF modulates angiogenesis by inducing endothelial cell proliferation [532]. To address antiangiogenic activity of ritonavir, exposed RPE culture medium was used to assess the proliferation of choroid-retina endothelial (RF/6A) cells. While proliferation of these cells increased significantly upon exposure to hypoxic medium, ritonavir inhibited such proliferation signifying its antiangiogenic properties. Cytotoxicity studies revealed that alterations in HIF-1α and VEGF expressions are not attributed to ritonavir toxicity.

Conclusions

Our studies have demonstrated that ritonavir inhibits the expression of HIF-1α mediated VEGF expression in RPE cells probably *via* inhibition of PI3K/AKT pathway (**Figure 61**). This inhibition may reduce retinal neovascularization. These findings shed new light on the possibility of incorporating ritonavir in the treatment regimen of ocular angiogenic diseases. Although many inhibitors of HIF-1α are in clinical trials, additional benefit of using ritonavir is that it has been given to HIV patients with relatively low toxicity. The process of traditional drug development could be fast tracked since ritonavir is clinically approved for human use. However, further preclinical and clinical experiments are necessary to determine the repositioning of ritonavir in the treatment of ocular neovascular diseases.

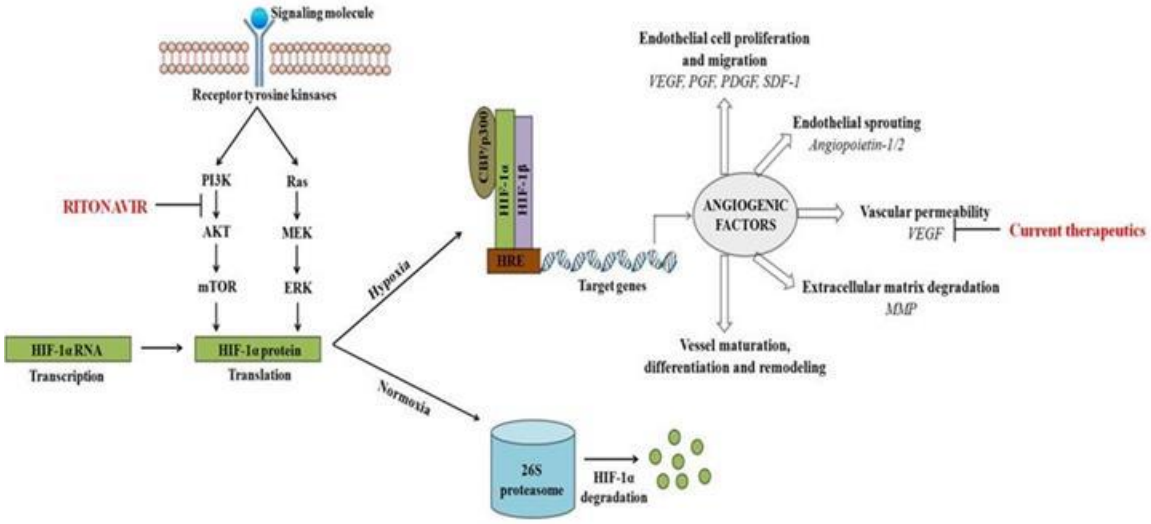

Figure 61. Schematic representation of inhibition of HIF-1α protein translation by ritonavir.

SUMMARY AND RECOMMENDATIONS

Summary

Cancer remains one of the major leading causes of death worldwide. Acquisition of multidrug resistance (MDR) remains a major impediment to successful chemotherapy. As the name implies, MDR is not limited only to one drug but often associated to structurally and functionally unrelated chemotherapeutics. Extensive research and investigations have identified several mechanisms underlying the development of MDR. This process of drug resistance is considered to be multifactorial including decreased drug accumulation, increased efflux, increased biotransformation, drug compartmentalization, modification of drug targets and defects in cellular pathways. Although the pathways can act independently, they are more often intertwined. Of the various mechanisms involved, upregulation of efflux transporters and metabolizing enzymes constitute a major resistance phenotype.

Interaction of gemifloxacin with efflux transporters is studied in polarized canine kidney cells overexpressing efflux transporters. Dose-dependent inhibition studies were performed with [^{14}C] erythromycin on MDCKII-MDR1 and MDCKII-MRP2 cells. Results from our study suggest that gemifloxacin inhibited both MDR1 and MRP2 mediated efflux with IC_{50} values of 123 ± 2 µM and 16 ± 2 µM, respectively. Transport studies were conducted to determine bidirectional permeability and to assess efflux ratio of gemifloxacin. The efflux ratio of [^{14}C] erythromycin lowered from 3.56 to 1.63 on MDCKII-MDR1 cells and 4.93 to 1.26 on MDCKII-MRP2 cells. This significant reduction in efflux ratio further confirmed the inhibitory potential of gemifloxacin towards MDR1 and MRP2. ATP determination assay was performed to examine ATP involvement in cellular translocation of gemifloxacin. No significant change in the ATP activity was observed suggesting that efflux by gemifloxacin is not affected by ATP activity.

Similarly, results obtained from cytotoxicity assay indicated that gemifloxacin tested at various concentrations do not elicit any cytotoxic effects in MDCKII-MDR1 and MRP2 cells.

Human colon adenocarcinoma cells (LS-180) were treated for 72 hours with vinblastine alone and in presence of ritonavir. RNA expression levels of efflux transporters (MDR1 and MRP2) and metabolizing enzyme (CYP3A4) were measured by quantitative real time PCR. The expression of MDR1, MRP2 and CYP3A4 was induced in response to vinblastine. This overexpression was completely neutralized when cells were cotreated with ritonavir. Uptake of [^{3}H] lopinavir and Vivid™ assay were performed to evaluate the functional activity of efflux transporters and metabolizing enzymes respectively. A reduction in lopinavir uptake and increased formation of vivid substrate further confirmed the functional activity of transcribed genes upon cotreatment. Cell proliferation, migration and apoptosis assays were performed on human breast cancer cells (T47D) and prostate cancer cells (PC-3) to better delineate the efficacy upon cotreatment of anticancer drug (doxorubicin, paclitaxel, tamoxifen or vinblastine) with ritonavir. Reduced cell proliferation and migration and increased apoptosis of cancer cells were indicative of enhanced activity of chemotherapeutics in the presence of ritonavir. In summary, combination therapy of anticancer drug with ritonavir may overcome drug resistance, by neutralizing overexpression of efflux transporters and metabolizing enzymes. Further, this combination may also increase the efficacy in a synergistic way due to antitumorigenic and antiangiogenic properties of ritonavir. Therefore, we anticipate that drug regimens containing ritonavir would enhance patient exposure to chemotherapeutics, thereby preventing or overcoming resistance and potentially improving chemotherapeutic efficacy.

A decrease in tissue oxygen levels (aka hypoxia) mediates a number of vascular retinal diseases. Despite introduction of novel therapeutics, treatment of retinal disorders remains

challenging, possibly due to complex nature of hypoxia signaling. Human retinal pigment epithelial (RPE) cells were cultured under normoxic and hypoxic conditions. Quantitative real time PCR analysis showed elevated expression of efflux transporters (MDR1, MRP2 and BCRP) and influx transporters (FR-α, $B^{(0,+)}$, SMVT) in a time-dependent manner. The expression of peptide transporters did not alter significantly in hypoxia while the expression of organic cation transporters was downregulated significantly. Immunoblot analysis further confirmed elevated expression of BCRP and SMVT. A decrease in the uptake of efflux transporter substrates (digoxin, lopinavir and abacavir) and enhanced uptake of influx transporter substrates (arginine, folic acid and biotin) in hypoxia relative to normoxia further confirmed elevated expression of the transporters, respectively. Our study demonstrates for the first time that hypoxic conditions may alter expression of efflux and influx transporters in RPE cells. These findings suggest that hypoxia may further alter disposition of ophthalmic drugs.

Retinal hypoxia mediated activation of the hypoxia-inducible factor (HIF pathway) leading to angiogenesis is a major signaling mechanism underlying a number of sight-threatening diseases. Inhibiting this signaling mechanism with an already approved therapeutic molecule may have promising antiangiogenic role with fewer side effects. Hence, we examined the expression of HIF-1a and VEGF in RPE cells treated with ritonavir under hypoxic and normoxic conditions. Presence of ritonavir in the culture medium strongly inhibited VEGF expression in a concentration-dependent manner under hypoxic conditions. Immunoblot analysis demonstrated a substantially reduced protein expression of HIF-1α in the presence of ritonavir. Further, hypoxic exposure induced VEGF secretion was also inhibited by ritonavir, as demonstrated using ELISA. Finally, ritonavir significantly diminished the proliferation of choroid-retinal endothelial (RF/6A) cells demonstrating potential antiangiogenic activity. Our study demonstrates for the

first time that ritonavir can inhibit HIF-1α and VEGF in RPE cells. Such inhibition may form a platform for application of ritonavir in the treatment of various ocular diseases.

Recommendations

The goal of my dissertation project was to identify a novel multidrug resistance modulator that can be used in both cancer and ocular diseases. A few recommendations can be suggested to continue with this project.

(1) Both the chemotherapeutic and ritonavir can be encapsulated in nanoparticles (NPs) for efficient drug delivery into the tumor tissues following systemic administration.

(2) Since ritonavir inhibits efflux and metabolism of the coadministered drug, it should be given at the same time the chemotherapeutic is administered. If co-formulation is not feasible, a cocktail of drug NPs and ritonavir NPs can be injected simultaneously.

(3) Ritonavir could be formulated into nanomicelles for promising delivery in ocular neovascular diseases. Also, nanomicelles can be targeted using specific targeting ligands attached to the micelle surface. Studies from this dissertation have shown the differential expression of various influx transporters in hypoxic conditions. Selection of an influx transporter highly expressed in hypoxia compared to normoxia enables targeted drug delivery into diseased tissues.

(4) Further, ritonavir can also be used as a preventive drug in the treatment of age-related eye diseases (ARED). Ritonavir inhibits HIF-pathway which is only activated in diseased conditions. Hence, using it as a preventive measure may reduce the risk of neovascularization in ARED.

APPENDIX

BENTHAM SCIENCE PUBLISHERS LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Bentham Science Publishers ("Bentham Science Publishers") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Bentham Science Publishers, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3314281315169
License date	Jan 22, 2014
Licensed content publisher	Bentham Science Publishers
Licensed content publication	Current Pharmaceutical Design
Licensed content title	Mechanisms of Drug Resistance in Cancer Chemotherapy: Coordinated Role and Regulation of Efflux Transporters and Metabolizing Enzymes
Licensed copyright line	Copyright © 2013, Eureka Science Ltd.
Licensed content author	Ramya Krishna Vadlapatla, Aswani Dutt Vadlapudi, Dhananjay Pal and Ashim K. Mitra
Licensed content date	December,2013
Type of Use	Thesis/Dissertation
Requestor type	Author of requested content
Format	Electronic
Portion	chapter/article
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
In the following language(s)	Original language of publication
With incidental promotional use	no
The lifetime unit quantity of new product	0 to 499
The requesting person/organization is:	Ramya Krishna Vadlapatla
Title of your thesis / dissertation	Ritonavir - A Novel Multi Drug Resistance Modulator in Cancer Chemotherapy and Ocular Neovascular Diseases
Expected completion date	Mar 2014
Expected size (number of	250

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314290045103
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	International Journal of Pharmaceutics
Licensed content title	Differential effect of P-gp and MRP2 on cellular translocation of gemifloxacin
Licensed content author	Ramya Krishna Vadlapatla,Aswani Dutt Vadlapudi,Deep Kwatra,Dhananjay Pal,Ashim K. Mitra
Licensed content date	25 November 2011
Licensed content volume number	420
Licensed content issue number	1
Number of pages	8
Start Page	26
End Page	33
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	full article
Format	electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your thesis/dissertation	Ritonavir - A Novel Multi Drug Resistance Modulator in Cancer Chemotherapy and Ocular Neovascular Diseases
Expected completion date	Mar 2014

BENTHAM SCIENCE PUBLISHERS LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Bentham Science Publishers ("Bentham Science Publishers") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Bentham Science Publishers, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3314280411047
License date	Jan 22, 2014
Licensed content publisher	Bentham Science Publishers
Licensed content publication	Current Drug Targets
Licensed content title	Hypoxia-Inducible Factor-1 (HIF-1): A Potential Target for Intervention in Ocular Neovascular Diseases
Licensed copyright line	Copyright © 2013, Eureka Science Ltd.
Licensed content author	Ramya Krishna Vadlapatla, Aswani Dutt Vadlapudi and Ashim K. Mitra
Licensed content date	July,2013
Type of Use	Thesis/Dissertation
Requestor type	Author of requested content
Format	Electronic
Portion	chapter/article
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
In the following language(s)	Original language of publication
With incidental promotional use	no
The lifetime unit quantity of new product	0 to 499
The requesting person/organization is:	Ramya Krishna Vadlapatla
Title of your thesis / dissertation	Ritonavir - A Novel Multi Drug Resistance Modulator in Cancer Chemotherapy and Ocular Neovascular Diseases
Expected completion date	Mar 2014
Expected size (number of	250

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314290272829
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	International Journal of Pharmaceutics
Licensed content title	Molecular expression and functional activity of efflux and influx transporters in hypoxia induced retinal pigment epithelial cells
Licensed content author	Ramya Krishna Vadlapatla,Aswani Dutt Vadlapudi,V.K. Chaithanya Ponnaluri,Dhananjay Pal,Mridul Mukherji,Ashim K. Mitra
Licensed content date	15 September 2013
Licensed content volume number	454
Licensed content issue number	1
Number of pages	9
Start Page	444
End Page	452
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	full article
Format	electronic
Are you the author of this Elsevier article?	Yes

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3314290739909
License date	Jan 22, 2014
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Cancer
Licensed content title	Multidrug resistance in cancer: role of ATP|[ndash]|dependent transporters
Licensed content author	Michael M. Gottesman,Tito FojoandSusan E. Bates
Licensed content date	Jan 1, 2002
Volume number	2
Issue number	1
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
High-res required	no
Figures	Figure 2
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Ritonavir - A Novel Multi Drug Resistance Modulator in Cancer Chemotherapy and Ocular Neovascular Diseases
Expected completion date	Mar 2014
Estimated size (number of pages)	250
Total	0.00 USD
Terms and Conditions	

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314290887660
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	European Journal of Pharmaceutical Sciences
Licensed content title	The power of the pump: Mechanisms of action of P-glycoprotein (ABCB1)
Licensed content author	Suresh V. Ambudkar,In-Wha Kim,Zuben E. Sauna
Licensed content date	April 2006
Licensed content volume number	27
Licensed content issue number	5
Number of pages	9
Start Page	392
End Page	400
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314291178223
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	Cancer Treatment Reviews
Licensed content title	Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer
Licensed content author	Irma Meijerman,Jos H. Beijnen,Jan H.M. Schellens
Licensed content date	October 2008
Licensed content volume number	34
Licensed content issue number	6
Number of pages	16
Start Page	505
End Page	520
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314291325015
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	Seminars in Neonatology
Licensed content title	Pathogenesis of retinopathy of prematurity
Licensed content author	Lois E.H Smith
Licensed content date	December 2003
Licensed content volume number	8
Licensed content issue number	6
Number of pages	5
Start Page	469
End Page	473
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ramya Krishna Vadlapatla
Customer address	2464 Charlotte street HSB 5258 KANSAS CITY, MO 64108
License number	3314291472034
License date	Jan 22, 2014
Licensed content publisher	Elsevier
Licensed content publication	Ageing Research Reviews
Licensed content title	Regulatory role of HIF-1α in the pathogenesis of age-related macular degeneration (AMD)
Licensed content author	Olli Arjamaa,Mikko Nikinmaa,Antero Salminen,Kai Kaarniranta
Licensed content date	October 2009
Licensed content volume number	8
Licensed content issue number	4
Number of pages	10
Start Page	349
End Page	358
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jan 22, 2014

This is a License Agreement between Ramya Krishna Vadlapatla ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3314300112051
License date	Jan 22, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Journal of Cellular and Molecular Medicine
Licensed content title	Deguelin inhibits retinal neovascularization by down-regulation of HIF-1α in oxygen-induced retinopathy
Licensed copyright line	© 2007 The Authors Journal compilation © 2007 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd
Licensed content author	J. H. Kim,J. H. Kim,Y. S. Yu,J. Y. Shin,H.-Y. Lee,K.-W. Kim
Licensed content date	Jan 19, 2008
Start page	2407
End page	2415
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1
Will you be translating?	No
Total	0.00 USD
Terms and Conditions	

REFERENCES

1. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013.* CA: A Cancer Journal for Clinicians, 2013. **63**(1): p. 11-30.
2. Szakacs, G., et al., *Targeting multidrug resistance in cancer.* Nature reviews. Drug discovery, 2006. **5**(3): p. 219-34.
3. Mellor, H.R. and R. Callaghan, *Resistance to chemotherapy in cancer: a complex and integrated cellular response.* Pharmacology, 2008. **81**(4): p. 275-300.
4. Dobson, P.D. and D.B. Kell, *Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule?* Nature reviews. Drug discovery, 2008. **7**(3): p. 205-20.
5. Flaten, G.E., et al., *Drug permeability across a phospholipid vesicle based barrier: a novel approach for studying passive diffusion.* European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2006. **27**(1): p. 80-90.
6. Hediger, M.A., et al., *The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction.* Pflugers Archiv : European journal of physiology, 2004. **447**(5): p. 465-8.
7. Assaraf, Y.G., *Molecular basis of antifolate resistance.* Cancer metastasis reviews, 2007. **26**(1): p. 153-81.
8. Farrell, J.J., et al., *Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer.* Gastroenterology, 2009. **136**(1): p. 187-95.
9. Okabe, M., et al., *Profiling SLCO and SLC22 genes in the NCI-60 cancer cell lines to identify drug uptake transporters.* Molecular cancer therapeutics, 2008. **7**(9): p. 3081-91.
10. Dean, M., A. Rzhetsky, and R. Allikmets, *The human ATP-binding cassette (ABC) transporter superfamily.* Genome research, 2001. **11**(7): p. 1156-66.
11. Guengerich, F.P., *Cytochrome p450 and chemical toxicology.* Chemical research in toxicology, 2008. **21**(1): p. 70-83.
12. Guengerich, F.P., *Mechanisms of cytochrome P450 substrate oxidation: MiniReview.* Journal of biochemical and molecular toxicology, 2007. **21**(4): p. 163-8.
13. Jancova, P., P. Anzenbacher, and E. Anzenbacherova, *Phase II drug metabolizing enzymes.* Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia, 2010. **154**(2): p. 103-16.
14. Ghezzi, P. and P. Di Simplicio, *Glutathionylation pathways in drug response.* Current opinion in pharmacology, 2007. **7**(4): p. 398-403.
15. Iyanagi, T., *Molecular mechanism of phase I and phase II drug-metabolizing enzymes: implications for detoxification.* International review of cytology, 2007. **260**: p. 35-112.
16. Deeley, R.G., C. Westlake, and S.P. Cole, *Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins.* Physiological reviews, 2006. **86**(3): p. 849-99.
17. Cummings, J., et al., *Enhanced clearance of topoisomerase I inhibitors from human colon cancer cells by glucuronidation.* Biochemical pharmacology, 2002. **63**(4): p. 607-13.
18. Meijerman, I., J.H. Beijnen, and J.H. Schellens, *Combined action and regulation of phase II enzymes and multidrug resistance proteins in multidrug resistance in cancer.* Cancer treatment reviews, 2008. **34**(6): p. 505-20.
19. Duvvuri, M. and J.P. Krise, *Intracellular drug sequestration events associated with the emergence of multidrug resistance: a mechanistic review.* Frontiers in bioscience : a journal and virtual library, 2005. **10**: p. 1499-509.

20. Safaei, R., et al., *Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells.* Molecular cancer therapeutics, 2005. **4**(10): p. 1595-604.
21. Safaei, R., et al., *Intracellular localization and trafficking of fluorescein-labeled cisplatin in human ovarian carcinoma cells.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2005. **11**(2 Pt 1): p. 756-67.
22. Liang, X.J., et al., *Trafficking and localization of platinum complexes in cisplatin-resistant cell lines monitored by fluorescence-labeled platinum.* Journal of cellular physiology, 2005. **202**(3): p. 635-41.
23. Chauhan, S.S., et al., *Reduced endocytosis and altered lysosome function in cisplatin-resistant cell lines.* British journal of cancer, 2003. **88**(8): p. 1327-34.
24. Chapuy, B., et al., *ABC transporter A3 facilitates lysosomal sequestration of imatinib and modulates susceptibility of chronic myeloid leukemia cell lines to this drug.* Haematologica, 2009. **94**(11): p. 1528-36.
25. Chapuy, B., et al., *Intracellular ABC transporter A3 confers multidrug resistance in leukemia cells by lysosomal drug sequestration.* Leukemia, 2008. **22**(8): p. 1576-86.
26. Steinbach, D., et al., *ABCA3 as a possible cause of drug resistance in childhood acute myeloid leukemia.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2006. **12**(14 Pt 1): p. 4357-63.
27. Cheong, N., et al., *Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome.* The Journal of biological chemistry, 2006. **281**(14): p. 9791-800.
28. Kellner, U., et al., *Culprit and victim -- DNA topoisomerase II.* The lancet oncology, 2002. **3**(4): p. 235-43.
29. Pommier, Y., *Topoisomerase I inhibitors: camptothecins and beyond.* Nature reviews. Cancer, 2006. **6**(10): p. 789-802.
30. Martincic, D. and K.R. Hande, *Topoisomerase II inhibitors.* Cancer chemotherapy and biological response modifiers, 2005. **22**: p. 101-21.
31. Fotoohi, A.K. and F. Albertioni, *Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells.* Leukemia & lymphoma, 2008. **49**(3): p. 410-26.
32. Morganti, M., et al., *Modulation of dihydrofolate reductase gene expression in methotrexate-resistant human leukemia CCRF-CEM/E cells by antisense oligonucleotides.* Anti-cancer drugs, 2000. **11**(4): p. 285-94.
33. Gillet, J.P. and M.M. Gottesman, *Mechanisms of multidrug resistance in cancer.* Methods in molecular biology, 2010. **596**: p. 47-76.
34. Hakem, R., *DNA-damage repair; the good, the bad, and the ugly.* The EMBO journal, 2008. **27**(4): p. 589-605.
35. Helleday, T., et al., *DNA repair pathways as targets for cancer therapy.* Nature reviews. Cancer, 2008. **8**(3): p. 193-204.
36. Youn, C.K., et al., *Oncogenic H-Ras up-regulates expression of ERCC1 to protect cells from platinum-based anticancer agents.* Cancer research, 2004. **64**(14): p. 4849-57.
37. Olaussen, K.A., et al., *DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy.* The New England journal of medicine, 2006. **355**(10): p. 983-91.
38. Hegi, M.E., et al., *MGMT gene silencing and benefit from temozolomide in glioblastoma.* The New England journal of medicine, 2005. **352**(10): p. 997-1003.
39. Esteller, M., et al., *Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents.* The New England journal of medicine, 2000. **343**(19): p. 1350-4.

40. Broker, L.E., F.A. Kruyt, and G. Giaccone, *Cell death independent of caspases: a review.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2005. **11**(9): p. 3155-62.
41. Okada, H. and T.W. Mak, *Pathways of apoptotic and non-apoptotic death in tumour cells.* Nature reviews. Cancer, 2004. **4**(8): p. 592-603.
42. Hail, N., Jr., et al., *Apoptosis effector mechanisms: a requiem performed in different keys.* Apoptosis : an international journal on programmed cell death, 2006. **11**(6): p. 889-904.
43. Kerr, J.F., *History of the events leading to the formulation of the apoptosis concept.* Toxicology, 2002. **181-182**: p. 471-4.
44. Buchholz, T.A., et al., *Chemotherapy-induced apoptosis and Bcl-2 levels correlate with breast cancer response to chemotherapy.* Cancer journal, 2003. **9**(1): p. 33-41.
45. Sjostrom, J., et al., *The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2002. **8**(3): p. 811-6.
46. Deng, X., et al., *Regulation of Bcl2 phosphorylation and potential significance for leukemic cell chemoresistance.* Journal of the National Cancer Institute. Monographs, 2001(28): p. 30-7.
47. Tredan, O., et al., *Drug resistance and the solid tumor microenvironment.* Journal of the National Cancer Institute, 2007. **99**(19): p. 1441-54.
48. Vaupel, P., *Tumor microenvironmental physiology and its implications for radiation oncology.* Seminars in radiation oncology, 2004. **14**(3): p. 198-206.
49. Cosse, J.P. and C. Michiels, *Tumour hypoxia affects the responsiveness of cancer cells to chemotherapy and promotes cancer progression.* Anti-cancer agents in medicinal chemistry, 2008. **8**(7): p. 790-7.
50. Heldin, C.H., et al., *High interstitial fluid pressure - an obstacle in cancer therapy.* Nature reviews. Cancer, 2004. **4**(10): p. 806-13.
51. Gerweck, L.E., S. Vijayappa, and S. Kozin, *Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics.* Molecular cancer therapeutics, 2006. **5**(5): p. 1275-9.
52. Cowan, D.S. and I.F. Tannock, *Factors that influence the penetration of methotrexate through solid tissue.* International journal of cancer. Journal international du cancer, 2001. **91**(1): p. 120-5.
53. Raghunand, N., B.P. Mahoney, and R.J. Gillies, *Tumor acidity, ion trapping and chemotherapeutics. II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents.* Biochemical pharmacology, 2003. **66**(7): p. 1219-29.
54. Semenza, G.L., *Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics.* Oncogene, 2010. **29**(5): p. 625-34.
55. Shannon, A.M., et al., *Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies.* Cancer treatment reviews, 2003. **29**(4): p. 297-307.
56. Barnhart, B.C. and M.C. Simon, *Metastasis and stem cell pathways.* Cancer metastasis reviews, 2007. **26**(2): p. 261-71.
57. Sullivan, R. and C.H. Graham, *Hypoxia-driven selection of the metastatic phenotype.* Cancer metastasis reviews, 2007. **26**(2): p. 319-31.
58. Chan, D.A. and A.J. Giaccia, *Hypoxia, gene expression, and metastasis.* Cancer metastasis reviews, 2007. **26**(2): p. 333-9.
59. Moeller, B.J., R.A. Richardson, and M.W. Dewhirst, *Hypoxia and radiotherapy: opportunities for improved outcomes in cancer treatment.* Cancer metastasis reviews, 2007. **26**(2): p. 241-8.
60. Bindra, R.S., M.E. Crosby, and P.M. Glazer, *Regulation of DNA repair in hypoxic cancer cells.* Cancer metastasis reviews, 2007. **26**(2): p. 249-60.

61. Swietach, P., R.D. Vaughan-Jones, and A.L. Harris, *Regulation of tumor pH and the role of carbonic anhydrase 9.* Cancer metastasis reviews, 2007. **26**(2): p. 299-310.
62. Lukashev, D., A. Ohta, and M. Sitkovsky, *Hypoxia-dependent anti-inflammatory pathways in protection of cancerous tissues.* Cancer metastasis reviews, 2007. **26**(2): p. 273-9.
63. Liao, D., et al., *Hypoxia-inducible factor-1alpha is a key regulator of metastasis in a transgenic model of cancer initiation and progression.* Cancer research, 2007. **67**(2): p. 563-72.
64. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression.* Nature, 2006. **441**(7092): p. 437-43.
65. Lluis, J.M., et al., *Critical role of mitochondrial glutathione in the survival of hepatocytes during hypoxia.* The Journal of biological chemistry, 2005. **280**(5): p. 3224-32.
66. Krishnamurthy, P., et al., *The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme.* The Journal of biological chemistry, 2004. **279**(23): p. 24218-25.
67. Comerford, K.M., et al., *Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene.* Cancer research, 2002. **62**(12): p. 3387-94.
68. Semenza, G.L., *Evaluation of HIF-1 inhibitors as anticancer agents.* Drug discovery today, 2007. **12**(19-20): p. 853-9.
69. Huang, G.W., L.Y. Yang, and W.Q. Lu, *Expression of hypoxia-inducible factor 1alpha and vascular endothelial growth factor in hepatocellular carcinoma: Impact on neovascularization and survival.* World journal of gastroenterology : WJG, 2005. **11**(11): p. 1705-8.
70. Theodoropoulos, V.E., et al., *Evaluation of hypoxia-inducible factor 1alpha overexpression as a predictor of tumour recurrence and progression in superficial urothelial bladder carcinoma.* BJU international, 2005. **95**(3): p. 425-31.
71. Kuwai, T., et al., *Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma.* International journal of cancer. Journal international du cancer, 2003. **105**(2): p. 176-81.
72. Gottesman, M.M. and V. Ling, *The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research.* FEBS letters, 2006. **580**(4): p. 998-1009.
73. Gottesman, M.M., T. Fojo, and S.E. Bates, *Multidrug resistance in cancer: role of ATP-dependent transporters.* Nature reviews. Cancer, 2002. **2**(1): p. 48-58.
74. Ambudkar, S.V., et al., *P-glycoprotein: from genomics to mechanism.* Oncogene, 2003. **22**(47): p. 7468-85.
75. Linton, K.J. and C.F. Higgins, *Structure and function of ABC transporters: the ATP switch provides flexible control.* Pflugers Archiv : European journal of physiology, 2007. **453**(5): p. 555-67.
76. Mohrmann, K., et al., *Absence of N-linked glycosylation does not affect plasma membrane localization of breast cancer resistance protein (BCRP/ABCG2).* Cancer chemotherapy and pharmacology, 2005. **56**(4): p. 344-50.
77. Zhang, P., et al., *Role of glycosylation in trafficking of Mrp2 in sandwich-cultured rat hepatocytes.* Molecular pharmacology, 2005. **67**(4): p. 1334-41.
78. Schinkel, A.H. and J.W. Jonker, *Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview.* Advanced drug delivery reviews, 2003. **55**(1): p. 3-29.
79. Dean, M., Y. Hamon, and G. Chimini, *The human ATP-binding cassette (ABC) transporter superfamily.* Journal of lipid research, 2001. **42**(7): p. 1007-17.
80. Sauna, Z.E., K. Nandigama, and S.V. Ambudkar, *Exploiting reaction intermediates of the ATPase reaction to elucidate the mechanism of transport by P-glycoprotein (ABCB1).* The Journal of biological chemistry, 2006. **281**(36): p. 26501-11.
81. Zaitseva, J., et al., *A molecular understanding of the catalytic cycle of the nucleotide-binding domain of the ABC transporter HlyB.* Biochemical Society transactions, 2005. **33**(Pt 5): p. 990-5.

82. Jones, P.M. and A.M. George, *The ABC transporter structure and mechanism: perspectives on recent research.* Cellular and molecular life sciences : CMLS, 2004. **61**(6): p. 682-99.
83. Kerr, I.D., *Structure and association of ATP-binding cassette transporter nucleotide-binding domains.* Biochimica et biophysica acta, 2002. **1561**(1): p. 47-64.
84. Juliano, R.L. and V. Ling, *A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants.* Biochimica et biophysica acta, 1976. **455**(1): p. 152-62.
85. Edwards, J.E., et al., *Role of P-glycoprotein in distribution of nelfinavir across the blood-mammary tissue barrier and blood-brain barrier.* Antimicrobial agents and chemotherapy, 2005. **49**(4): p. 1626-8.
86. Gil, S., et al., *P-glycoprotein expression of the human placenta during pregnancy.* Placenta, 2005. **26**(2-3): p. 268-70.
87. Melaine, N., et al., *Multidrug resistance genes and p-glycoprotein in the testis of the rat, mouse, Guinea pig, and human.* Biology of reproduction, 2002. **67**(6): p. 1699-707.
88. Sharom, F.J., *ABC multidrug transporters: structure, function and role in chemoresistance.* Pharmacogenomics, 2008. **9**(1): p. 105-27.
89. Seelig, A., *A general pattern for substrate recognition by P-glycoprotein.* European journal of biochemistry / FEBS, 1998. **251**(1-2): p. 252-61.
90. Cianchetta, G., et al., *A pharmacophore hypothesis for P-glycoprotein substrate recognition using GRIND-based 3D-QSAR.* Journal of medicinal chemistry, 2005. **48**(8): p. 2927-35.
91. Pajeva, I.K. and M. Wiese, *Pharmacophore model of drugs involved in P-glycoprotein multidrug resistance: explanation of structural variety (hypothesis).* Journal of medicinal chemistry, 2002. **45**(26): p. 5671-86.
92. Wiese, M. and I.K. Pajeva, *Structure-activity relationships of multidrug resistance reversers.* Current medicinal chemistry, 2001. **8**(6): p. 685-713.
93. Callaghan, R., R.C. Ford, and I.D. Kerr, *The translocation mechanism of P-glycoprotein.* FEBS letters, 2006. **580**(4): p. 1056-63.
94. Ambudkar, S.V., I.W. Kim, and Z.E. Sauna, *The power of the pump: mechanisms of action of P-glycoprotein (ABCB1).* European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2006. **27**(5): p. 392-400.
95. Higgins, C.F. and K.J. Linton, *The ATP switch model for ABC transporters.* Nature structural & molecular biology, 2004. **11**(10): p. 918-26.
96. Sauna, Z.E. and S.V. Ambudkar, *Characterization of the catalytic cycle of ATP hydrolysis by human P-glycoprotein. The two ATP hydrolysis events in a single catalytic cycle are kinetically similar but affect different functional outcomes.* The Journal of biological chemistry, 2001. **276**(15): p. 11653-61.
97. Riordan, J.R., *CFTR function and prospects for therapy.* Annual review of biochemistry, 2008. **77**: p. 701-26.
98. Burke, M.A., R.K. Mutharasan, and H. Ardehali, *The sulfonylurea receptor, an atypical ATP-binding cassette protein, and its regulation of the KATP channel.* Circulation research, 2008. **102**(2): p. 164-76.
99. Gillet, J.P., T. Efferth, and J. Remacle, *Chemotherapy-induced resistance by ATP-binding cassette transporter genes.* Biochimica et biophysica acta, 2007. **1775**(2): p. 237-62.
100. Borst, P., et al., *A family of drug transporters: the multidrug resistance-associated proteins.* Journal of the National Cancer Institute, 2000. **92**(16): p. 1295-302.
101. Cole, S.P., et al., *Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.* Science, 1992. **258**(5088): p. 1650-4.

102. Taniguchi, K., et al., *A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation.* Cancer research, 1996. **56**(18): p. 4124-9.
103. Stride, B.D., et al., *Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter.* Molecular pharmacology, 1996. **49**(6): p. 962-71.
104. Bakos, E., et al., *Membrane topology and glycosylation of the human multidrug resistance-associated protein.* The Journal of biological chemistry, 1996. **271**(21): p. 12322-6.
105. Kast, C. and P. Gros, *Topology mapping of the amino-terminal half of multidrug resistance-associated protein by epitope insertion and immunofluorescence.* The Journal of biological chemistry, 1997. **272**(42): p. 26479-87.
106. Hipfner, D.R., et al., *Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus.* The Journal of biological chemistry, 1997. **272**(38): p. 23623-30.
107. St-Pierre, M.V., et al., *Expression of members of the multidrug resistance protein family in human term placenta.* American journal of physiology. Regulatory, integrative and comparative physiology, 2000. **279**(4): p. R1495-503.
108. Wijnholds, J., et al., *Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage.* The Journal of experimental medicine, 1998. **188**(5): p. 797-808.
109. Flens, M.J., et al., *Tissue distribution of the multidrug resistance protein.* The American journal of pathology, 1996. **148**(4): p. 1237-47.
110. Meyer zu Schwabedissen, H.E., et al., *Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation.* Drug metabolism and disposition: the biological fate of chemicals, 2005. **33**(7): p. 896-904.
111. Sandusky, G.E., et al., *Expression of multidrug resistance-associated protein 2 (MRP2) in normal human tissues and carcinomas using tissue microarrays.* Histopathology, 2002. **41**(1): p. 65-74.
112. Rost, D., et al., *Expression and localization of the multidrug resistance proteins MRP2 and MRP3 in human gallbladder epithelia.* Gastroenterology, 2001. **121**(5): p. 1203-8.
113. Schaub, T.P., et al., *Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma.* Journal of the American Society of Nephrology : JASN, 1999. **10**(6): p. 1159-69.
114. Paulusma, C.C., et al., *A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome.* Hepatology, 1997. **25**(6): p. 1539-42.
115. Chan, H.S., et al., *Multidrug resistance protein (MRP) expression in retinoblastoma correlates with the rare failure of chemotherapy despite cyclosporine for reversal of P-glycoprotein.* Cancer research, 1997. **57**(12): p. 2325-30.
116. Norris, M.D., et al., *Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma.* The New England journal of medicine, 1996. **334**(4): p. 231-8.
117. Kim, S.J., et al., *Detection of breast cancer micrometastases in peripheral blood using immunomagnetic separation and immunocytochemistry.* Breast cancer, 2001. **8**(1): p. 63-9.
118. Wright, S.R., et al., *Immunohistochemical detection of multidrug resistance protein in human lung cancer and normal lung.* Clinical cancer research : an official journal of the American Association for Cancer Research, 1998. **4**(9): p. 2279-89.
119. Steinbach, D., et al., *Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood*

acute myeloid leukemia. Clinical cancer research : an official journal of the American Association for Cancer Research, 2003. **9**(3): p. 1083-6.
120. Burger, H., et al., *RNA expression of breast cancer resistance protein, lung resistance-related protein, multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2003. **9**(2): p. 827-36.
121. Ohishi, Y., et al., *ATP-binding cassette superfamily transporter gene expression in human primary ovarian carcinoma.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2002. **8**(12): p. 3767-75.
122. Nies, A.T., et al., *Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma.* International journal of cancer. Journal international du cancer, 2001. **94**(4): p. 492-9.
123. Hinoshita, E., et al., *Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2000. **6**(6): p. 2401-7.
124. Nakagawa, M., et al., *Reduced intracellular drug accumulation in the absence of P-glycoprotein (mdr1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells.* Cancer research, 1992. **52**(22): p. 6175-81.
125. Doyle, L.A., et al., *A multidrug resistance transporter from human MCF-7 breast cancer cells.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(26): p. 15665-70.
126. Allikmets, R., et al., *A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance.* Cancer research, 1998. **58**(23): p. 5337-9.
127. Wang, H., et al., *Membrane topology of the human breast cancer resistance protein (BCRP/ABCG2) determined by epitope insertion and immunofluorescence.* Biochemistry, 2008. **47**(52): p. 13778-87.
128. McDevitt, C.A., et al., *Purification and 3D structural analysis of oligomeric human multidrug transporter ABCG2.* Structure, 2006. **14**(11): p. 1623-32.
129. Henriksen, U., et al., *Identification of intra- and intermolecular disulfide bridges in the multidrug resistance transporter ABCG2.* The Journal of biological chemistry, 2005. **280**(44): p. 36926-34.
130. Fetsch, P.A., et al., *Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues.* Cancer letters, 2006. **235**(1): p. 84-92.
131. Rocchi, E., et al., *The product of the ABC half-transporter gene ABCG2 (BCRP/MXR/ABCP) is expressed in the plasma membrane.* Biochemical and biophysical research communications, 2000. **271**(1): p. 42-6.
132. Natarajan, K., et al., *Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance.* Biochemical pharmacology, 2012. **83**(8): p. 1084-103.
133. Nakanishi, T. and D.D. Ross, *Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression.* Chinese journal of cancer, 2012. **31**(2): p. 73-99.
134. Diestra, J.E., et al., *Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material.* The Journal of pathology, 2002. **198**(2): p. 213-9.
135. Sim, S.C. and M. Ingelman-Sundberg, *The Human Cytochrome P450 (CYP) Allele Nomenclature website: a peer-reviewed database of CYP variants and their associated effects.* Human genomics, 2010. **4**(4): p. 278-81.
136. Denisov, I.G., et al., *Structure and chemistry of cytochrome P450.* Chemical reviews, 2005. **105**(6): p. 2253-77.

137. Williams, P.A., et al., *Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone.* Science, 2004. **305**(5684): p. 683-6.
138. Liu, M., P.D. Hurn, and N.J. Alkayed, *Cytochrome P450 in neurological disease.* Current drug metabolism, 2004. **5**(3): p. 225-34.
139. Du, L., S.M. Hoffman, and D.S. Keeney, *Epidermal CYP2 family cytochromes P450.* Toxicology and applied pharmacology, 2004. **195**(3): p. 278-87.
140. Zhao, X. and J.D. Imig, *Kidney CYP450 enzymes: biological actions beyond drug metabolism.* Current drug metabolism, 2003. **4**(1): p. 73-84.
141. Ding, X. and L.S. Kaminsky, *Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts.* Annual review of pharmacology and toxicology, 2003. **43**: p. 149-73.
142. Guengerich, F.P., *Cytochrome P450s and other enzymes in drug metabolism and toxicity.* The AAPS journal, 2006. **8**(1): p. E101-11.
143. McFadyen, M.C., W.T. Melvin, and G.I. Murray, *Cytochrome P450 enzymes: novel options for cancer therapeutics.* Molecular cancer therapeutics, 2004. **3**(3): p. 363-71.
144. Lewis, D.F., *P450 structures and oxidative metabolism of xenobiotics.* Pharmacogenomics, 2003. **4**(4): p. 387-95.
145. Bu, H.Z., *A literature review of enzyme kinetic parameters for CYP3A4-mediated metabolic reactions of 113 drugs in human liver microsomes: structure-kinetics relationship assessment.* Current drug metabolism, 2006. **7**(3): p. 231-49.
146. Rodriguez-Antona, C. and M. Ingelman-Sundberg, *Cytochrome P450 pharmacogenetics and cancer.* Oncogene, 2006. **25**(11): p. 1679-91.
147. Miyoshi, Y., et al., *Prediction of response to docetaxel by immunohistochemical analysis of CYP3A4 expression in human breast cancers.* Breast cancer, 2005. **12**(1): p. 11-5.
148. Dhaini, H.R., et al., *Cytochrome P450 CYP3A4/5 expression as a biomarker of outcome in osteosarcoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2003. **21**(13): p. 2481-5.
149. Azzariti, A., et al., *The coordinated role of CYP450 enzymes and P-gp in determining cancer resistance to chemotherapy.* Current drug metabolism, 2011. **12**(8): p. 713-21.
150. Wacher, V.J., C.Y. Wu, and L.Z. Benet, *Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy.* Molecular carcinogenesis, 1995. **13**(3): p. 129-34.
151. Harmsen, S., et al., *The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology.* Cancer treatment reviews, 2007. **33**(4): p. 369-80.
152. Perissi, V. and M.G. Rosenfeld, *Controlling nuclear receptors: the circular logic of cofactor cycles.* Nature reviews. Molecular cell biology, 2005. **6**(7): p. 542-54.
153. Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors.* Genes & development, 2000. **14**(2): p. 121-41.
154. Chen, Y., et al., *Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters.* Biochemical pharmacology, 2012. **83**(8): p. 1112-26.
155. Tolson, A.H. and H. Wang, *Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR.* Advanced drug delivery reviews, 2010. **62**(13): p. 1238-49.
156. Blumberg, B., et al., *SXR, a novel steroid and xenobiotic-sensing nuclear receptor.* Genes & development, 1998. **12**(20): p. 3195-205.
157. Bertilsson, G., et al., *Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(21): p. 12208-13.

158. Kliewer, S.A., et al., *An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway.* Cell, 1998. **92**(1): p. 73-82.
159. Squires, E.J., T. Sueyoshi, and M. Negishi, *Cytoplasmic localization of pregnane X receptor and ligand-dependent nuclear translocation in mouse liver.* The Journal of biological chemistry, 2004. **279**(47): p. 49307-14.
160. Kawana, K., et al., *Molecular mechanism of nuclear translocation of an orphan nuclear receptor, SXR.* Molecular pharmacology, 2003. **63**(3): p. 524-31.
161. Bhalla, S., et al., *Ligand-activated pregnane X receptor interferes with HNF-4 signaling by targeting a common coactivator PGC-1alpha. Functional implications in hepatic cholesterol and glucose metabolism.* The Journal of biological chemistry, 2004. **279**(43): p. 45139-47.
162. Wang, H. and E.L. LeCluyse, *Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes.* Clinical pharmacokinetics, 2003. **42**(15): p. 1331-57.
163. Watkins, R.E., et al., *The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity.* Science, 2001. **292**(5525): p. 2329-33.
164. Chen, Y. and D. Nie, *Pregnane X receptor and its potential role in drug resistance in cancer treatment.* Recent patents on anti-cancer drug discovery, 2009. **4**(1): p. 19-27.
165. Rosenfeld, J.M., et al., *Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor.* Molecular endocrinology, 2003. **17**(7): p. 1268-82.
166. Xie, W., et al., *Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(7): p. 4150-5.
167. Maglich, J.M., et al., *Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification.* Molecular pharmacology, 2002. **62**(3): p. 638-46.
168. Kast, H.R., et al., *Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor.* The Journal of biological chemistry, 2002. **277**(4): p. 2908-15.
169. Geick, A., M. Eichelbaum, and O. Burk, *Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin.* The Journal of biological chemistry, 2001. **276**(18): p. 14581-7.
170. di Masi, A., et al., *Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects.* Molecular aspects of medicine, 2009. **30**(5): p. 297-343.
171. Albermann, N., et al., *Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver.* Biochemical pharmacology, 2005. **70**(6): p. 949-58.
172. Lamba, V., et al., *PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators.* Toxicology and applied pharmacology, 2004. **199**(3): p. 251-65.
173. Bauer, B., et al., *Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood-brain barrier.* Molecular pharmacology, 2004. **66**(3): p. 413-9.
174. Kliewer, S.A., B. Goodwin, and T.M. Willson, *The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism.* Endocrine reviews, 2002. **23**(5): p. 687-702.
175. Gupta, D., et al., *Expanding the roles for pregnane X receptor in cancer: proliferation and drug resistance in ovarian cancer.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. **14**(17): p. 5332-40.
176. Zhou, J., et al., *The antiapoptotic role of pregnane X receptor in human colon cancer cells.* Molecular endocrinology, 2008. **22**(4): p. 868-80.

177. Chen, Y., et al., *Human pregnane X receptor and resistance to chemotherapy in prostate cancer.* Cancer research, 2007. **67**(21): p. 10361-7.
178. Masuyama, H., et al., *Down-regulation of pregnane X receptor contributes to cell growth inhibition and apoptosis by anticancer agents in endometrial cancer cells.* Molecular pharmacology, 2007. **72**(4): p. 1045-53.
179. Mensah-Osman, E.J., et al., *Expression levels and activation of a PXR variant are directly related to drug resistance in osteosarcoma cell lines.* Cancer, 2007. **109**(5): p. 957-65.
180. Miki, Y., et al., *Expression of the steroid and xenobiotic receptor and its possible target gene, organic anion transporting polypeptide-A, in human breast carcinoma.* Cancer research, 2006. **66**(1): p. 535-42.
181. Coley, H.M., *Overcoming multidrug resistance in cancer: clinical studies of p-glycoprotein inhibitors.* Methods in molecular biology, 2010. **596**: p. 341-58.
182. Ozben, T., *Mechanisms and strategies to overcome multiple drug resistance in cancer.* FEBS letters, 2006. **580**(12): p. 2903-9.
183. Xiang, Q., et al., *Cabozantinib reverses multidrug resistance of human hepatoma HepG2/adr cells by modulating the function of P-glycoprotein.* Liver international : official journal of the International Association for the Study of the Liver, 2014.
184. Liu, K.J., et al., *Saracatinib (AZD0530) is a potent modulator of ABCB1-mediated multidrug resistance in vitro and in vivo.* International journal of cancer. Journal international du cancer, 2013. **132**(1): p. 224-35.
185. Owen, A., et al., *Expression of pregnane-X-receptor transcript in peripheral blood mononuclear cells and correlation with MDR1 mRNA.* Antivir Ther, 2004. **9**(5): p. 819-21.
186. Masuyama, H., et al., *Expression and potential roles of pregnane X receptor in endometrial cancer.* J Clin Endocrinol Metab, 2003. **88**(9): p. 4446-54.
187. Synold, T.W., I. Dussault, and B.M. Forman, *The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux.* Nat Med, 2001. **7**(5): p. 584-90.
188. Takeshita, A., et al., *Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics.* The Journal of biological chemistry, 2002. **277**(36): p. 32453-8.
189. Huang, H., et al., *Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole.* Oncogene, 2007. **26**(2): p. 258-68.
190. Healan-Greenberg, C., et al., *A human immunodeficiency virus protease inhibitor is a novel functional inhibitor of human pregnane X receptor.* Drug metabolism and disposition: the biological fate of chemicals, 2008. **36**(3): p. 500-7.
191. Zhou, C., et al., *The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor.* Molecular pharmacology, 2007. **71**(1): p. 220-9.
192. Raynal, C., et al., *Pregnane X Receptor (PXR) expression in colorectal cancer cells restricts irinotecan chemosensitivity through enhanced SN-38 glucuronidation.* Mol Cancer, 2010. **9**: p. 46.
193. Chen, Y., et al., *Camptothecin attenuates cytochrome P450 3A4 induction by blocking the activation of human pregnane X receptor.* J Pharmacol Exp Ther, 2010. **334**(3): p. 999-1008.
194. Liu, C.L., Y.P. Lim, and M.L. Hu, *Fucoxanthin attenuates rifampin-induced cytochrome P450 3A4 (CYP3A4) and multiple drug resistance 1 (MDR1) gene expression through pregnane X receptor (PXR)-mediated pathways in human hepatoma HepG2 and colon adenocarcinoma LS174T cells.* Mar Drugs, 2012. **10**(1): p. 242-57.
195. Das, B.C., et al., *Synthesis of novel ketoconazole derivatives as inhibitors of the human Pregnane X Receptor (PXR; NR1I2; also termed SXR, PAR).* Bioorg Med Chem Lett, 2008. **18**(14): p. 3974-7.

196. Venkatesh, M., et al., *In vivo and in vitro characterization of a first-in-class novel azole analog that targets pregnane X receptor activation.* Molecular pharmacology, 2011. **80**(1): p. 124-35.
197. Mani, S., W. Dou, and M.R. Redinbo, *PXR antagonists and implication in drug metabolism.* Drug Metab Rev, 2013. **45**(1): p. 60-72.
198. Dvorak, Z., *Drug-drug interactions by azole antifungals: Beyond a dogma of CYP3A4 enzyme activity inhibition.* Toxicol Lett, 2011. **202**(2): p. 129-32.
199. Wang, H., et al., *The phytoestrogen coumestrol is a naturally occurring antagonist of the human pregnane X receptor.* Molecular endocrinology, 2008. **22**(4): p. 838-57.
200. Andriole, V.T., *The quinolones: past, present, and future.* Clin Infect Dis, 2005. **41 Suppl 2**: p. S113-9.
201. Zhanel, G.G. and A.M. Noreddin, *Pharmacokinetics and pharmacodynamics of the new fluoroquinolones: focus on respiratory infections.* Current opinion in pharmacology, 2001. **1**(5): p. 459-63.
202. Turnidge, J., *Pharmacokinetics and pharmacodynamics of fluoroquinolones.* Drugs, 1999. **58 Suppl 2**: p. 29-36.
203. Hooper, D.C., *Mode of action of fluoroquinolones.* Drugs, 1999. **58 Suppl 2**: p. 6-10.
204. Alvarez, A.I., et al., *Fluoroquinolone efflux mediated by ABC transporters.* J Pharm Sci, 2008. **97**(9): p. 3483-93.
205. Saravolatz, L.D. and J. Leggett, *Gatifloxacin, gemifloxacin, and moxifloxacin: the role of 3 newer fluoroquinolones.* Clin Infect Dis, 2003. **37**(9): p. 1210-5.
206. Appelbaum, P.C. and P.A. Hunter, *The fluoroquinolone antibacterials: past, present and future perspectives.* Int J Antimicrob Agents, 2000. **16**(1): p. 5-15.
207. Goldstein, M.H., R.P. Kowalski, and Y.J. Gordon, *Emerging fluoroquinolone resistance in bacterial keratitis: a 5-year review.* Ophthalmology, 1999. **106**(7): p. 1313-8.
208. Yoo, B.K., et al., *Gemifloxacin: a new fluoroquinolone approved for treatment of respiratory infections.* Ann Pharmacother, 2004. **38**(7-8): p. 1226-35.
209. Ramji, J.V., et al., *The disposition of gemifloxacin, a new fluoroquinolone antibiotic, in rats and dogs.* Drug metabolism and disposition: the biological fate of chemicals, 2001. **29**(4 Pt 1): p. 435-42.
210. Hooper, D.C., *Mechanisms of fluoroquinolone resistance.* Drug Resist Updat, 1999. **2**(1): p. 38-55.
211. Heaton, V.J., J.E. Ambler, and L.M. Fisher, *Potent antipneumococcal activity of gemifloxacin is associated with dual targeting of gyrase and topoisomerase IV, an in vivo target preference for gyrase, and enhanced stabilization of cleavable complexes in vitro.* Antimicrobial agents and chemotherapy, 2000. **44**(11): p. 3112-7.
212. Vousden, M., et al., *Evaluation of phototoxic potential of gemifloxacin in healthy volunteers compared with ciprofloxacin.* Chemotherapy, 1999. **45**(6): p. 512-20.
213. Blondeau, J.M., *Expanded activity and utility of the new fluoroquinolones: a review.* Clin Ther, 1999. **21**(1): p. 3-40; discussion 1-2.
214. Lipsky, B.A. and C.A. Baker, *Fluoroquinolone toxicity profiles: a review focusing on newer agents.* Clin Infect Dis, 1999. **28**(2): p. 352-64.
215. Domagala, J.M., *Structure-activity and structure-side-effect relationships for the quinolone antibacterials.* J Antimicrob Chemother, 1994. **33**(4): p. 685-706.
216. Hohl, A.F., et al., *International multicenter investigation of LB20304, a new fluoronaphthyridone.* Clin Microbiol Infect, 1998. **4**(5): p. 280-284.
217. Johnson, D.M., R.N. Jones, and M.E. Erwin, *Anti-streptococcal activity of SB-265805 (LB20304), a novel fluoronaphthyridone, compared with five other compounds, including quality control guidelines.* Diagn Microbiol Infect Dis, 1999. **33**(2): p. 87-91.

218. Cormican, M.G. and R.N. Jones, *Antimicrobial activity and spectrum of LB20304, a novel fluoronaphthyridone.* Antimicrobial agents and chemotherapy, 1997. **41**(1): p. 204-11.
219. Oh, J.I., et al., *In vitro and in vivo evaluations of LB20304, a new fluoronaphthyridone.* Antimicrobial agents and chemotherapy, 1996. **40**(6): p. 1564-8.
220. Goldstein, E.J., et al., *In vitro activity of gemifloxacin compared to seven other oral antimicrobial agents against aerobic and anaerobic pathogens isolated from antral sinus puncture specimens from patients with sinusitis.* Diagn Microbiol Infect Dis, 2002. **42**(2): p. 113-8.
221. Koeth, L.M., et al., *Comparative in vitro activity of gemifloxacin to other fluoroquinolones and non-quinolone agents against Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis in the United States in 1999-2000.* Int J Antimicrob Agents, 2002. **19**(1): p. 33-7.
222. Fuchs, P.C., A.L. Barry, and S.D. Brown, *In vitro activity of gemifloxacin against contemporary clinical bacterial isolates from eleven North American medical centers, and assessment of disk diffusion test interpretive criteria.* Diagn Microbiol Infect Dis, 2000. **38**(4): p. 243-53.
223. Rittenhouse, S., et al., *In vitro antibacterial activity of gemifloxacin and comparator compounds against common respiratory pathogens.* J Antimicrob Chemother, 2000. **45 Suppl 1**: p. 23-7.
224. King, A., et al., *Comparative in vitro activity of gemifloxacin.* J Antimicrob Chemother, 2000. **45 Suppl 1**: p. 1-12.
225. Berry, V., et al., *Comparative in vivo activity of gemifloxacin in a rat model of respiratory tract infection.* J Antimicrob Chemother, 2000. **45 Suppl 1**: p. 79-85.
226. Erwin, M.E. and R.N. Jones, *Studies to establish quality control ranges for SB-265805 (LB2030) when using National Committee for Laboratory Standards antimicrobial susceptibility test methods. Quality Control Study Group.* J Clin Microbiol, 1999. **37**(1): p. 279-80.
227. Allen, A., et al., *Pharmacokinetics and tolerability of gemifloxacin (SB-265805) after administration of single oral doses to healthy volunteers.* Antimicrobial agents and chemotherapy, 2000. **44**(6): p. 1604-8.
228. Pal, D., et al., *Efflux transporters- and cytochrome P-450-mediated interactions between drugs of abuse and antiretrovirals.* Life Sci, 2010.
229. Luo, S., et al., *Effect of HEPES buffer on the uptake and transport of P-glycoprotein substrates and large neutral amino acids.* Mol Pharm, 2010. **7**(2): p. 412-20.
230. Pal, D. and A.K. Mitra, *MDR- and CYP3A4-mediated drug-drug interactions.* J Neuroimmune Pharmacol, 2006. **1**(3): p. 323-39.
231. Sikri, V., et al., *Cotransport of macrolide and fluoroquinolones, a beneficial interaction reversing P-glycoprotein efflux.* Am J Ther, 2004. **11**(6): p. 433-42.
232. Glavinas, H., et al., *The role of ABC transporters in drug resistance, metabolism and toxicity.* Curr Drug Deliv, 2004. **1**(1): p. 27-42.
233. Katragadda, S., et al., *Role of efflux pumps and metabolising enzymes in drug delivery.* Expert Opin Drug Deliv, 2005. **2**(4): p. 683-705.
234. Braun, A., et al., *Cell cultures as tools in biopharmacy.* European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2000. **11 Suppl 2**: p. S51-60.
235. Evers, R., et al., *Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA.* J Clin Invest, 1998. **101**(7): p. 1310-9.
236. Evers, R., et al., *Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1.* FEBS letters, 1997. **419**(1): p. 112-6.
237. Hariharan, S., et al., *Enhanced corneal absorption of erythromycin by modulating P-glycoprotein and MRP mediated efflux with corticosteroids.* Pharm Res, 2009. **26**(5): p. 1270-82.

238. Dey, S., S. Gunda, and A.K. Mitra, *Pharmacokinetics of erythromycin in rabbit corneas after single-dose infusion: role of P-glycoprotein as a barrier to in vivo ocular drug absorption.* J Pharmacol Exp Ther, 2004. **311**(1): p. 246-55.
239. Anand, B.S., J. Patel, and A.K. Mitra, *Interactions of the dipeptide ester prodrugs of acyclovir with the intestinal oligopeptide transporter: competitive inhibition of glycylsarcosine transport in human intestinal cell line-Caco-2.* J Pharmacol Exp Ther, 2003. **304**(2): p. 781-91.
240. Evers, R., et al., *Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport.* British journal of cancer, 2000. **83**(3): p. 366-74.
241. Essodaigui, M., H.J. Broxterman, and A. Garnier-Suillerot, *Kinetic analysis of calcein and calcein-acetoxymethylester efflux mediated by the multidrug resistance protein and P-glycoprotein.* Biochemistry, 1998. **37**(8): p. 2243-50.
242. Boumendjel, A., et al., *Recent advances in the discovery of flavonoids and analogs with high-affinity binding to P-glycoprotein responsible for cancer cell multidrug resistance.* Med Res Rev, 2002. **22**(5): p. 512-29.
243. Luders, A.K., R. Saborowski, and U. Bickmeyer, *Inhibition of multidrug/xenobiotic resistance transporter by MK571 improves dye (Fura 2) accumulation in crustacean tissues from lobster, shrimp, and isopod.* Comp Biochem Physiol C Toxicol Pharmacol, 2009. **150**(3): p. 368-71.
244. Agarwal, S., D. Pal, and A.K. Mitra, *Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor.* Int J Pharm, 2007. **339**(1-2): p. 139-47.
245. Eneroth, A., et al., *Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction.* European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 2001. **12**(3): p. 205-14.
246. Polli, J.W., et al., *Rational use of in vitro P-glycoprotein assays in drug discovery.* J Pharmacol Exp Ther, 2001. **299**(2): p. 620-8.
247. Fairchild, C.R. and K.H. Cowan, *Keynote address: multidrug resistance: a pleiotropic response to cytotoxic drugs.* Int J Radiat Oncol Biol Phys, 1991. **20**(2): p. 361-7.
248. Raucy, J.L. and J.M. Lasker, *Current in vitro high throughput screening approaches to assess nuclear receptor activation.* Current drug metabolism, 2010. **11**(9): p. 806-14.
249. Dussault, I. and B.M. Forman, *The nuclear receptor PXR: a master regulator of "homeland" defense.* Critical reviews in eukaryotic gene expression, 2002. **12**(1): p. 53-64.
250. Wilcox, M.H., et al., *Fluoroquinolones in the treatment of severe community-acquired.* British journal of hospital medicine, 2011. **Suppl**: p. S1-7.
251. Blondeau, J.M., *Fluoroquinolones: mechanism of action, classification, and development of resistance.* Survey of ophthalmology, 2004. **49 Suppl 2**: p. S73-8.
252. Vadlapatla, R.K., et al., *Differential effect of P-gp and MRP2 on cellular translocation of gemifloxacin.* International journal of pharmaceutics, 2011. **420**(1): p. 26-33.
253. Kwatra, D., et al., *Interaction of gatifloxacin with efflux transporters: a possible mechanism for drug resistance.* International journal of pharmaceutics, 2010. **395**(1-2): p. 114-21.
254. Terashi, K., et al., *Interactions of ofloxacin and erythromycin with the multidrug resistance protein (MRP) in MRP-overexpressing human leukemia cells.* Antimicrobial agents and chemotherapy, 2000. **44**(6): p. 1697-700.
255. Ito, T., et al., *Transport of quinolone antibacterial drugs by human P-glycoprotein expressed in a kidney epithelial cell line, LLC-PK1.* The Journal of pharmacology and experimental therapeutics, 1997. **282**(2): p. 955-60.

256. Saravolatz, L.D. and J. Leggett, *Gatifloxacin, gemifloxacin, and moxifloxacin: the role of 3 newer fluoroquinolones.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2003. **37**(9): p. 1210-5.
257. Vadlapatla, R.K., et al., *Mechanisms of Drug Resistance in Cancer Chemotherapy: Coordinated Role and Regulation of Efflux Transporters and Metabolizing Enzymes.* Current pharmaceutical design, 2013.
258. Rees, D.C., E. Johnson, and O. Lewinson, *ABC transporters: the power to change.* Nature reviews. Molecular cell biology, 2009. **10**(3): p. 218-27.
259. Choudhuri, S. and C.D. Klaassen, *Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters.* International journal of toxicology, 2006. **25**(4): p. 231-59.
260. Kliewer, S.A., *The nuclear pregnane X receptor regulates xenobiotic detoxification.* The Journal of nutrition, 2003. **133**(7 Suppl): p. 2444S-2447S.
261. Peloquin, C.A., et al., *Population pharmacokinetics of levofloxacin, gatifloxacin, and moxifloxacin in adults with pulmonary tuberculosis.* Antimicrobial agents and chemotherapy, 2008. **52**(3): p. 852-7.
262. Allen, A., et al., *Multiple-dose pharmacokinetics and tolerability of gemifloxacin administered orally to healthy volunteers.* Antimicrobial agents and chemotherapy, 2001. **45**(2): p. 540-5.
263. Fish, D.N. and A.T. Chow, *The clinical pharmacokinetics of levofloxacin.* Clinical pharmacokinetics, 1997. **32**(2): p. 101-19.
264. Haslam, I.S., et al., *Rifampin and digoxin induction of MDR1 expression and function in human intestinal (T84) epithelial cells.* British journal of pharmacology, 2008. **154**(1): p. 246-55.
265. Harmsen, S., et al., *PXR-mediated induction of P-glycoprotein by anticancer drugs in a human colon adenocarcinoma-derived cell line.* Cancer chemotherapy and pharmacology, 2010. **66**(4): p. 765-71.
266. Harmsen, S., et al., *Comparison of two immortalized human cell lines to study nuclear receptor-mediated CYP3A4 induction.* Drug metabolism and disposition: the biological fate of chemicals, 2008. **36**(6): p. 1166-71.
267. Alvarez, A.I., et al., *Fluoroquinolone efflux mediated by ABC transporters.* Journal of pharmaceutical sciences, 2008. **97**(9): p. 3483-93.
268. Konig, S.K., et al., *Impact of drug transporters on cellular resistance towards saquinavir and darunavir.* The Journal of antimicrobial chemotherapy, 2010. **65**(11): p. 2319-28.
269. Gupta, A., et al., *Intestinal human colon adenocarcinoma cell line LS180 is an excellent model to study pregnane X receptor, but not constitutive androstane receptor, mediated CYP3A4 and multidrug resistance transporter 1 induction: studies with anti-human immunodeficiency virus protease inhibitors.* Drug metabolism and disposition: the biological fate of chemicals, 2008. **36**(6): p. 1172-80.
270. Pascussi, J.M., et al., *The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences.* Annual review of pharmacology and toxicology, 2008. **48**: p. 1-32.
271. Greenbaum, D., et al., *Comparing protein abundance and mRNA expression levels on a genomic scale.* Genome biology, 2003. **4**(9): p. 117.
272. Tian, Q., et al., *Integrated genomic and proteomic analyses of gene expression in Mammalian cells.* Molecular & cellular proteomics : MCP, 2004. **3**(10): p. 960-9.
273. Vallet, C.M., et al., *Modulation of the expression of ABC transporters in murine (J774) macrophages exposed to large concentrations of the fluoroquinolone antibiotic moxifloxacin.* Toxicology, 2011. **290**(2-3): p. 178-86.

274. Sampath, D., et al., *Pharmacodynamics of cytarabine alone and in combination with 7-hydroxystaurosporine (UCN-01) in AML blasts in vitro and during a clinical trial.* Blood, 2006. **107**(6): p. 2517-24.
275. Zahreddine, H. and K.L. Borden, *Mechanisms and insights into drug resistance in cancer.* Frontiers in pharmacology, 2013. **4**: p. 28.
276. Patel, M., et al., *Recent patents and emerging therapeutics for HIV infections: a focus on protease inhibitors.* Pharmaceutical patent analyst, 2013. **2**(4): p. 513-38.
277. Vadlapatla, R.K., et al., *Molecular expression and functional activity of efflux and influx transporters in hypoxia induced retinal pigment epithelial cells.* International journal of pharmaceutics, 2013.
278. Ikezoe, T., et al., *HIV-1 protease inhibitor, ritonavir: a potent inhibitor of CYP3A4, enhanced the anticancer effects of docetaxel in androgen-independent prostate cancer cells in vitro and in vivo.* Cancer research, 2004. **64**(20): p. 7426-31.
279. Srirangam, A., et al., *The human immunodeficiency virus protease inhibitor ritonavir inhibits lung cancer cells, in part, by inhibition of survivin.* Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer, 2011. **6**(4): p. 661-70.
280. Srirangam, A., et al., *Effects of HIV protease inhibitor ritonavir on Akt-regulated cell proliferation in breast cancer.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2006. **12**(6): p. 1883-96.
281. Kumar, S., et al., *Ritonavir blocks AKT signaling, activates apoptosis and inhibits migration and invasion in ovarian cancer cells.* Molecular cancer, 2009. **8**: p. 26.
282. Pati, S., et al., *Antitumorigenic effects of HIV protease inhibitor ritonavir: inhibition of Kaposi sarcoma.* Blood, 2002. **99**(10): p. 3771-9.
283. Vadlapatla, R.K., et al., *Ritonavir inhibits HIF-1alpha-mediated VEGF expression in retinal pigment epithelial cells in vitro.* Eye, 2014. **28**(1): p. 93-101.
284. Semenza, G.L., *Oxygen homeostasis.* Wiley Interdiscip Rev Syst Biol Med, 2010. **2**(3): p. 336-61.
285. Semenza, G.L., *Life with oxygen.* Science, 2007. **318**(5847): p. 62-4.
286. Giaccia, A.J., M.C. Simon, and R. Johnson, *The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease.* Genes & development, 2004. **18**(18): p. 2183-94.
287. Lopez-Barneo, J., R. Pardal, and P. Ortega-Saenz, *Cellular mechanism of oxygen sensing.* Annu Rev Physiol, 2001. **63**: p. 259-87.
288. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(13): p. 5680-4.
289. Goldberg, M.A., S.P. Dunning, and H.F. Bunn, *Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein.* Science, 1988. **242**(4884): p. 1412-5.
290. Loenarz, C., et al., *The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, Trichoplax adhaerens.* EMBO Rep, 2011. **12**(1): p. 63-70.
291. Wang, G.L., et al., *Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(12): p. 5510-4.
292. Reyes, H., S. Reisz-Porszasz, and O. Hankinson, *Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor.* Science, 1992. **256**(5060): p. 1193-5.
293. Greer, S.N., et al., *The updated biology of hypoxia-inducible factor.* The EMBO journal, 2012. **31**(11): p. 2448-60.
294. Li, Y. and D. Ye, *Cancer therapy by targeting hypoxia-inducible factor-1.* Curr Cancer Drug Targets, 2010. **10**(7): p. 782-96.

295. Jiang, B.H., et al., *Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1.* The Journal of biological chemistry, 1996. **271**(30): p. 17771-8.
296. Huang, L.E., et al., *Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(14): p. 7987-92.
297. Ruas, J.L., L. Poellinger, and T. Pereira, *Functional analysis of hypoxia-inducible factor-1 alpha-mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein.* The Journal of biological chemistry, 2002. **277**(41): p. 38723-30.
298. Jiang, B.H., et al., *Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension.* The Journal of biological chemistry, 1997. **272**(31): p. 19253-60.
299. Kallio, P.J., et al., *Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha.* The EMBO journal, 1998. **17**(22): p. 6573-86.
300. Tian, H., et al., *The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development.* Genes & development, 1998. **12**(21): p. 3320-4.
301. Ema, M., et al., *A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(9): p. 4273-8.
302. Tian, H., S.L. McKnight, and D.W. Russell, *Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells.* Genes & development, 1997. **11**(1): p. 72-82.
303. Hara, S., et al., *Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha.* Biochemical and biophysical research communications, 2001. **287**(4): p. 808-13.
304. Gu, Y.Z., et al., *Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha.* Gene Expr, 1998. **7**(3): p. 205-13.
305. Makino, Y., et al., *Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus.* The Journal of biological chemistry, 2002. **277**(36): p. 32405-8.
306. Makino, Y., et al., *Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.* Nature, 2001. **414**(6863): p. 550-4.
307. Wiesener, M.S., et al., *Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha.* Blood, 1998. **92**(7): p. 2260-8.
308. Kallio, P.J., et al., *Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(11): p. 5667-72.
309. Brahimi-Horn, C., N. Mazure, and J. Pouyssegur, *Signalling via the hypoxia-inducible factor-1alpha requires multiple posttranslational modifications.* Cell Signal, 2005. **17**(1): p. 1-9.
310. Welsh, S.J. and G. Powis, *Hypoxia inducible factor as a cancer drug target.* Curr Cancer Drug Targets, 2003. **3**(6): p. 391-405.
311. Ivan, M., et al., *HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing.* Science, 2001. **292**(5516): p. 464-8.

312. Jaakkola, P., et al., *Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation.* Science, 2001. **292**(5516): p. 468-72.
313. Masson, N. and P.J. Ratcliffe, *HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O(2) levels.* J Cell Sci, 2003. **116**(Pt 15): p. 3041-9.
314. Masson, N., et al., *Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation.* The EMBO journal, 2001. **20**(18): p. 5197-206.
315. Srinivas, V., et al., *Characterization of an oxygen/redox-dependent degradation domain of hypoxia-inducible factor alpha (HIF-alpha) proteins.* Biochemical and biophysical research communications, 1999. **260**(2): p. 557-61.
316. Huang, J., et al., *Sequence determinants in hypoxia-inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3.* The Journal of biological chemistry, 2002. **277**(42): p. 39792-800.
317. Schofield, C.J. and Z. Zhang, *Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes.* Curr Opin Struct Biol, 1999. **9**(6): p. 722-31.
318. Bruick, R.K. and S.L. McKnight, *A conserved family of prolyl-4-hydroxylases that modify HIF.* Science, 2001. **294**(5545): p. 1337-40.
319. Lando, D., et al., *Oxygen-dependent regulation of hypoxia-inducible factors by prolyl and asparaginyl hydroxylation.* European journal of biochemistry / FEBS, 2003. **270**(5): p. 781-90.
320. Yuan, F., et al., *Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(25): p. 14765-70.
321. Epstein, A.C., et al., *C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation.* Cell, 2001. **107**(1): p. 43-54.
322. Berra, E., et al., *HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia.* The EMBO journal, 2003. **22**(16): p. 4082-90.
323. Metzen, E., et al., *Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing.* J Cell Sci, 2003. **116**(Pt 7): p. 1319-26.
324. Hon, W.C., et al., *Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL.* Nature, 2002. **417**(6892): p. 975-8.
325. Min, J.H., et al., *Structure of an HIF-1alpha -pVHL complex: hydroxyproline recognition in signaling.* Science, 2002. **296**(5574): p. 1886-9.
326. Ivan, M. and W.G. Kaelin, Jr., *The von Hippel-Lindau tumor suppressor protein.* Curr Opin Genet Dev, 2001. **11**(1): p. 27-34.
327. Kamura, T., et al., *Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(19): p. 10430-5.
328. Groulx, I. and S. Lee, *Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor requires nuclear-cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein.* Mol Cell Biol, 2002. **22**(15): p. 5319-36.
329. Berra, E., et al., *Hypoxia-inducible factor-1 alpha (HIF-1 alpha) escapes O(2)-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm.* EMBO Rep, 2001. **2**(7): p. 615-20.
330. Yu, F., et al., *Dynamic, site-specific interaction of hypoxia-inducible factor-1alpha with the von Hippel-Lindau tumor suppressor protein.* Cancer research, 2001. **61**(10): p. 4136-42.
331. Krieg, M., et al., *Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function.* Oncogene, 2000. **19**(48): p. 5435-43.

332. Cockman, M.E., et al., *Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein.* The Journal of biological chemistry, 2000. **275**(33): p. 25733-41.
333. Jeong, J.W., et al., *Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation.* Cell, 2002. **111**(5): p. 709-20.
334. Kim, M.S., et al., *Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes.* Nat Med, 2001. **7**(4): p. 437-43.
335. Tanimoto, K., et al., *Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein.* The EMBO journal, 2000. **19**(16): p. 4298-309.
336. Sang, N., et al., *Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent, hydroxylation-regulated association with p300/CBP.* Mol Cell Biol, 2002. **22**(9): p. 2984-92.
337. Lando, D., et al., *Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch.* Science, 2002. **295**(5556): p. 858-61.
338. Lando, D., et al., *FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor.* Genes & development, 2002. **16**(12): p. 1466-71.
339. Hewitson, K.S., et al., *Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family.* The Journal of biological chemistry, 2002. **277**(29): p. 26351-5.
340. Mahon, P.C., K. Hirota, and G.L. Semenza, *FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity.* Genes & development, 2001. **15**(20): p. 2675-86.
341. Freedman, S.J., et al., *Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(8): p. 5367-72.
342. Dames, S.A., et al., *Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(8): p. 5271-6.
343. Minet, E., et al., *Transduction pathways involved in Hypoxia-Inducible Factor-1 phosphorylation and activation.* Free Radic Biol Med, 2001. **31**(7): p. 847-55.
344. Sodhi, A., et al., *The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha.* Cancer research, 2000. **60**(17): p. 4873-80.
345. Richard, D.E., et al., *p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1.* The Journal of biological chemistry, 1999. **274**(46): p. 32631-7.
346. Suzuki, H., A. Tomida, and T. Tsuruo, *Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia.* Oncogene, 2001. **20**(41): p. 5779-88.
347. Hur, E., et al., *Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha.* Molecular pharmacology, 2001. **59**(5): p. 1216-24.
348. Gradin, K., et al., *The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine.* The Journal of biological chemistry, 2002. **277**(26): p. 23508-14.
349. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE.* Sci STKE, 2005. **2005**(306): p. re12.

350. Huang, L.E., et al., *Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit.* The Journal of biological chemistry, 1996. **271**(50): p. 32253-9.
351. Pugh, C.W., et al., *Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(23): p. 10553-7.
352. Conway, E.M., D. Collen, and P. Carmeliet, *Molecular mechanisms of blood vessel growth.* Cardiovascular research, 2001. **49**(3): p. 507-21.
353. Giordano, F.J. and R.S. Johnson, *Angiogenesis: the role of the microenvironment in flipping the switch.* Curr Opin Genet Dev, 2001. **11**(1): p. 35-40.
354. Berra, E., et al., *Signaling angiogenesis via p42/p44 MAP kinase and hypoxia.* Biochemical pharmacology, 2000. **60**(8): p. 1171-8.
355. Forsythe, J.A., et al., *Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.* Mol Cell Biol, 1996. **16**(9): p. 4604-13.
356. Bunn, H.F. and R.O. Poyton, *Oxygen sensing and molecular adaptation to hypoxia.* Physiological reviews, 1996. **76**(3): p. 839-85.
357. Levy, A.P., et al., *Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia.* The Journal of biological chemistry, 1995. **270**(22): p. 13333-40.
358. Josko, J., et al., *Vascular endothelial growth factor (VEGF) and its effect on angiogenesis.* Med Sci Monit, 2000. **6**(5): p. 1047-52.
359. Harris, A.L., *von Hippel-Lindau syndrome: target for anti-vascular endothelial growth factor (VEGF) receptor therapy.* Oncologist, 2000. **5 Suppl 1**: p. 32-6.
360. Neufeld, G., et al., *Vascular endothelial growth factor (VEGF) and its receptors.* FASEB J, 1999. **13**(1): p. 9-22.
361. Nguyen, S.V. and W.C. Claycomb, *Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes.* Biochemical and biophysical research communications, 1999. **265**(2): p. 382-6.
362. Hu, J., et al., *Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand.* Biochemical and biophysical research communications, 1998. **245**(3): p. 894-9.
363. Eckhart, A.D., et al., *Characterization of the alpha1B-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(17): p. 9487-92.
364. Lee, P.J., et al., *Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia.* The Journal of biological chemistry, 1997. **272**(9): p. 5375-81.
365. Melillo, G., et al., *A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter.* The Journal of experimental medicine, 1995. **182**(6): p. 1683-93.
366. Lima e Silva, R., et al., *The SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization.* FASEB J, 2007. **21**(12): p. 3219-30.
367. Rakic, J.M., et al., *Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization.* Investigative ophthalmology & visual science, 2003. **44**(7): p. 3186-93.
368. Seo, M.S., et al., *Photoreceptor-specific expression of platelet-derived growth factor-B results in traction retinal detachment.* The American journal of pathology, 2000. **157**(3): p. 995-1005.
369. Hackett, S.F., et al., *Angiopoietin 2 expression in the retina: upregulation during physiologic and pathologic neovascularization.* Journal of cellular physiology, 2000. **184**(3): p. 275-84.

370. Ben-Yosef, Y., et al., *Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation.* Circulation research, 2002. **90**(7): p. 784-91.
371. Takahashi, Y., et al., *Hypoxic induction of prolyl 4-hydroxylase alpha (I) in cultured cells.* The Journal of biological chemistry, 2000. **275**(19): p. 14139-46.
372. Kietzmann, T., U. Roth, and K. Jungermann, *Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes.* Blood, 1999. **94**(12): p. 4177-85.
373. Ames, A., 3rd, *Energy requirements of CNS cells as related to their function and to their vulnerability to ischemia: a commentary based on studies on retina.* Can J Physiol Pharmacol, 1992. **70 Suppl**: p. S158-64.
374. Campochiaro, P.A., *Ocular neovascularization.* Journal of molecular medicine, 2013. **91**(3): p. 311-21.
375. Caprara, C. and C. Grimm, *From oxygen to erythropoietin: relevance of hypoxia for retinal development, health and disease.* Progress in retinal and eye research, 2012. **31**(1): p. 89-119.
376. Sung, C.H. and J.Z. Chuang, *The cell biology of vision.* J Cell Biol, 2010. **190**(6): p. 953-63.
377. Baccus, S.A., *Timing and computation in inner retinal circuitry.* Annu Rev Physiol, 2007. **69**: p. 271-90.
378. Okawa, H., et al., *ATP consumption by mammalian rod photoreceptors in darkness and in light.* Curr Biol, 2008. **18**(24): p. 1917-21.
379. Stone, J., et al., *The locations of mitochondria in mammalian photoreceptors: relation to retinal vasculature.* Brain Res, 2008. **1189**: p. 58-69.
380. Flammer, J. and M. Mozaffarieh, *Autoregulation, a balancing act between supply and demand.* Can J Ophthalmol, 2008. **43**(3): p. 317-21.
381. Pournaras, C.J., et al., *Regulation of retinal blood flow in health and disease.* Progress in retinal and eye research, 2008. **27**(3): p. 284-330.
382. Saint-Geniez, M. and P.A. D'Amore, *Development and pathology of the hyaloid, choroidal and retinal vasculature.* Int J Dev Biol, 2004. **48**(8-9): p. 1045-58.
383. Kocur, I. and S. Resnikoff, *Visual impairment and blindness in Europe and their prevention.* Br J Ophthalmol, 2002. **86**(7): p. 716-22.
384. Ferris, F.L., 3rd, M.D. Davis, and L.M. Aiello, *Treatment of diabetic retinopathy.* The New England journal of medicine, 1999. **341**(9): p. 667-78.
385. Crawford, T.N., et al., *Diabetic retinopathy and angiogenesis.* Curr Diabetes Rev, 2009. **5**(1): p. 8-13.
386. Zimmet, P., K.G. Alberti, and J. Shaw, *Global and societal implications of the diabetes epidemic.* Nature, 2001. **414**(6865): p. 782-7.
387. Bhavsar, A.R., *Diabetic retinopathy: the latest in current management.* Retina, 2006. **26**(6 Suppl): p. S71-9.
388. Arden, G.B. and S. Sivaprasad, *Hypoxia and oxidative stress in the causation of diabetic retinopathy.* Current diabetes reviews, 2011. **7**(5): p. 291-304.
389. Wilkinson-Berka, J.L., *Angiotensin and diabetic retinopathy.* The international journal of biochemistry & cell biology, 2006. **38**(5-6): p. 752-65.
390. Engerman, R.L. and T.S. Kern, *Retinopathy in animal models of diabetes.* Diabetes/metabolism reviews, 1995. **11**(2): p. 109-20.
391. Stitt, A.W., et al., *Diabetic retinopathy: quantitative variation in capillary basement membrane thickening in arterial or venous environments.* The British journal of ophthalmology, 1994. **78**(2): p. 133-7.

392. Bursell, S.E., et al., *Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy.* Investigative ophthalmology & visual science, 1996. **37**(5): p. 886-97.
393. Josifova, T., I. Plestina-Borjan, and P.B. Henrich, *Proliferative diabetic retinopathy: predictive and preventive measures at hypoxia induced retinal changes.* The EPMA journal, 2010. **1**(1): p. 73-7.
394. Morello, C.M., *Etiology and natural history of diabetic retinopathy: an overview.* American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists, 2007. **64**(17 Suppl 12): p. S3-7.
395. Mohamed, Q., M.C. Gillies, and T.Y. Wong, *Management of diabetic retinopathy: a systematic review.* JAMA : the journal of the American Medical Association, 2007. **298**(8): p. 902-16.
396. Watkins, P.J., *Retinopathy.* BMJ, 2003. **326**(7395): p. 924-6.
397. Holekamp, N.M., Y.B. Shui, and D. Beebe, *Lower intraocular oxygen tension in diabetic patients: possible contribution to decreased incidence of nuclear sclerotic cataract.* Am J Ophthalmol, 2006. **141**(6): p. 1027-32.
398. Lim, J.I., C. Spee, and D.R. Hinton, *A comparison of hypoxia-inducible factor-alpha in surgically excised neovascular membranes of patients with diabetes compared with idiopathic epiretinal membranes in nondiabetic patients.* Retina, 2010. **30**(9): p. 1472-8.
399. Abu El-Asrar, A.M., L. Missotten, and K. Geboes, *Expression of hypoxia-inducible factor-1alpha and the protein products of its target genes in diabetic fibrovascular epiretinal membranes.* The British journal of ophthalmology, 2007. **91**(6): p. 822-6.
400. Lin, M., et al., *Ischaemia-induced retinal neovascularisation and diabetic retinopathy in mice with conditional knockout of hypoxia-inducible factor-1 in retinal Muller cells.* Diabetologia, 2011. **54**(6): p. 1554-66.
401. Gilbert, C., *Retinopathy of prematurity: a global perspective of the epidemics, population of babies at risk and implications for control.* Early human development, 2008. **84**(2): p. 77-82.
402. Smith, L.E., *Pathogenesis of retinopathy of prematurity.* Seminars in neonatology : SN, 2003. **8**(6): p. 469-73.
403. Kuriyama, H., et al., *Involvement of oxygen free radicals in experimental retinal ischemia and the selective vulnerability of retinal damage.* Ophthalmic research, 2001. **33**(4): p. 196-202.
404. Hardy, P., et al., *Oxidants, nitric oxide and prostanoids in the developing ocular vasculature: a basis for ischemic retinopathy.* Cardiovascular research, 2000. **47**(3): p. 489-509.
405. Hardy, P., et al., *Free radicals in retinal and choroidal blood flow autoregulation in the piglet: interaction with prostaglandins.* Investigative ophthalmology & visual science, 1994. **35**(2): p. 580-91.
406. Flynn, J.T., et al., *A cohort study of transcutaneous oxygen tension and the incidence and severity of retinopathy of prematurity.* The New England journal of medicine, 1992. **326**(16): p. 1050-4.
407. Sapieha, P., et al., *Retinopathy of prematurity: understanding ischemic retinal vasculopathies at an extreme of life.* The Journal of clinical investigation, 2010. **120**(9): p. 3022-32.
408. Kermorvant-Duchemin, E., et al., *Understanding ischemic retinopathies: emerging concepts from oxygen-induced retinopathy.* Documenta ophthalmologica. Advances in ophthalmology, 2010. **120**(1): p. 51-60.
409. Smith, L.E., *Through the eyes of a child: understanding retinopathy through ROP the Friedenwald lecture.* Investigative ophthalmology & visual science, 2008. **49**(12): p. 5177-82.
410. Chen, J. and L.E. Smith, *Retinopathy of prematurity.* Angiogenesis, 2007. **10**(2): p. 133-40.
411. Mowat, F.M., et al., *HIF-1alpha and HIF-2alpha are differentially activated in distinct cell populations in retinal ischaemia.* PloS one, 2010. **5**(6): p. e11103.
412. Jiang, J., et al., *Inhibition of retinal neovascularization by gene transfer of small interfering RNA targeting HIF-1alpha and VEGF.* Journal of cellular physiology, 2009. **218**(1): p. 66-74.

413. Gehrs, K.M., et al., *Age-related macular degeneration--emerging pathogenetic and therapeutic concepts.* Annals of medicine, 2006. **38**(7): p. 450-71.
414. Adams, M.K., et al., *Abdominal obesity and age-related macular degeneration.* American journal of epidemiology, 2011. **173**(11): p. 1246-55.
415. Reynolds, R., B. Rosner, and J.M. Seddon, *Serum lipid biomarkers and hepatic lipase gene associations with age-related macular degeneration.* Ophthalmology, 2010. **117**(10): p. 1989-95.
416. Klein, R., et al., *The prevalence of age-related macular degeneration and associated risk factors.* Archives of ophthalmology, 2010. **128**(6): p. 750-8.
417. Tolppanen, A.M., et al., *Single nucleotide polymorphisms of the tenomodulin gene (TNMD) in age-related macular degeneration.* Molecular vision, 2009. **15**: p. 762-70.
418. van Leeuwen, R., et al., *Blood pressure, atherosclerosis, and the incidence of age-related maculopathy: the Rotterdam Study.* Investigative ophthalmology & visual science, 2003. **44**(9): p. 3771-7.
419. Cook, H.L., P.J. Patel, and A. Tufail, *Age-related macular degeneration: diagnosis and management.* British medical bulletin, 2008. **85**: p. 127-49.
420. Algvere, P.V. and S. Seregard, *Drusen maculopathy: a risk factor for AMD. Can we prevent visual loss?* Acta ophthalmologica Scandinavica, 2003. **81**(5): p. 427-9.
421. Ambati, J., et al., *Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies.* Survey of ophthalmology, 2003. **48**(3): p. 257-93.
422. Bhutto, I. and G. Lutty, *Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex.* Molecular aspects of medicine, 2012. **33**(4): p. 295-317.
423. Ayoub, T. and N. Patel, *Age-related macular degeneration.* Journal of the Royal Society of Medicine, 2009. **102**(2): p. 56-61.
424. Donoso, L.A., et al., *The role of inflammation in the pathogenesis of age-related macular degeneration.* Survey of ophthalmology, 2006. **51**(2): p. 137-52.
425. Beatty, S., et al., *The role of oxidative stress in the pathogenesis of age-related macular degeneration.* Survey of ophthalmology, 2000. **45**(2): p. 115-34.
426. Arjamaa, O., et al., *Regulatory role of HIF-1alpha in the pathogenesis of age-related macular degeneration (AMD).* Ageing research reviews, 2009. **8**(4): p. 349-58.
427. Taylor, C.T., *Interdependent roles for hypoxia inducible factor and nuclear factor-kappaB in hypoxic inflammation.* The Journal of physiology, 2008. **586**(Pt 17): p. 4055-9.
428. Metelitsina, T.I., et al., *Foveolar choroidal circulation and choroidal neovascularization in age-related macular degeneration.* Investigative ophthalmology & visual science, 2008. **49**(1): p. 358-63.
429. Grunwald, J.E., et al., *Reduced foveolar choroidal blood flow in eyes with increasing AMD severity.* Investigative ophthalmology & visual science, 2005. **46**(3): p. 1033-8.
430. Schlingemann, R.O., *Role of growth factors and the wound healing response in age-related macular degeneration.* Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 2004. **242**(1): p. 91-101.
431. Yuan, G., et al., *Induction of HIF-1alpha expression by intermittent hypoxia: involvement of NADPH oxidase, Ca2+ signaling, prolyl hydroxylases, and mTOR.* Journal of cellular physiology, 2008. **217**(3): p. 674-85.
432. Frede, S., U. Berchner-Pfannschmidt, and J. Fandrey, *Regulation of hypoxia-inducible factors during inflammation.* Methods in enzymology, 2007. **435**: p. 405-19.
433. Sheridan, C.M., et al., *Expression of hypoxia-inducible factor-1alpha and -2alpha in human choroidal neovascular membranes.* Graefe's archive for clinical and experimental ophthalmology

= Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 2009. **247**(10): p. 1361-7.
434. Inoue, Y., et al., *Expression of hypoxia-inducible factor 1alpha and 2alpha in choroidal neovascular membranes associated with age-related macular degeneration.* The British journal of ophthalmology, 2007. **91**(12): p. 1720-1.
435. Quigley, H.A. and A.T. Broman, *The number of people with glaucoma worldwide in 2010 and 2020.* The British journal of ophthalmology, 2006. **90**(3): p. 262-7.
436. Liu, Y. and R.R. Allingham, *Molecular genetics in glaucoma.* Experimental eye research, 2011. **93**(4): p. 331-9.
437. Quigley, H.A., *Glaucoma.* Lancet, 2011. **377**(9774): p. 1367-77.
438. Flammer, J., et al., *The impact of ocular blood flow in glaucoma.* Progress in retinal and eye research, 2002. **21**(4): p. 359-93.
439. Osborne, N.N., et al., *A hypothesis to explain ganglion cell death caused by vascular insults at the optic nerve head: possible implication for the treatment of glaucoma.* The British journal of ophthalmology, 2001. **85**(10): p. 1252-9.
440. Tezel, G. and M.B. Wax, *Hypoxia-inducible factor 1alpha in the glaucomatous retina and optic nerve head.* Archives of ophthalmology, 2004. **122**(9): p. 1348-56.
441. Ng, E.W. and A.P. Adamis, *Targeting angiogenesis, the underlying disorder in neovascular age-related macular degeneration.* Canadian journal of ophthalmology. Journal canadien d'ophtalmologie, 2005. **40**(3): p. 352-68.
442. Lashkari, K., et al., *Vascular endothelial growth factor and hepatocyte growth factor levels are differentially elevated in patients with advanced retinopathy of prematurity.* The American journal of pathology, 2000. **156**(4): p. 1337-44.
443. Tripathi, R.C., et al., *Increased level of vascular endothelial growth factor in aqueous humor of patients with neovascular glaucoma.* Ophthalmology, 1998. **105**(2): p. 232-7.
444. Aiello, L.P., et al., *Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders.* The New England journal of medicine, 1994. **331**(22): p. 1480-7.
445. Ferrara, N., *Vascular endothelial growth factor: basic science and clinical progress.* Endocrine reviews, 2004. **25**(4): p. 581-611.
446. Famiglietti, E.V., et al., *Immunocytochemical localization of vascular endothelial growth factor in neurons and glial cells of human retina.* Brain research, 2003. **969**(1-2): p. 195-204.
447. Takagi, H., G.L. King, and L.P. Aiello, *Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes.* Diabetes, 1996. **45**(8): p. 1016-23.
448. Aiello, L.P., et al., *Hypoxic regulation of vascular endothelial growth factor in retinal cells.* Archives of ophthalmology, 1995. **113**(12): p. 1538-44.
449. Thieme, H., et al., *Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells.* Diabetes, 1995. **44**(1): p. 98-103.
450. Kimoto, K. and T. Kubota, *Anti-VEGF Agents for Ocular Angiogenesis and Vascular Permeability.* Journal of ophthalmology, 2012. **2012**: p. 852183.
451. Tolentino, M., *Systemic and ocular safety of intravitreal anti-VEGF therapies for ocular neovascular disease.* Survey of ophthalmology, 2011. **56**(2): p. 95-113.
452. Campa, C. and S.P. Harding, *Anti-VEGF compounds in the treatment of neovascular age related macular degeneration.* Current drug targets, 2011. **12**(2): p. 173-81.
453. Pieramici, D.J. and M.D. Rabena, *Anti-VEGF therapy: comparison of current and future agents.* Eye, 2008. **22**(10): p. 1330-6.
454. Quaggin, S.E., *Turning a blind eye to anti-VEGF toxicities.* The Journal of clinical investigation, 2012. **122**(11): p. 3849-51.

455. Kurihara, T., et al., *Targeted deletion of Vegfa in adult mice induces vision loss.* The Journal of clinical investigation, 2012. **122**(11): p. 4213-7.
456. Young, R.M., et al., *Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism.* The Journal of biological chemistry, 2008. **283**(24): p. 16309-19.
457. Chen, P., et al., *Inhibition of VEGF expression and corneal neovascularization by shRNA targeting HIF-1alpha in a mouse model of closed eye contact lens wear.* Molecular vision, 2012. **18**: p. 864-73.
458. Zhang, H., et al., *Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(50): p. 19579-86.
459. Yoshida, T., et al., *Digoxin inhibits retinal ischemia-induced HIF-1alpha expression and ocular neovascularization.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2010. **24**(6): p. 1759-67.
460. Buchler, P., et al., *Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression.* Cancer, 2004. **100**(1): p. 201-10.
461. Wang, B., et al., *Genistein inhibited retinal neovascularization and expression of vascular endothelial growth factor and hypoxia inducible factor 1alpha in a mouse model of oxygen-induced retinopathy.* Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 2005. **21**(2): p. 107-13.
462. Neckers, L., *Heat shock protein 90: the cancer chaperone.* Journal of biosciences, 2007. **32**(3): p. 517-30.
463. Isaacs, J.S., et al., *Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway.* The Journal of biological chemistry, 2002. **277**(33): p. 29936-44.
464. Kim, W.Y., et al., *Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radiation-induced stabilization of hypoxia-inducible factor-1alpha.* Cancer research, 2009. **69**(4): p. 1624-32.
465. Kim, J.H., et al., *Deguelin inhibits retinal neovascularization by down-regulation of HIF-1alpha in oxygen-induced retinopathy.* Journal of cellular and molecular medicine, 2008. **12**(6A): p. 2407-15.
466. Lee, K., et al., *Anthracycline chemotherapy inhibits HIF-1 transcriptional activity and tumor-induced mobilization of circulating angiogenic cells.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(7): p. 2353-8.
467. Kong, D., et al., *Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity.* Cancer research, 2005. **65**(19): p. 9047-55.
468. Kung, A.L., et al., *Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway.* Cancer cell, 2004. **6**(1): p. 33-43.
469. Gibson, J.S., A.R. Cossins, and J.C. Ellory, *Oxygen-sensitive membrane transporters in vertebrate red cells.* J Exp Biol, 2000. **203**(Pt 9): p. 1395-407.
470. Semenza, G.L., *HIF-1: mediator of physiological and pathophysiological responses to hypoxia.* J Appl Physiol, 2000. **88**(4): p. 1474-80.
471. Arjamaa, O. and M. Nikinmaa, *Oxygen-dependent diseases in the retina: role of hypoxia-inducible factors.* Experimental eye research, 2006. **83**(3): p. 473-83.
472. Wang, G.L. and G.L. Semenza, *Purification and characterization of hypoxia-inducible factor 1.* The Journal of biological chemistry, 1995. **270**(3): p. 1230-7.
473. Jewell, U.R., et al., *Induction of HIF-1alpha in response to hypoxia is instantaneous.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2001. **15**(7): p. 1312-4.

474. Semenza, G.L., *Hypoxia-inducible factors in physiology and medicine.* Cell, 2012. **148**(3): p. 399-408.
475. Ke, Q. and M. Costa, *Hypoxia-inducible factor-1 (HIF-1).* Molecular pharmacology, 2006. **70**(5): p. 1469-80.
476. Hirota, K. and G.L. Semenza, *Regulation of angiogenesis by hypoxia-inducible factor 1.* Crit Rev Oncol Hematol, 2006. **59**(1): p. 15-26.
477. Hayreh, S.S., *Role of retinal hypoxia in diabetic macular edema: a new concept.* Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 2008. **246**(3): p. 353-61.
478. Stefansson, E., *The therapeutic effects of retinal laser treatment and vitrectomy. A theory based on oxygen and vascular physiology.* Acta ophthalmologica Scandinavica, 2001. **79**(5): p. 435-40.
479. Penn, J.S., S. Li, and M.I. Naash, *Ambient hypoxia reverses retinal vascular attenuation in a transgenic mouse model of autosomal dominant retinitis pigmentosa.* Investigative ophthalmology & visual science, 2000. **41**(12): p. 4007-13.
480. Pierce, E.A., et al., *Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(3): p. 905-9.
481. Mannermaa, E., et al., *Efflux protein expression in human retinal pigment epithelium cell lines.* Pharm Res, 2009. **26**(7): p. 1785-91.
482. Kennedy, B.G. and N.J. Mangini, *P-glycoprotein expression in human retinal pigment epithelium.* Molecular vision, 2002. **8**: p. 422-30.
483. Katragadda, S., R.S. Talluri, and A.K. Mitra, *Modulation of P-glycoprotein-mediated efflux by prodrug derivatization: an approach involving peptide transporter-mediated influx across rabbit cornea.* Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 2006. **22**(2): p. 110-20.
484. Majumdar, S., S. Duvvuri, and A.K. Mitra, *Membrane transporter/receptor-targeted prodrug design: strategies for human and veterinary drug development.* Advanced drug delivery reviews, 2004. **56**(10): p. 1437-52.
485. Anand, B.S., S. Dey, and A.K. Mitra, *Current prodrug strategies via membrane transporters/receptors.* Expert Opin Biol Ther, 2002. **2**(6): p. 607-20.
486. Ganapathy, M.E. and V. Ganapathy, *Amino Acid Transporter ATB0,+ as a delivery system for drugs and prodrugs.* Curr Drug Targets Immune Endocr Metabol Disord, 2005. **5**(4): p. 357-64.
487. Smith, S.B., et al., *Expression of folate receptor alpha in the mammalian retinol pigmented epithelium and retina.* Invest Ophthalmol Vis Sci, 1999. **40**(5): p. 840-8.
488. Vadlapudi, A.D., R.K. Vadlapatla, and A.K. Mitra, *Sodium dependent multivitamin transporter (SMVT): a potential target for drug delivery.* Current drug targets, 2012. **13**(7): p. 994-1003.
489. Ponnaluri, V.K., et al., *Hypoxia induced expression of histone lysine demethylases: implications in oxygen-dependent retinal neovascular diseases.* Biochemical and biophysical research communications, 2011. **415**(2): p. 373-7.
490. Janoria, K.G., et al., *Vitreal pharmacokinetics of biotinylated ganciclovir: role of sodium-dependent multivitamin transporter expressed on retina.* Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 2009. **25**(1): p. 39-49.
491. Vavilala, D.T., et al., *Honokiol inhibits HIF pathway and hypoxia-induced expression of histone lysine demethylases.* Biochemical and biophysical research communications, 2012. **422**(3): p. 369-74.

492. Tan, S.C., et al., *Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere-derived cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors.* Mol Biol Rep, 2012. **39**(4): p. 4857-67.
493. Vadlapudi, A.D., et al., *Functional and molecular aspects of biotin uptake via SMVT in human corneal epithelial (HCEC) and retinal pigment epithelial (D407) cells.* The AAPS journal, 2012. **14**(4): p. 832-42.
494. Jwala, J., et al., *Differential expression of folate receptor-alpha, sodium-dependent multivitamin transporter, and amino acid transporter (B (0, +)) in human retinoblastoma (Y-79) and retinal pigment epithelial (ARPE-19) cell lines.* Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 2012. **28**(3): p. 237-44.
495. Constable, P.A., et al., *P-Glycoprotein expression in human retinal pigment epithelium cell lines.* Experimental eye research, 2006. **83**(1): p. 24-30.
496. Geisen, P., et al., *Characterization of barrier properties and inducible VEGF expression of several types of retinal pigment epithelium in medium-term culture.* Curr Eye Res, 2006. **31**(9): p. 739-48.
497. Slomiany, M.G. and S.A. Rosenzweig, *IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 alpha expression and activity in retinal pigment epithelial cell line D407.* Invest Ophthalmol Vis Sci, 2004. **45**(8): p. 2838-47.
498. Davis, A.A., et al., *A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture.* Invest Ophthalmol Vis Sci, 1995. **36**(5): p. 955-64.
499. Krishnamurthy, P. and J.D. Schuetz, *The ABC transporter Abcg2/Bcrp: role in hypoxia mediated survival.* Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, 2005. **18**(4): p. 349-58.
500. Staud, F. and P. Pavek, *Breast cancer resistance protein (BCRP/ABCG2).* The international journal of biochemistry & cell biology, 2005. **37**(4): p. 720-5.
501. Cheng, G.M. and K.K. To, *Adverse Cell Culture Conditions Mimicking the Tumor Microenvironment Upregulate ABCG2 to Mediate Multidrug Resistance and a More Malignant Phenotype.* ISRN Oncol, 2012. **2012**: p. 746025.
502. Boidot, R., et al., *Regulation of monocarboxylate transporter MCT1 expression by p53 mediates inward and outward lactate fluxes in tumors.* Cancer research, 2012. **72**(4): p. 939-48.
503. Soh, H., M. Wasa, and M. Fukuzawa, *Hypoxia upregulates amino acid transport in a human neuroblastoma cell line.* J Pediatr Surg, 2007. **42**(4): p. 608-12.
504. Mannermaa, E., K.S. Vellonen, and A. Urtti, *Drug transport in corneal epithelium and blood-retina barrier: emerging role of transporters in ocular pharmacokinetics.* Advanced drug delivery reviews, 2006. **58**(11): p. 1136-63.
505. Kadam, R.S., et al., *Hypoxia alters ocular drug transporter expression and activity in rat and calf models: implications for drug delivery.* Molecular pharmaceutics, 2013. **10**(6): p. 2350-61.
506. Chang, T.T., et al., *Hypoxia-mediated down-regulation of OCTN2 and PPARalpha expression in human placentas and in BeWo cells.* Molecular pharmaceutics, 2011. **8**(1): p. 117-25.
507. Wu, W.C., et al., *Geldanamycin, a HSP90 inhibitor, attenuates the hypoxia-induced vascular endothelial growth factor expression in retinal pigment epithelium cells in vitro.* Experimental eye research, 2007. **85**(5): p. 721-31.
508. Kaur, C., W.S. Foulds, and E.A. Ling, *Hypoxia-ischemia and retinal ganglion cell damage.* Clinical ophthalmology, 2008. **2**(4): p. 879-89.
509. Weidemann, A. and R.S. Johnson, *Biology of HIF-1alpha.* Cell death and differentiation, 2008. **15**(4): p. 621-7.

510. Rohwer, N. and T. Cramer, *Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways.* Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy, 2011. **14**(3): p. 191-201.
511. Rankin, E.B. and A.J. Giaccia, *The role of hypoxia-inducible factors in tumorigenesis.* Cell death and differentiation, 2008. **15**(4): p. 678-85.
512. Hewitson, K.S. and C.J. Schofield, *The HIF pathway as a therapeutic target.* Drug discovery today, 2004. **9**(16): p. 704-11.
513. Andriolo, R.B., et al., *Bevacizumab for ocular neovascular diseases: a systematic review.* Sao Paulo medical journal = Revista paulista de medicina, 2009. **127**(2): p. 84-91.
514. Kourlas, H. and P. Abrams, *Ranibizumab for the treatment of neovascular age-related macular degeneration: a review.* Clinical therapeutics, 2007. **29**(9): p. 1850-61.
515. Slevin, M., et al., *New VEGF antagonists as possible therapeutic agents in vascular disease.* Expert opinion on investigational drugs, 2008. **17**(9): p. 1301-14.
516. Simo, R. and C. Hernandez, *Intravitreous anti-VEGF for diabetic retinopathy: hopes and fears for a new therapeutic strategy.* Diabetologia, 2008. **51**(9): p. 1574-80.
517. Maggiorella, L., et al., *Combined radiation sensitizing and anti-angiogenic effects of ionizing radiation and the protease inhibitor ritonavir in a head and neck carcinoma model.* Anticancer research, 2005. **25**(6B): p. 4357-62.
518. Xu, Y., et al., *A novel antiangiogenic peptide derived from hepatocyte growth factor inhibits neovascularization in vitro and in vivo.* Molecular vision, 2010. **16**: p. 1982-95.
519. Pugh, C.W. and P.J. Ratcliffe, *Regulation of angiogenesis by hypoxia: role of the HIF system.* Nature medicine, 2003. **9**(6): p. 677-84.
520. Das, A. and P.G. McGuire, *Retinal and choroidal angiogenesis: pathophysiology and strategies for inhibition.* Progress in retinal and eye research, 2003. **22**(6): p. 721-48.
521. Oh, J.H., et al., *Effects of Ginkgo biloba Extract on Cultured Human Retinal Pigment Epithelial Cells under Chemical Hypoxia.* Current eye research, 2013.
522. DeNiro, M., et al., *Pleiotropic effects of YC-1 selectively inhibit pathological retinal neovascularization and promote physiological revascularization in a mouse model of oxygen-induced retinopathy.* Molecular pharmacology, 2010. **77**(3): p. 348-67.
523. Zhao, W., et al., *Inhibition of proliferation, migration and tube formation of choroidal microvascular endothelial cells by targeting HIF-1alpha with short hairpin RNA-expressing plasmid DNA in human RPE cells in a coculture system.* Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 2008. **246**(10): p. 1413-22.
524. Zhang, P., et al., *Inhibition of VEGF expression by targeting HIF-1 alpha with small interference RNA in human RPE cells.* Ophthalmologica. Journal international d'ophtalmologie. International journal of ophthalmology. Zeitschrift fur Augenheilkunde, 2007. **221**(6): p. 411-7.
525. Wang, B., et al., *Genistein inhibited hypoxia-inducible factor-1alpha expression induced by hypoxia and cobalt chloride in human retinal pigment epithelium cells.* Methods and findings in experimental and clinical pharmacology, 2005. **27**(3): p. 179-84.
526. Forooghian, F., R. Razavi, and L. Timms, *Hypoxia-inducible factor expression in human RPE cells.* The British journal of ophthalmology, 2007. **91**(10): p. 1406-10.
527. Vadlapatla, R.K., A.D. Vadlapudi, and A.K. Mitra, *Hypoxia-Inducible Factor-1 (HIF-1): A Potential Target for Intervention in Ocular Neovascular Diseases.* Current drug targets, 2013. **14**(8): p. 919-35.
528. Pore, N., et al., *Nelfinavir down-regulates hypoxia-inducible factor 1alpha and VEGF expression and increases tumor oxygenation: implications for radiotherapy.* Cancer research, 2006. **66**(18): p. 9252-9.

529. Jacot, J.L. and D. Sherris, *Potential Therapeutic Roles for Inhibition of the PI3K/Akt/mTOR Pathway in the Pathophysiology of Diabetic Retinopathy.* Journal of ophthalmology, 2011. **2011**: p. 589813.
530. Yang, X.M., et al., *Role of PI3K/Akt and MEK/ERK in mediating hypoxia-induced expression of HIF-1alpha and VEGF in laser-induced rat choroidal neovascularization.* Investigative ophthalmology & visual science, 2009. **50**(4): p. 1873-9.
531. Witmer, A.N., et al., *Vascular endothelial growth factors and angiogenesis in eye disease.* Progress in retinal and eye research, 2003. **22**(1): p. 1-29.
532. Carmeliet, P., *Mechanisms of angiogenesis and arteriogenesis.* Nature medicine, 2000. **6**(4): p. 389-95.

VITA

Ramya Krishna Vadlapatla was born on October 6th, 1986 in Vijayawada, Andhra Pradesh, India. She completed her secondary education from Nirmala High School at Vijayawada. Thereafter, she pursued her Bachelor's degree in Pharmaceutical Sciences from Andhra University College of Pharmaceutical Sciences, Visakhapatnam, Andhra Pradesh, India during 2004-2008. Ms. Vadlapatla joined the Department of Pharmaceutical Sciences at the University of Missouri-Kansas City School of Pharmacy in August 2008 in pursuit of a doctorate degree. Ms. Vadlapatla is a member of American Association of Pharmaceutical Scientists (AAPS), Association of Research in Vision and Ophthalmology (ARVO) and Pharmaceutical Sciences Graduate Student Association (PSGSA). She has been awarded the prestigious Chancellor's Doctoral Fellowship for academic excellence during 2013-14. She has also been awarded with Judith Hemberger Graduate Scholarship (2012 and 2011), Women's Council Graduate Assistant Fund (2013 and 2012) and Best Poster Award at UMKC Health Sciences Student Research Summit 2011. She also received travel award from AAPS Clinical Pharmacology and Translational Research section (2013, 2012 and 2011). During her graduate career, she has authored/co-authored more than 24 peer reviewed research and review articles in reputed international journals and 6 book chapters.