Enzyme and transporter-mediated drug-drug and herb-drug interactions of the antimalarials and HIV antiretrovirals

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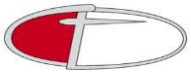

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Enzyme and transporter-mediated drug-drug and herb-drug interactions of the antimalarials and HIV antiretrovirals by Enoche Florence OGA

A Thesis submitted to the Graduate School of Pharmaceutical Sciences, Chiba University, Japan in partial fulfillment of the requirements for the Degree of a Doctor of Philosophy (Pharmaceutical Sciences)

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PAPERS DISCUSSED

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals assigned below:

I. **E F Oga**, S Sekine, Y Shitara and T Horie (2012). Potential P-glycoprotein mediated drug-drug interaction of some anti-malarials in Caco-2 cells. *American Journal of Tropical Medicine and Hygiene. 87 (1);64-69*

II. **E F Oga**, S Sekine, Y Shitara and T Horie (2012). P-glycoprotein mediated efflux in Caco-2 cell monolayers: the influence of herbals on digoxin transport. *Journal of Ethnopharmacology.* 144 (3); 612-617

III. **E F Oga**, S Sekine and T Horie. *Ex vivo* and *in vivo* investigations of extracts of *Vernonia amygdalina*, *Carica papaya* and *Tapinanthus sessilifolius* on digoxin transport and pharmacokinetics: Assessing the significance on p-glycoprotein efflux (*Drug Metabolism and Pharmacokinetics,* accepted manuscript in press)

IV. **E F Oga**, Y Shitara, S Sekine and T Horie. Inhibition of CYPP450 enzymes as a mechanism for drug-drug interactions between artemisinin and HIV antiretrovirals (manuscript in preparation)

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Enzyme and transporter-mediated drug-drug and herb-drug interactions of the antimalarials and HIV antiretrovirals
（抗マラリア薬、抗 HIV レトロウイルス薬による代謝酵素、トランスポーターを介した薬物間、ハーブ・薬物間相互作用に関する研究）
Enoche Florence OGA

ABSTRACT

Drug-drug interactions (DDIs) are a major cause of concern during drug treatment and may result in clinically relevant or sometimes serious adverse events. Besides conventional drug therapy, patients are increasingly utilizing herbal medicines, with a resultant increase in the incidence of herb-drug interactions (HDIs) on their concurrent use. These DDIs/HDIs may result in changed systemic exposure leading to variations in drug response of the co-administered drugs including diminished therapeutic effect or toxicity. Although DDIs involving the cytochrome P450 (CYP) enzymes are more widely recognized, drug transporters make a significant contribution to drug disposition, exerting their effect through pharmacokinetic alterations. Induction, inhibition and inactivation of CYP enzymes and transporters (especially P-glycoprotein; P-gp, an efflux transporter expressed on the apical membrane of intestinal epithelial cells) are the major pathways underlying drug–drug interactions. Malaria and HIV/AIDS have become enormous public health concerns and are known to share a wide overlap in their socio-economic and geographical areas of occurrence, routinely resulting in drug co-administration for their management. Also, because of their pandemicity and the need to treat concurrent ailments, multidrug therapy is common. However, there is a paucity of research on HDIs in malaria or DDIs in malaria and HIV/AIDS co-morbidity. In this regard, the study aimed at predicting the likelihood of encountering P-gp-mediated and CYP-based DDIs/HDIs during the drug management of malaria and HIV/AIDS. It made use of various *in vitro*, *ex vivo* and *in vivo* approaches which have their individual merits as well as limitations.

The study brings new light to the fact that altered therapeutic outcomes might be caused by DDIs between the antimalarials (amodiaquin and artesunate) and digoxin or other drugs that interact with P-gp. These interactions may be of clinical relevance given the high incidence of co-administration of P-gp substrate drugs with antimalarials. The HDI study shows that *Vernonia amygdalina* co-administration poses a more clinically significant risk for P-gp-mediated HDIs than *Carica papaya* or *Tapinanthus sessilifolius*. Importantly, significant *in vitro* P-gp inhibition may not always be replicated in animal models. The findings point to the need for comprehensive assessments of HDIs in order to obtain more detailed understanding of their impact in human subjects. This would ensure appropriate

clinical intervention on co-administration with P-gp substrate drugs, especially those with narrow therapeutic index. Finally, the influence of ritonavir and nevirapine on the CYP2B6 and -3A4 mediated inhibition of the metabolism of artemisinin is strongly suggestive of their DDI likelihood. However, an evaluation of their influence on CYP induction might be necessary to obtain their net effect on CYPs. The present study indicates the likelihood of P-gp and CYP –mediated DDIs/HDIs occurring in malaria and HIV/AIDS therapy, suggesting the need for caution in clinical instances. However, further in-depth clinical studies are recommended in order to obtain the precise therapeutic consequence.

Keywords

Artemisinin, Cytochrome P450, Drug-drug interactions, Drug-herb interactions, Drug metabolism, Drug transporters, HIV/AIDS, Malaria, P-glycoprotein, Pharmacokinetics

LIST OF ABBREVIATIONS

ABC, ATP binding cassette

ACT, artemisinin-based combination therapy

ADQ, amodiaquin

ANOVA, analysis of variance

ART, artesunate

ASN, artemisinin

AUC, area under the plasma concentration-time curve

BCRP, Breast cancer resistance protein

CAM, Complementary and alternative medicine

CP, *Carica papaya*

C_p, plasma concentration

C_{max}, maximum plasma concentration

CUR, *Curcuma longa*

CYP, cytochrome P450

Caco-2、human epithelial colorectal adenocarcinoma cells

DDI, drug-drug interaction

DIN, Drug interaction number (ratio of inhibitor dose to inhibition constant)

ER, efflux ratio

HAART, highly active antiretroviral therapy

HBSS, Hank's balanced salt solution

HDI, herb-drug interaction

HIM, human intestinal microsomes

HIV, human immunodeficiency virus

HT-29 cells, human colon adenocarcinoma cell line

K_a, absorption rate constant

K_{el}, elimination rate constant

K_m, Michaelis-Menten constant

KRBB, Krebs-Ringer bicarbonate buffer

LC-MS/MS, Liquid chromatography tandem mass spectroscopy

LG, *Cymbopogon citratus* (Lemon grass)

LUM, lumefantrine

MATE, Multidrug and toxic compound extrusion

MDCK, Madin-Darby canine kidney cells

ML, *Tapinanthus sessilifolius* (mistletoe leaves)

MLB, *Morinda lucida*

MRP Multidrug-associated resistance protein

NL, *Azadiractha indica* (Neem leaves)

NLF, nelfinavir

NNRTI, non-nucleoside reverse transcriptase inhibitor

NVP, nevirapine

OATP, Organic anion transporting polypeptide

P_{app}, Apparent permeability coefficient

P-gp, Permeability glycoprotein

PEPT1, Peptide transporter 1

PI, anti-HIV protease inhibitor

QD, quinidine

RTV, ritonavir

SEM, standard error of mean

SLC, solute carrier

SQV, saquinavir

T84 cells, cells derived by lung metastasis of a human colon carcinoma

TEER, Trans-epithelial electrical resistance

VA, *Vernonia amygdalina* (Bitter leaves)

VER, verapamil

V_{max}, maximal velocity

pHLM, pooled human liver microsomes

AIMS OF THE THESIS

- To investigate the potential of certain antimalarial drugs to undergo intestinal P-gp-related DDIs when co-administered with agents that are transported by the glycoprotein, using rhodamine-123, fexofenadine and digoxin as probe P-gp substrates (PAPER I)
- To examine the interaction of selected herbal extracts with digoxin with the view to predict the potential of P-glycoprotein (P-gp) mediated herb-drug interactions occurring with P-gp substrate drugs using an *in vitro* model (Caco-2 cell monolayer), an *ex vivo* (Ussing chamber) and an *in vivo* (Sprague Dawley rats) model (PAPERS II and III)
- To predict the likelihood of encountering clinically significant cytochrome-based drug-drug interactions through CYP inhibition, when selected HIV antiretrovirals are co-administered with artemisinin in co-morbid malaria and HIV (PAPER IV)

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Malaria

1.1.1 Pathophysiology and epidemiology

Malaria is an enormous public health concern, with approximately one-half of the world's population currently at risk. Globally, about 250 million people are infected with malaria. Although malaria is a preventable and treatable disease, it has a high mortality rate being responsible for an estimated 655 000 malaria deaths in 2010 [1]. As illustrated in Figure 1A, sub-Saharan Africa accounts for the majority of cases, whereas Asia, Latin America, and some parts of the Middle East and Europe are also affected. About 86 % of the victims of malaria deaths globally were of children under 5 years of age [2, 3]. Apart from its impact on the health of the populace, malaria imposes a heavy economic burden on individuals and entire economies [4-6] . Malaria is an infectious disease whose parasite is transmitted through the bites of infected *Anopheles* mosquitoes. Malaria is mainly caused by the Plasmodium species; *P. vivax, P. ovale, P. malariae and P. falciparum*. However, most cases of severe malaria and related mortality are caused by *P. falciparum* infection [7, 8]. There are reports of complications occurring in non-falciparum infections as well including severe infection and even deaths reported following infections with *P. vivax* and *P. knowlesi* [9-11].

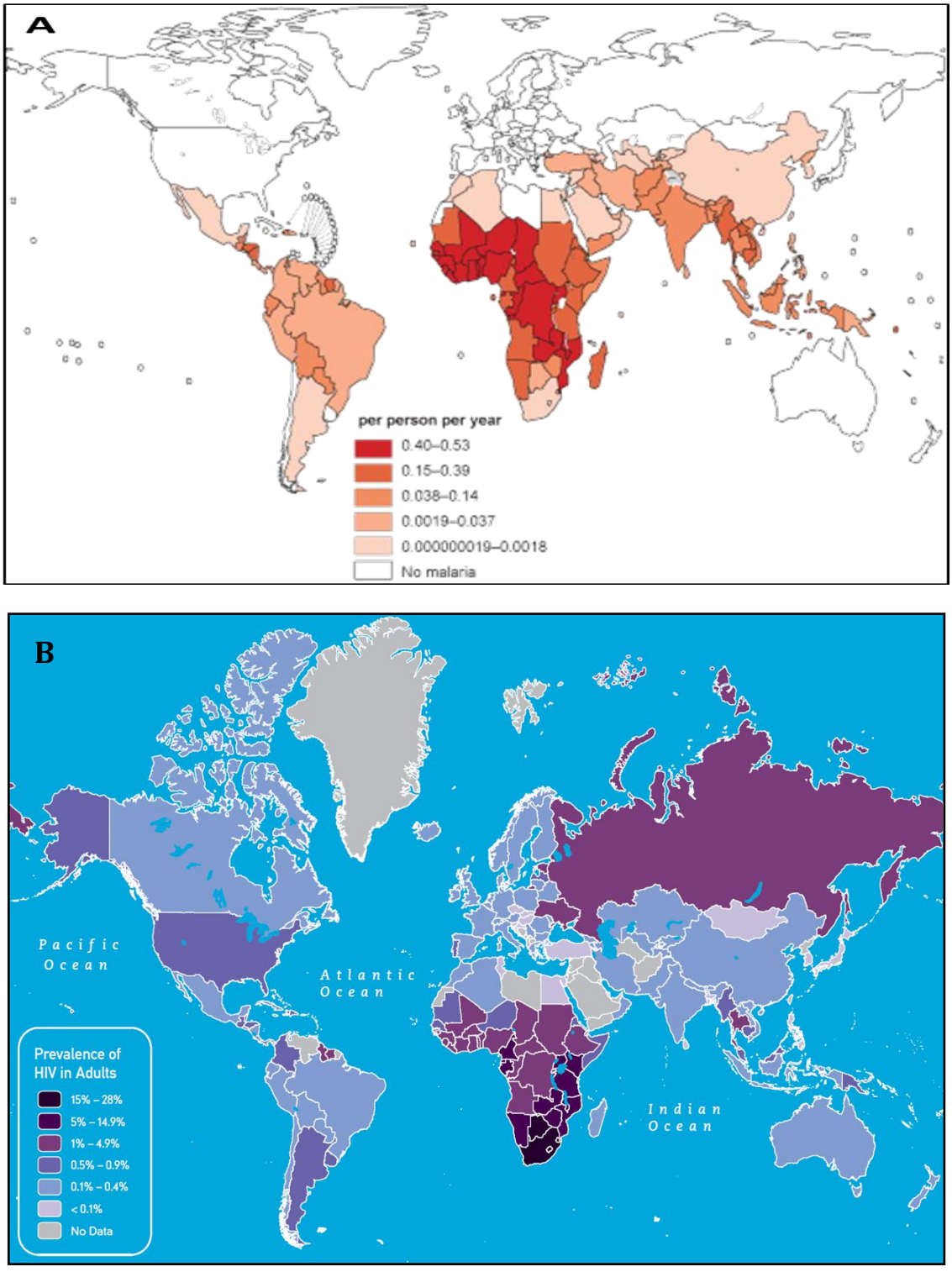

Figure 1. A) Adult prevalence of malaria B) Global HIV adult prevalence rate

Source; [12, 13)]

1.1.2 Conventional Malaria management

Despite the major advances made, malaria is still regarded as a major global infectious disease, with a high pediatric mortality toll, especially in the developing world [1]. Conventional medicines are a central tool for its management. However, other measures are also incorporated. Effective antimalarial treatment includes the artemisinin-based combination therapy (ACTs) which form the WHO recommended standard treatment for uncomplicated malaria in combination with other antimalarials. These artemisinin compounds include artemisinin, artesunate, artemether, arteether and dihydroartemisinin. Other antimalarial drug classes include the 4-aminoquinolines (chloroquine, amodiaquin), arylaminoalcohols (halofantrine, lumefantrine, mefloquine, quinine), 8-aminoquinolines (tafenoquine, primaquin), antifolates (sulphadoxine, dapsone, pyrimethamine, proguanil, chlorproguanil), respiratory chain inhibitors (atovaquone which is commonly combined with proguanil) and antibiotics (azithromycin, doxycycline, clindamycin) [14]. Because ACTs are the first-line antimalarial treatment, clinical antimalarial drugs are usually a combination spanning several of these drug classes such as; artemether/lumefantrine, artesunate/amodiaquin, artesunate/mefloquine, artesunate/sulphadoxin-pyrimethamine, dihydroartemisinin/piperaquine, dapsone/chlorproguanil/artesunate, pyronaridine/artesunate etc. Newer antimalarial drug classes are evolving including the spiroindolones and the imidazolopiperizines [15, 16]. Intermittent preventive treatment is widely used among pregnant women, who are a special population easily susceptible to malaria [17]. This measure provides pregnant women with at least two doses of an anti-malarial drug (usually

sulphadoxine-pyrimethamine), at each scheduled antenatal visit after the first trimester, whether they show symptoms of infection with malaria or not [18]. This preventive treatment has been shown to substantially reduce the risk of anaemia in the mother and low birth weight in the newborn [19]. Several non-drug measures are also being utilized in the management of malaria. Insecticide-treated bed nets (ITNs) are now a major intervention for the control of malaria [20]. They are a form of personal protection that has been shown to reduce malaria illness, severe disease and death due to malaria in endemic regions. They vary according to the net materials and the specific insecticides embedded. Vector control through indoor residual spraying interventions and measures that target the larval stages of the mosquito are used a common control intervention.

1.2 HIV/AIDS

Around the world, the HIV epidemic rages on, emerging the greatest challenge in global health. However, AIDS-related deaths are decreasing largely due to increased access to treatment. By the end of 2011, 34 million people were living with HIV globally (Figure 1B). 0.8% of adults aged 15-49 years worldwide are living with HIV, although the burden of the epidemic differs between countries and regions [13]. HIV/AIDS is a life-long condition and antiretroviral drug therapy is typically used. Table 1 illustrates the classification of currently used antiretroviral agents. Because standard treatment for HIV/AIDS comprises a combination or different classes of antiretrovirals, there is a higher likelihood of encountering DDIs. Table 2 illustrates some established and potential drug-drug interactions with HIV antiretrovirals

Table 1. Main classes of HIV antiretrovirals

	Class	Examples	Mechanism of action	Reference
1	Nucleoside Reverse Transcriptase inhibitors	Abacavir, Didanosine, Lamivudine, Stavudine, Tenofovir, Zalcitabine, Zidovudine	They interrupt HIV replication cycle through competitive inhibition of HIV reverse transcriptase and termination of the DNA chain	21)
2	Non-nucleoside Reverse Transcriptase inhibitors	Delavirdine, Efavirenz, Etravirine, Nevirapine	They noncompetitively bind the p66 subunit at a hydrophobic pocket distant from the active site of the enzyme, inducing a conformational change in the enzyme that alters the active site and limits its activity	22)
3	Protease Inhibitors	Indinavir, Nelfinavir, Ritonavir, Saquinavir, Atazanavir, Darunavir, Lopinavir, Tipranavir, Fosamprenavir	HIV protease inhibitors function as competitive inhibitors that directly bind to HIV protease and prevent subsequent cleavage of polypeptides.	23)
4	Integrase inhibitors	Raltegravir, Elvitegravir	They block the integrase enzyme which is used to integrate genetic material of the virus into the host cell of the target	24)
5	Fusion/Entry inhibitors	Enfuvirtide	Fusion inhibitors act extracellularly to prevent the fusion of HIV to the CD4 or other target cell.	25)
6	Chemokine receptor antagonists	Maraviroc	Selectively and reversibly binds the CCR5 chemokine co-receptor, blocking the V3 loop interaction and inhibiting fusion of the cellular membranes	26)

Table 2. Established and potential drug-drug interactions with HIV antiretrovirals

ARV	Co-administered drug	Effect on ARV/co-administered drug	Mechanism	Reference
Ritonavir	Digoxin	↑ in AUC and ↓ oral clearance of digoxin	Inhibition of P-gp	27)
Lopinavir/ ritonavir	Rosuvastatin	2-fold ↑ in rosuvastatin's AUC, 5-fold ↑ in C_{max}	Inhibition of OATP1B1	28, 29)
Tipranavir/ ritonavir	Atorvastatin	9-fold ↑ in $AUC_{0-\infty}$ and C_{max} of atorvastatin	Inhibition of OATP1B1	30)
	Rosuvastatin	↑ of 37 and 123 % in $AUC_{0-\infty}$ and C_{max} of rosuvastatin	Inhibition of OATP1B1 and BCRP	30)
Atazanavir/ ritonavir	Telaprevir	20 % ↓ in AUC and 15% ↓ in C_{min} of telaprevir 17% ↑ in AUC and 85% ↑ in C_{min} of atazanavir	Modulation of P-gp and CYP3A4	31)
Ritonavir	Quinine	3.4-fold ↑ in AUC, 2.8-fold ↑ in C_{max} of quinine	Modulation of P-gp and CYP3A4	32)
Nevirapine	Quinine	↓ of 33.4, 36 and 24.8% in AUC, Cmax and $T_{½}$, respectively of quinine 33.3 % ↑ in oral clearance of quinine	Modulation of CYP3A4	33)
Nevirapine	Artemether /lumefantrine	Nevirapine significantly induces their CYP metabolism	Modulation of CYP3A4 metabolism of the antimalarials	32)
Didanosine	Quinolones	↓ absorption of didanosine	Chelation reaction	34)
Atazanavir	Bosentan	↓ plasma concentration of atazanavir	Modulation of CYP3A4	35)
Lopinavir /Ritonavir	Bosentan	↑ bosentan concentration of up to 48-fold	Modulation of CYP3A4	36, 37)
Saquinavir	Ketoconazole	Significant ↑ in AUC, Plasma concentration and $T_{½}$ of saquinavir by 37, 94 and 38% respectively	Modulation of P-gp and CYP3A4	38)

ARV	Co-administered drug	Effect on ARV/co-administered drug	Mechanism	Reference
Ritonavir	Ketoconazole	Significant ↑ in AUC, C_p and $T_{½}$ of ritonavir by 29, 62 and 31% respectively	Modulation of P-gp and CYP3A4	38)
Delaviridine	Rifabutin	50-60 % ↓ in C_p of delaviridine	Induction of CYP metabolism	39, 40)
Nevirapine	Rifampicin	37% ↓in C_p of nevirapine		
	Rifabutin	16% ↓in C_p of nevirapine		
Saquinavir	Rifampicin	80% ↓in AUC of saquinavir		
Nelfinavir	Rifampicin	82% ↓in AUC of nelfinavir		
Amprenavir	Rifampicin	81% ↓in AUC of amprenavir		
Indinavir	Bosentan	↑ clearance and ↓ drug concentrations and loss of therapeutic efficacy of indinavir	CYP3A4 and CYP2C9 inductive effects of bosentan	41)
Indinavir	Carbamazepine	↓ indinavir C_p to 4–25% of mean population values	Induction of CYP metabolism	42)
Ritonavir	Carbamazepine	70–85% ↑ in serum carbamazepine levels	Inhibition of CYP metabolism	43, 44)
Indinavir	St Johns wort	↓ in indinavir AUC by 57%	Induction of CYP metabolism	45)
Nelfinavir	Ethinyl estradiol	47, 28 and 62 % ↓ in AUC, C_{max} and C_{min} of ethinyl estradiol	Induction of CYP metabolism	46)
Ritonavir	Ethinyl estradiol	41 and 32% ↓ in ethinyl estradiol AUC and C_{max} 31% ↑ in terminal rate constant	Induction of CYP metabolism	47, 48)
Tenofovir	Amphotericin B	↑ in nephrotoxicity	Synergy and additive nephrotoxic effect of the individual drugs	49)
Ritonavir	Docetaxel	↓ in plasma clearance of Docetaxel ↑ in severe docetaxel-related toxicity	Ritonavir-induced inhibition of CYP3A4	50-52)

ARV	Co-administered drug	Effect on ARV/co-administered drug	Mechanism	Reference
Nelfinavir	Phenytoin	29, 21 and 39 % ↓ in AUC, C_{max} and C_{min} of phenytoin	Induction of CYP enzymes	53, 54)
Efavirenz	Ketoconazole	72 % ↓ in ketoconazole C_p	Efavirenz and ketoconazole act as respective inducer and inhibitor of CYP3A4	49, 55, 56)
Nelfinavir	Omeprazole	36, 37 and 39 % ↓ in AUC, C_{max} and C_{min} of omeprazole		53, 57)
Nelfinavir	Atorvastatin	74, 122 and 39 % ↑ in AUC, C_{max} and C_{min} of atorvastatin	Inhibition of CYP3A4 by nelfinavir	53, 58)
Nelfinavir	Simvastatin	505 and 517 % ↑ in AUC and C_{max} of simvastatin	Inhibition of CYP3A4 by nelfinavir	53, 58, 59)
Nevirapine	Ketoconazole	63 % ↓ in ketoconazole C_p		49)
Efavirenz	Bexarotene	↓ AUC of efavirenz 50% ↓ in AUC of bexarotene	Induction of CYP3A and P-gp by bexarotene	60)

1.3 Co-morbidity of Malaria and HIV

Together malaria and HIV/AIDS cause more than 4 million deaths each year [61]. There is increasing evidence that where they occur together, malaria and HIV infections interact. Malaria worsens HIV by increasing viral load in adults and pregnant women; possibly accelerating progression to AIDS; and potentially increasing the risk of HIV transmission between adults, and between a mother and her child. In adults with low CD4 cell counts and pregnant women, HIV infection appears to make malaria worse. Because of the wide overlap of the regions in which these diseases occur, several HIV patients take antimalarials either as prophylaxis or for treatment. This increases the likelihood of interactions with drugs used in their management.

1.4 General overview of herbal medicine use

The use of complementary and alternative medicines (CAM) is becoming very popular with a widespread increase in its interest and reports of over 40 % of the adult population utilizing it. In several cases, complementary medicine is used in addition to conventional medicine. Therefore there is an increased tendency for DDIs to occur between conventional medicine and the herbal medicines. The scope of CAM is wide encompassing naturopathy, ancient healing systems, homeopathy, mind-body medicines and biologically based practices. Herbal medicine comprises the use of plant parts including the seeds, stems, rhizomes, berries, roots, leaves, bark, or flowers for medicinal purposes. Unlike conventional drugs, most herbal remedies are a complex mixture of chemical constituents whose bioactive components in many instances have not been fully characterized, but have been reported to be medicinally efficacious [62]. There is a long history of the use of

phytomedicines, with herbalism having a long tradition of use outside of conventional medicine. Their use has become more established as improvements in analysis and quality control as well as advances in clinical research has shown the value of herbal medicine in treating and preventing disease [63]. In some developing countries, up to 80 % of the indigenous populace depend on traditional systems of medicine and medicinal plants as their primary source of healthcare [64, 65]. Reports show that over 70% of German physicians prescribe phytomedicines and over 75% of the German populace have used complementary or natural medicine [66]. In countries like the United States, an increase in its use has been reported due to dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies [67]. Likewise, plant-based medicines are used in several African countries because of their low cost, perceived safety and efficacy and lower incidence of adverse effects when compared to orthodox medicines [68]. About 148 kinds of Japanese herbal medicines (Kampo medicines) have been approved and are enlisted on the National Health Insurance Drug Tariff. These medicines are reportedly prescribed by 72-78% of Japanese physicians [69, 70]. While herbal medicine use is on the increase in some countries, stringent regulatory requirements in other countries make it difficult to register herbal medicines, therefore their reported limited use [71]. For example in the United Kingdom, industrial herbal preparations are required to conform to the same regulations as conventional medicines, hindering license acquisition for numerous herbals [72]. Several conventional medicines in use evolved from medicinal plant research from where they are synthesized as pharmaceutical preparations. Of note are; vincristine (*Catharanthus roseus*), atropine (*Atropa belladonna*), quinine (*Cinchona*

sp.), emetine (*Cephaelis ipecacuanha*), digitoxin and digoxin (*Digitalis purpurea*), ephedrine (*Ephedra sinica*), artemisinin (*artemisia annua*), reserpine (*Rauwolfia serpentina*), etoposide (*Podophyllum peltatum*) among several others.

1.4.1 Herbal medicine use and interactions in Malaria and HIV/AIDS

Malaria is an important global public health issue with high morbidity and mortality. Majority of malarial endemic regions are from the world's developing economies. As a result of the relatively high costs for conventional antimalarials, many patients are known to take herbal remedies for its prevention and treatment [73]. Herbal remedies commonly used in the management of malaria include *Vernonia amygdalina, Piper longum, Tapinanthus sessilifolius, Carica papaya*, *Azadiractha indica, Curcuma longa, Echinacea purpurea* and *Phyllanthus niruri*. Sometimes these herbs are used alone or in combination with orthodox medicines, with reports on the wide use of herbal remedies in the management of malaria [74-78]. For instance, in certain communities, extracts from plants may be used, together with chloroquine, for the treatment of malaria [79]. Acupuncture has also been reported to be a means of managing malaria offering a relatively cheap and simple treatment method without side-effects [80]. Our study based on a human Caco-2 model revealed that extracts of *Vernonia amygdalina, Tapinanthus sessilifolius* and *Carica papaya* which are herbals commonly used in traditional malaria therapy significantly inhibited P-gp mediated digoxin transport [81]. Findings from that study suggested that caution should be observed when those herbs are concomitantly used with P-gp substrate drugs as they may enhance their absorptive transport [81]. Frequently, antagonistic effects have been reported on co-administration of herbals and orthodox medicines. For instance, although the

leaves of *Carica papaya* are known to exhibit antimalarial effect, the antagonistic antimalarial properties when used in combination with artesunic acid in *Plasmodium berghei*-infected mice has been reported [82]. In the study, the extracts of *Carica papaya* when solely administered had good antimalarial activity. In a similar study, the influence of *Eurycoma longifolia* extract (a herbal remedy commonly used in malaria therapy) was investigated on co-administration with artemisinin (WHO's recommended first-line antimalarial) in experimental mice. Findings from that study revealed the suppression of parasitemia in *Plasmodium yoelii*-infected mice. This is suggestive of a promising, potential antimalarial candidate by both oral and subcutaneous routes [83]. Similar synergistic herb-drug interactions involving goniothalamin (*Goniothalamus scortechinii)*-chloroquine, *Vernonia amygdalina*-chloroquine and curcumin (*Curcuma longa*)-artemisinin combinations have been documented with the suggestion that they be considered for future trials in the search for malaria combination therapy [84-86]. Also, herbs can be combined for their additive effect as shown by the mixture of a *Khaya ivorensis -Alstonia boonei* extract mixture as an antimalarial prophylactic remedy [87].

Numerous HIV/AIDS patients look towards complementary and alternative medicine, and are known to take herbal medicines in order to boost immunity, treat complications, or ease side effects from HIV drugs [88]. However, it is of importance to thoroughly investigate the potential alterations in pharmacokinetic and toxicological profiles when co-administration occurs as their concurrent use has resulted in both beneficial and detrimental effects. Their use varies across geographical and economic regions, with patients being more likely to incorporate traditional and conventional medicine (when available) in areas where there is

limited access to these antiretrovirals [89]. However, these medicines may not be completely safe as some detrimental herb-drug interactions involving the antiretrovirals have been reported. In addition, because the current treatment for HIV/AIDS comprises a cocktail of individual antiretroviral drug therapies, complicated interactions may occur. For instance, St Johns wort and garlic are known to interfere with HIV medications. Several other herbal medicines are used by HIV/AIDS patients including *Hypoxis hemerocallidea* (African potato), *Sutherlandia frutescens, Allium sativum*, *Eucalyptus globulus, Aloe vera, Trigonostema xyphophylloides, Vatica astrotricha*, *Vernonia amygdalina, Lippia javanica, Bidens pilosa, Peltoforum africanum, Hypoxis hemerocallidea* and *Moringa oleifera* [90-93]. However, the likelihood of their interactions occurring with conventional medicines has not been fully elucidated. For instance, *Sutherlandia frutescens* has been reported to inhibit the metabolism of atazanavir in human liver microsomes which may have important implications on the absorption and metabolism and the overall oral bioavailability of atazanavir [94]. Also the protease inhibitor; ritonavir on co-administration with some herbal components has been shown to modulate P-gp as well as CYP 3A4. In particular, the herbal constituents; kaempferol and quercetin (from several plants), hypericin (*Hypericum perforatum*) and allicin (*Allium sativum*) inhibit the efflux and CYP3A4-mediated metabolism of xenobiotics *in vitro* and may interact with HIV antiretrovirals that are P-gp or CYP 3A4 substrates, such as ritonavir [95, 96]. St. John's wort has been reported to potently alter the pharmacokinetics of indinavir (a protease inhibitor and a known CYP450 substrate) via induction of CYP 3A by St. John's wort. A study revealed a large reduction in indinavir concentrations by concomitant St John's wort. This finding

has important clinical implications for HIV-infected patients receiving the two agents since low plasma concentrations of protease inhibitors are a cause of antiretroviral resistance and treatment failure [45]. Garlic is another herbal remedy commonly utilized by HIV/AIDS patients. Although some studies have shown that garlic has the potential to induce CYP isoenzymes, consequently reducing the effectiveness of antiretroviral drugs, *in vitro* assessments of its effect on CYP450 are conflicting [97, 98]. In a clinical study, garlic was shown to reduce the plasma pharmacokinetic concentrations of saquinavir by altering CYP3A4 isoform of the CYP450 enzyme system, the isozyme through which saquinavir is metabolized recommending patient caution on concomitant administration [99]. Table 3 illustrates some theoretical and clinically relevant HDIs involving the antimalarials and HIV antiretrovirals.

Table 3. Herb-drug interactions involving the antimalarials and HIV antiretrovirals

S/No.	Indication	Conventional drug	Herbal medicine	Effect	Reference
1	Malaria	Chloroquine Halofantrine	*Khaya grandifoliola*	Enhanced antiplasmodial effect in infected mice Significant ↑ in parasite clearance	100)
		Quinine Mefloquine	Artemisinin from *Artemisia annua*	Synergistic effect in an *in vitro* assay	101)
2	HIV/AIDS	Saquinavir	Garlic (*Allium sativum*)	Significantly decreases the systemic exposure in humans ↓ mean saquinavir AUC by 51% ↓ C_{max} by 54%	45)
		Indinavir	St. Johns wort	↓ AUC_{0-8} of indinavir decreased by a mean of 57% in humans 49-99% ↓ in concentration 8 hr post-dosing ↓ in the mean C_{max} of indinavir from 12·3μg/mL to 8·9 μg/mL	99)
		Nevirapine	St. Johns wort	Clearance of nevirapine ↑ by 35% # in humans	102)
		Protease inhibitors	SP-303 (an extract of *Croton lechler*)	Significant reduction of stool weight and stool frequency in AIDS patients who had diarrhea	103)

1.5 Enzyme and Transporter-mediated drug interaction

1.5.1 Xenobiotic Transporters

Drug transporters are expressed in various tissues and play a significant role in the influx and efflux of drugs, xenobiotics and toxins across cells. They influence drug disposition by modulating their pharmacokinetics [104]. Several studies have focused on the identification and characterization of drug transporters with regards to their expression in tissues, mechanisms of transport, characterization of their substrates and inhibitors, species differences, and genetic polymorphisms [105-107]. The ATP-binding cassette (ABC) and solute carrier (SLC) transporters are the main divisions of drug transporters. The ABC superfamily comprises the largest class of transporters. These transporters use the binding and hydrolysis of ATP to power the translocation of a vast substrate range across membranes [108]. Clinically relevant ABC transporters include breast cancer resistance protein, BCRP (ABCG2); bile salt export pump, BSEP (ABCB11); P-glycoprotein, P-gp/multidrug resistant protein 1, MDR1 (ABCB1); multidrug resistant protein 3, MDR3 (ABCB4); multidrug resistance-associated protein 2, MRP2 (ABCC2); multidrug resistance-associated protein 3, MRP3 (ABCC3) and multidrug resistance-associated protein 4, MRP4 (ABCC4) [28]. The SLC superfamily consists of major uptake transporters responsible for xenobiotic transport and includes organic anion–transporting polypeptide 1B1, OATP1B1 (SLCO1B1); organic anion–transporting polypeptide 1B3, OATP1B3 (SLCO1B3); organic anion transporter 1, OAT1 (SLC22A6); organic anion transporter 3, OAT3 (SLC22A8); organic cation transporter 2, OCT2 (SLC22A2); organic anion–transporting polypeptide 1A2, OATP1A2 (SLCO1A2); organic anion–transporting polypeptide 2B1, OATP2B1 (SLCO2B1); organic cation transporter 1, OCT1 (SLC22A1); Peptide transporter 1, PEPT1 (SLC15A1); Peptide

transporter 2, PEPT2 (SLC15A2); multidrug and toxin extrusions 1, MATE1 (SLC47A1) and multidrug and toxin extrusions 2, MATE2-K (SLC47A2) [109, 110]. ATP-binding cassette transporters are known to function as barrier proteins extruding toxins and xenobiotics out of cells. Of these, P-glycoprotein (P-gp) is an efflux transporter expressed on barrier epithelia including the intestine, kidney, liver and blood-brain barrier where it mediates transport across cell membranes [111]. P-gp is the transporter of interest in this study. It is a determinant in the pharmacokinetics, efficacy and toxicity of xenobiotics. Figure 2 illustrates the localization of transporters in the intestinal epithelial cells, hepatocytes and renal tubular cells, which are in the main organs influencing drug disposition. With specific reference to the antimalarials, several transporters have been implicated in the disposition of some of them. Mefloquine has been shown to inhibit the function of P-glycoprotein in drug-resistant individuals [112]. In mediating the final excretion step of cationic drugs into bile and urine, of particular importance are the Multidrug and toxin extrusion (MATE; SLC47A) proteins which mediate the translocation of organic cations in exchange with protons across membranes proteins. These transporters are known to influence the disposition of several drugs including the antimalarial drugs; chloroquine and quinine [113]. Also, the antimalarial agent pyrimethamine is a potent MATE1 and MATE2-K inhibitor [114].

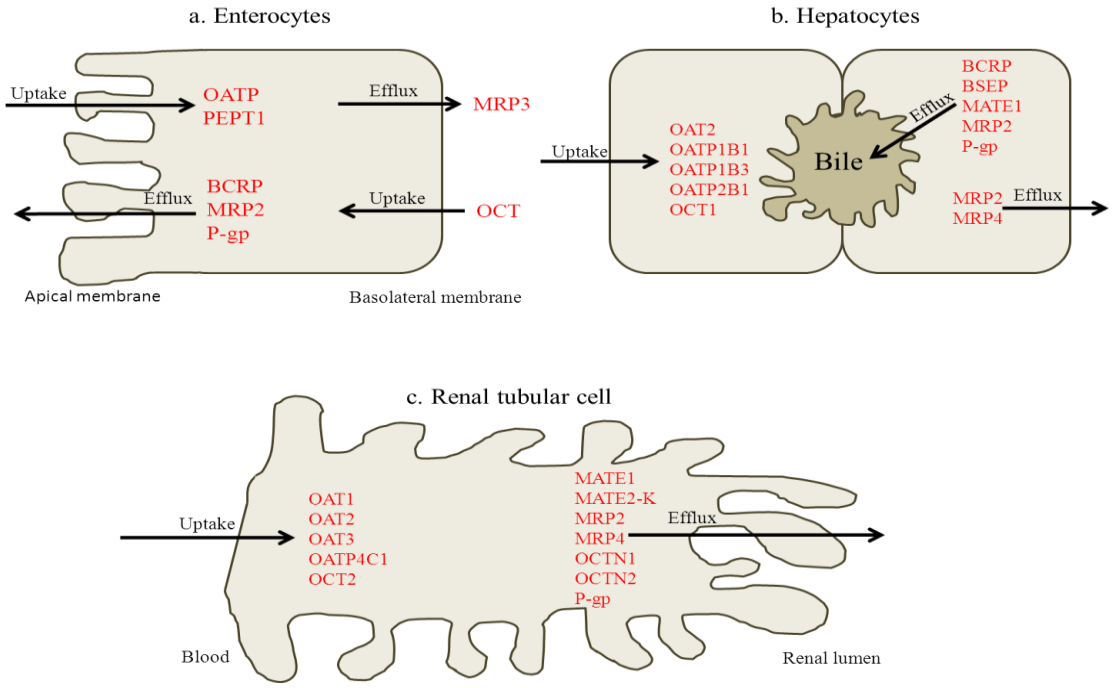

Figure 2. Localization of transporters in major drug disposition organs

1.5.2 Cytochrome P450 enzymes

Cytochrome P450 is a large and diverse group of isozymes responsible for the catalysis and biotransformation of several drugs. They perform this function by oxidizing, hydrolyzing or reducing the drugs, enabling conjugation enzymes to attach polar groups to make the metabolites water soluble easy excretion. Of the CYP isoforms, CYP3A4 is the most abundant. There is increasing evidence that the CYP isozymes contribute to the variability in drug response (incomplete cure, relapse, or resistance) or toxicity experienced with antimalarial drugs [115]. As instances, there is a clear association between concentrations of proguanil within plasma and certain genetic polymorphisms of CYP2C19 [116], and genetically established levels of CYP2C8 might have important clinical implications in the toxicity of amodiaquin [117, 118]. Variation in the expression of drug-metabolizing enzymes

and transport proteins affects the pharmacology of antimalarial drugs. Exploration of pharmacogenetics might help to optimize the use of antimalarial drugs.

1.5.3 Influence of transcriptional regulators

Several CYPs and transporters that influence drug disposition can be induced by xenobiotics and herbs [119]. The nuclear receptors; pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) which are present in the small intestine and liver have emerged as transcriptional regulators of cytochrome P450 isoenzymes (especially CYP 3A, -2B6, -2C8, -2C9 and -2C19) and drug transporters: P-gp, MRP2 and OATPs [120, 121]. Besides the various xenobiotics that have been reported to activate PXR, herbals including St. Johns Wort are known to potently induce it [122]. These nuclear receptors also enhance the expression of phase II conjugating enzymes like UDP-glucuronosyl transferase, sulfotransferase and glutathione-S-transferase enzymes [123, 124]. As a result of an increased induction of these nuclear receptors, there is an enhanced expression of P-gp and CYP enzymes which are likely to reduce the rate of absorption and increase the rate of elimination of drug substrates.

1.6 Models of drug absorption and transport

The intestine plays a vital role in the absorption of xenobiotics including drugs and toxins. In the intestine, uptake transporters including OATPs and PEPT1 are involved in drug absorption whereas P-gp, BCRP and MRPs function as efflux transporters. Co-administered drugs may inhibit or induce these transporters. After oral administration, the inhibitor concentration in the intestine can be much higher than that in systemic circulation. This may cause DDIs in the intestine rather than

in the liver on oral administration even at therapeutic doses [125]. Several methods for predicting the likelihood of occurrence of intestinal enzyme and transporter-mediated DDIs have been suggested [126-128]. Figure 3 depicts the main transport mechanisms across the intestinal wall. Several transport models have been developed which simulate the different transport mechanisms. These experimental models of permeability routinely utilize cell monolayers, artificial membranes (e.g. parallel artificial membrane permeability assay), excised tissues and animals. These studies are of vital importance especially in drug discovery and development as they allow prediction of *in vivo* intestinal absorption [129]. Different assay techniques, variability in animal species, tissue sources, and cell types usually give differing correlation between the measured human effective permeability values and the extent of absorption of drugs in humans determined by pharmacokinetic studies [129, 130]

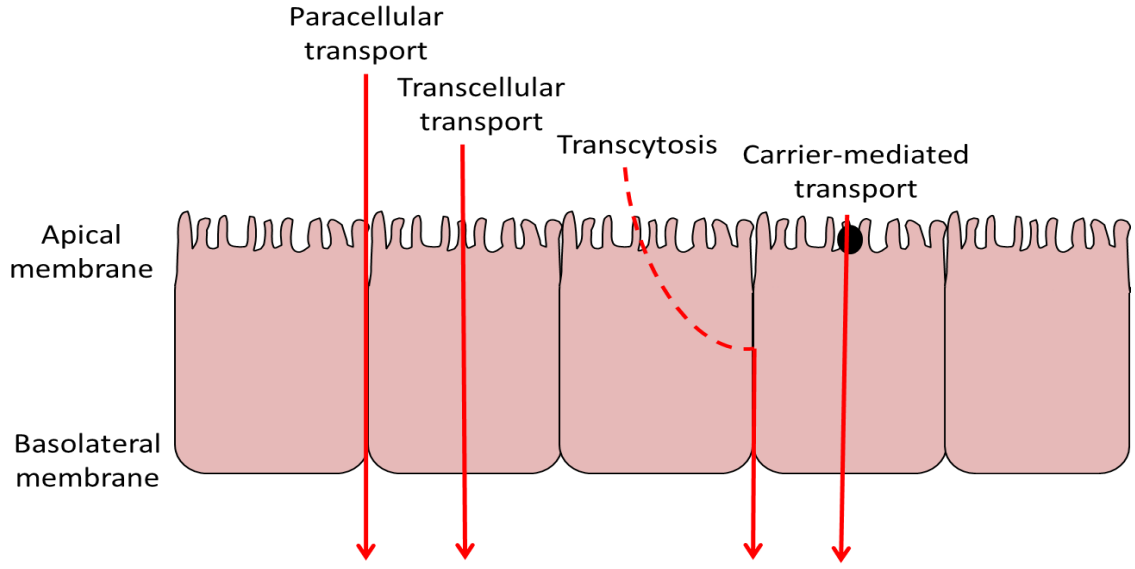

Figure 3. Transport mechanisms across intestinal epithelial cells

1.6.1 Cell monolayers

Although several cell monolayers (MDCK, HT-29, T-84 and Caco-2) can be used to study intestinal absorption as well as other functions of differentiated intestinal cells, Caco-2 cell monolayers are a widely accepted model for such studies including P-gp investigation [131, 132]. The human intestinal Caco-2 cell line has been extensively used over the last twenty years as a model of the intestinal barrier [133]. Although they originated from human colon adenocarcinoma cells, they closely resemble intestinal epithelial cells as demonstrated by the formation of a polarized monolayer, tight junctions, well-defined brush border on the apical surface [134]. As illustrated in Figure 4, because of the Transwell™ inserts cultured with Caco-2 cells closely resemble *in vivo* intestinal conditions, Caco-2 cells are widely used to predict drug absorption patterns because they retain several morphological and functional properties of enterocytes [135].

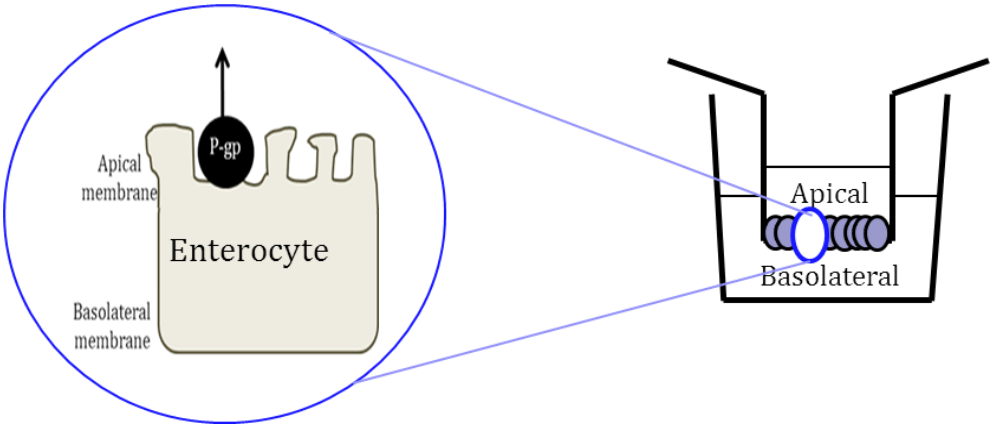

Figure 4. P-gp localization in the enterocyte and Caco-2 cell monolayer
An illustration of the similarity in P-gp expression between a Caco-2 cell monolayer grown on a Transwell insert and the intestinal epithelia

However, drug transporter expression and the tightness of tight junctions may differ between Caco-2 cells and the human intestine, which may affect drug permeability. [136] . Several permeability assays have been conducted for predicting the *in vivo* absorption of drugs across the intestinal wall by measuring the rate of transport of a compound using the Caco-2 cell model [137, 138]. In this, bidirectional studies of transporter interactions across the cell monolayer (apical-to-basolateral and basolateral-to-apical) can be assessed as illustrated in Figure 5. This enables the computation of the efflux ratio which is an indicator as to whether a compound undergoes active efflux. Caco-2 monolayers show good expression of transporters, including phase I and phase II enzymes. However, CYP3A, which is present in almost all intestinal cells, is very weakly expressed in Caco-2 cells [134]. P-gp is the transporter most studied using Caco-2 cells. Most P-gp studies utilize digoxin or rhodamine-123 as probe substrate. Verapamil, cyclosporin A or quinidine are commonly included as inhibitors to identify whether active transport is mediated by P-gp [139].

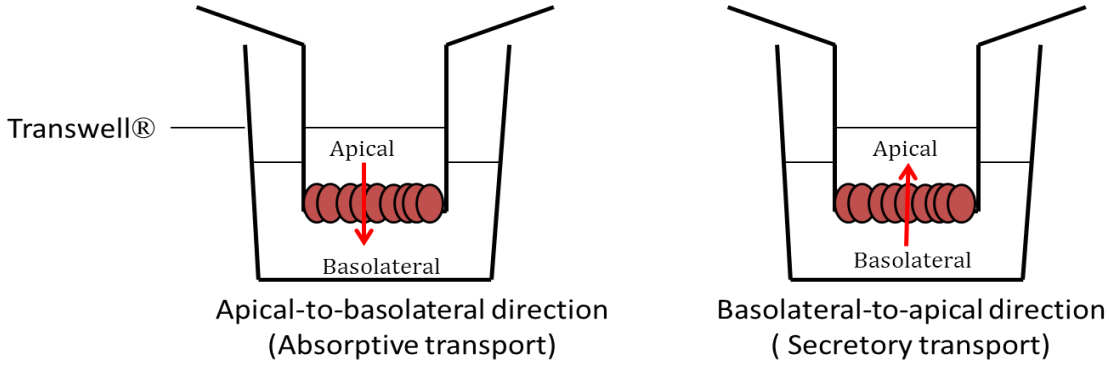

Figure 5. Techniques for transporter interactions in Caco-2 cell monolayers

1.6.2 Membrane vesicles expressing transporter cDNA

ABC transporters transport substrates across the cell membrane against their concentration gradient. These transporters include BCRP, BSEP, MRP1, MRP2, MRP3, MRP4 and P-gp. In order for this transport to be achieved, ATP hydrolysis is needed as an energy source. Inside-out vesicles are now commonly being used to study of the interaction of drugs with the transporter of interest. As depicted in Figure 6, these vesicular assays enable the evaluation of transporter activity by measuring transport of drug substrates from the reaction medium into the vesicles [140]. The drugs can then be quantified by fluorescence, liquid scintillation counting or chromatographic analysis.

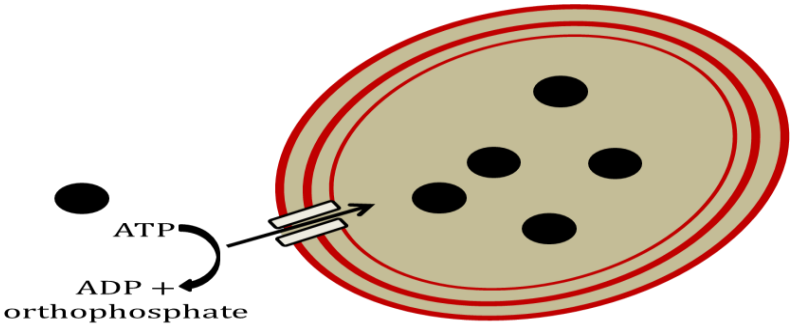

Figure 6. An inside-out vesicle

1.6.2 Artificial membranes

Artificial membranes are used as a model for intestinal permeability as they are high throughput, reproducible and low cost alternative for *in vitro* assessment [141]. The immobilized artificial membrane (IAM) and parallel artificial membrane permeation assay (PAMPA) are the commonly used models. The lack of enzymes, a lipid bilayer, transporters and active transport pathways are their main limitation. In addition, the UV-based PAMPA model requires UV absorbance, which many

compounds do not exhibit. PAMPA enables test compounds to be ranked based on a simple permeability property (passive, transcellular permeation) as it avoids the complexities of active transport. It is more commonly used and has become popular in the pharmaceutical industry as a tool for primary pharmacokinetic screening of research compounds [142].

1.6.3 The Ussing chamber

The Ussing chamber is a model used to examine absorption across various epithelial membranes. Besides being used for transport and toxicology studies across excised tissue sections, it can also be utilized on filter grown cell monolayers. Attempts have been made to develop *in situ* and *ex vivo* models that more closely replicate intestinal absorption in humans; however, the data obtained from studies using such models may be limited [130]. The likelihood of DDIs/HDIs occurring can be studied by monitoring drug absorption when co-administered with various herbs. Sections of intestinal mucosa mounted on Ussing chambers are useful tools for analyzing such changes in drug absorption, and the results obtained using this model are reported to have more merits than those obtained using Caco-2 cells or parallel artificial membrane permeability assay models. Furthermore, the expression levels of drug transporters and drug-metabolizing enzymes in the Ussing chamber system are comparable to those *in vivo* and the morphological structure of the intestine is preserved [143, 144]. Our study utilized excised rat ileal segments in which P-gp is highly expressed on the apical side of the epithelial cells [145, 146]. The ileum was specifically chosen as the level of absorptive-directed transport is higher in this segment than in other intestinal segments [147]. Figure 7 is a diagrammatic

representation of the basic design of the Ussing system. The Ussing chamber closely depicts the physiological system as the presence of a gas manifold provides air regulation to both sides of each chamber. Temperature is maintained utilizing a heater block, connected to a circulating water bath. It basically comprises 2 chambers- a mucosal and a serosal compartment. The excised intestinal tissue is carefully pinned in such a way to be bathed by the buffer (commonly Krebs Ringers bicarbonate buffer). Detailed description of the Ussing system and its application has been reported [148, 149].

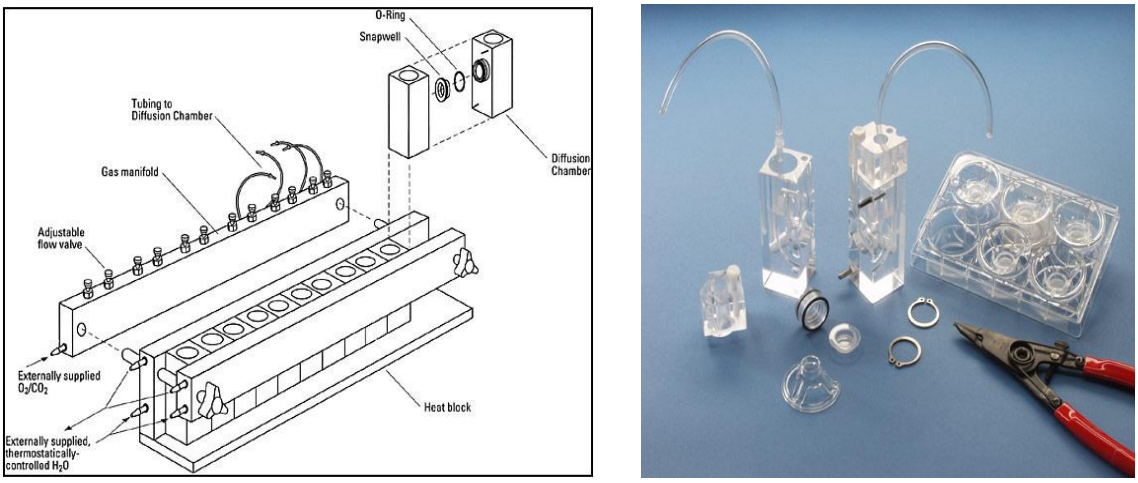

Figure 7. Ussings chambers
Source: [150]

1.6.4 *In situ* single-pass perfusion models

In situ models of intestinal perfusion of the intestine are commonly used as models to determine and compare the absorption characteristics of drugs. It enables easy determination of regional drug disposition. Normally, the animal of choice is anaesthetized and laparotomy conducted to provide access to the abdominal cavity (Figure 8). The investigated drug can then be introduced into the intestinal segment of interest either through a closed or opened loop. Comparison of the relative

contribution of intestine versus liver in the disposition of a specific compound can be determined using this model, further allowing the enteric and enterohepatic recycling of drugs to be examined [151].

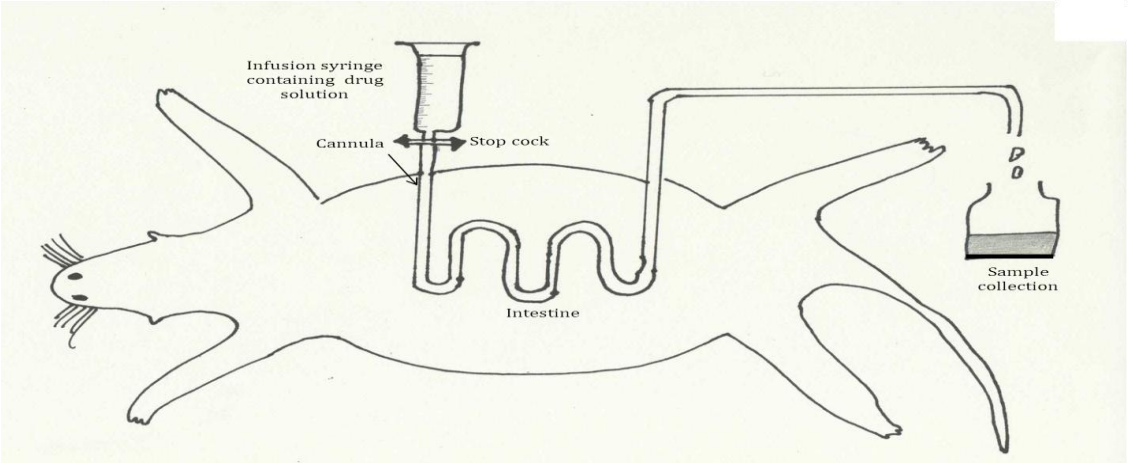

Figure 8. Close-loop perfusion model

1.6.5 *In vivo* animal models

Drug absorption studies in animals are widely believed to be good predictors of human absorption. The rat is the most commonly used animal model in the transport studies, mainly because it better reflects the human situation with respect to paracellular space and metabolism [152, 153]. Rats are a valuable animal model to assess pharmacokinetic drug interactions and data obtained from studies using rats has been used to justify the clinical use of specific drugs [154, 155]. *In vivo* animal models normally provide good correlation with human absorption patterns as they incorporate the dynamic components of the mesenteric blood circulation, the mucous layer and all the other factors that can influence drug dissolution [134]. However, studies utilizing animals also have limitations including the provision of little mechanistic information on drug absorption; therefore they tend to provide

false-positive results. Because of these demerits the best model for human experiments are actually humans.

1.7 Models of drug metabolism

1.7.1 Microsomal and sub-cellular fraction assays

Currently, several studies utilize microsomes, S9 and cytosol fractions as a major tool for studying CYP inhibition, CYP phenotyping, clearance, and metabolite characterization. Microsomes are responsible for the biotransformation of drugs, toxins and xenobiotics, enabling for metabolic investigations. Phase I and phase II reactions are the main pathways for enzyme reactions in drug metabolism. Phase I enzymes include the CYPs processes of oxidation, reduction and/or hydrolysis and require NADPH as a cofactor. In addition to factors for phase I metabolism, microsomes also possess Phase II enzymes such as glutathione S-transferases, epoxide hydrolase, and UDP-glucuronyl transferase which act predominantly on Phase I altered compounds to facilitate excretion. They can therefore be used to evaluate Phase I and Phase II metabolism. In addition to factors for phase I metabolism, microsomes also possess Phase II enzymes such as glutathione S-transferases, epoxide hydrolase, UDP-glucuronyl transferase, flavin-monooxygenases and carboxylesterases which act predominantly on phase I altered compounds to facilitate excretion. They can therefore be used to evaluate phase I and phase II metabolism. These microsomes may be extracted from the liver or extrahepatic tissues like the intestine, lungs or kidney. Hepatic and intestinal-derived tissues evaluate the potential of pre-systemic metabolism which may ultimately influence the bioavailability of a drug. The intestinal mucosa has

been reported to be the most important extrahepatic site of drug biotransformation, appreciably expressing CYPs [156]. Renal and pulmonary-derived cells allow for an examination of extrahepatic metabolism. Depending on which species they are derived from, the microsomal system allows for the investigation of interspecies differences in metabolism, permitting a comparison of metabolic profiling in guinea pigs, minipigs, rats, mice, trout, rabbits, dogs, monkeys and humans.

1.7.2 Hepatocyte assays

Liver hepatocytes are valuable and effective tools for the *in vitro* evaluation of drug interactions, intrinsic clearance, metabolism, metabolic profiling, hepatotoxicity, and transporter activity. In addition to the evaluations which microsomal assays cover, they also allow for studies of transporter activity and drug induction, which cannot be assessed in microsomal systems. Hepatocytes are known to possess all the hepatic drug metabolizing enzymes within an intact cell. Interspecies differences can also be assessed by the use of species-specific cryopreserved. Because hepatocytes have *in vivo*-like enzyme expression levels, it is easier to correlate *in vitro*/*in vivo* data. Fresh hepatocytes, cryopreserved hepatocytes are routinely used. HepG2 cells are also widely used as they are a suitable *in vitro* model system for the study of polarized human hepatocytes. Recently systems, including HepaRG™ cells (terminally differentiated hepatic cells derived from a human liver progenitor cell line) are gaining interest, as they maintain the morphology, expression of key metabolic enzymes, nuclear receptors, and drug transporters which are exhibited by primary human hepatocytes [157].

1.8 Mechanisms of herb-drug interactions

Herb-drug interactions (HDIs) can occur when herbal and conventional medicines are co-administered, and the possible changes in pharmacokinetic disposition as well as the pharmacodynamic impact of such interactions need to be investigated [158, 159]. Several HDIs have been reported including the influences of St. John's wort, ginseng, gingko, and milk thistle when they are co-administered with conventional medicines [160, 161]. The majority of these HDIs interfere with cytochrome P450 enzymes [162]. Although reports concerning the influence of HDIs on xenobiotic transporters are limited, the impact of HDIs on permeability glycoprotein (P-gp) is slowly gaining interest [163-165]. P-gp is an efflux protein that pumps its substrates out of cells in an ATP-dependent manner, thereby reducing the intracellular accumulation of these substrates [28]. P-gp is expressed on the apical membrane of epithelial cells where it extrudes several orally administered drugs into the intestinal lumen and thereby interferes with drug disposition [166, 167]. Inhibition of P-gp is of great clinical interest as this can enhance the oral bioavailability of specific drugs and reverse multidrug resistance (MDR). However, the mechanisms and potential influence of HDIs on P-gp are not fully understood.

Pharmacokinetic and pharmacodynamic alterations are the two mechanistic pathways through which herb-drug interactions (HDIs) occur, resulting in beneficial or toxic responses. Figure 9 illustrates the mechanisms of HDIs. Majority of the HDIs manifest as a result of pharmacokinetic alterations. This arises from changes in the function and expression of transporters or enzyme that mediate the absorption and elimination of drugs in the small intestine, kidney and liver [92]. Numerous pharmacokinetic interactions involving herbal remedies which are

mediated by transporters and enzymes have been documented. In addition to the cytochrome enzymes, membrane transporters are known play an important role in the modulation of absorption, distribution, metabolism and excretion of drugs [28].

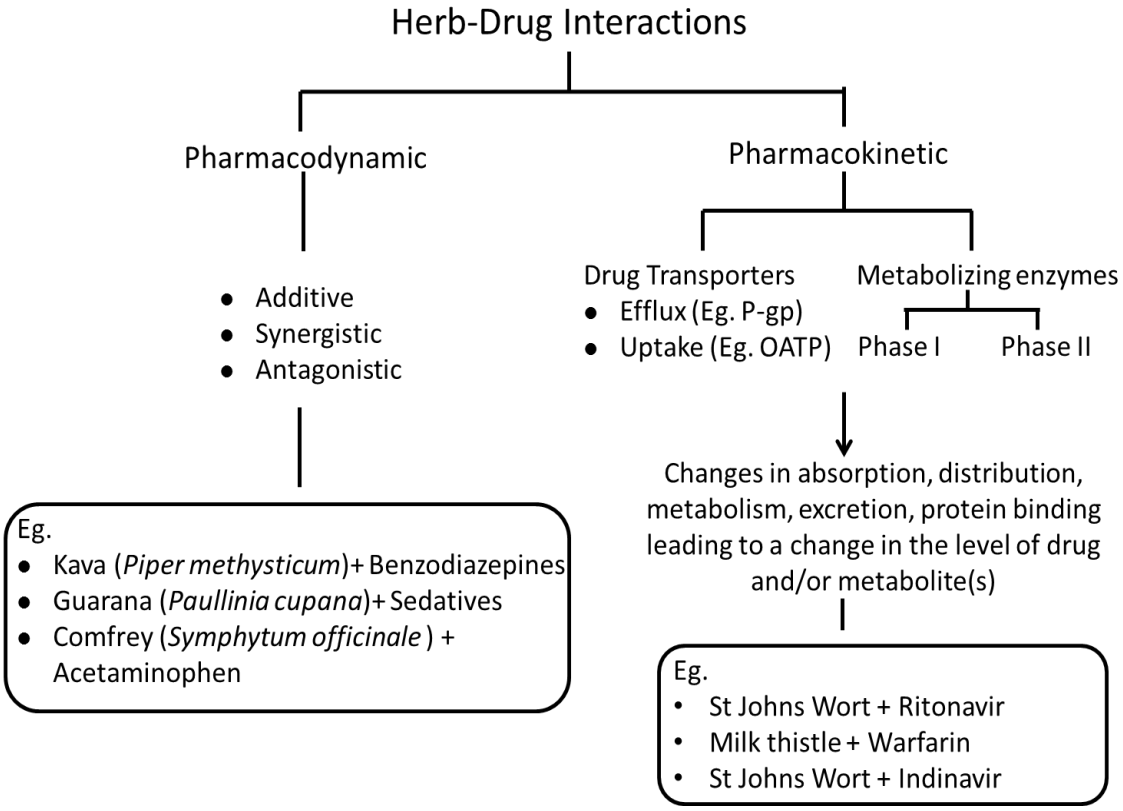

Figure 9. Main mechanisms for herb-drug interaction

1.8.1 Enzyme-mediated HDIs

One of the most important causes of clinically-relevant HDIs is the inhibition or induction of the activity of cytochrome P450, a superfamily of enzymes catalyzing extremely diverse and often complex reactions in the metabolism of numerous drugs, phytomedicines and xenobiotics. Majority of the drugs are metabolized by or interact with the isoform CYP 3A. For instance, several intestinal CYP 3A inhibitors (including the triterpenes; maslinic acid, corosolic acid, and ursolic acid) have been

isolated from *Vaccinium macrocarpon* (cranberry). Cranberry is now commonly taken as a treatment for urinary tract infections due to a group of proanthocyanidins which exhibit bacterial anti-adhesion activity against both antibiotic susceptible and resistant strains of uropathogenic *Escherichia coli* bacteria [168]. Another study revealed that cranberry juice inhibits the CYP3A-mediated metabolism of nifedipine altering its pharmacokinetics by increasing the concentration of nifedipine in rat plasma. The study demonstrated that the oxidation activities of nifedipine in rat intestinal and human hepatic microsomes were inhibited after pre-incubation with cranberry juice [169]. Similarly, the widely used herbal remedy, *Echinacea purpurea* has been reported to significantly induce CYP 3A [170]. It has been shown to selectively modulate the catalytic activity of CYP3A at hepatic and intestinal sites suggesting that CYP 3A substrates with relatively high bioavailability may be more susceptible to *Echinacea*-mediated interactions [171, 172]. As CYP 3A is the main drug metabolizing enzyme, the potential of HDIs occurring with *Echinacea purpurea* may be high.

1.8.2 Transporter-mediated HDIs

Although most studies have focused on cytochrome-based drug interactions, the influence of transporters as a mechanism for DDIs is increasingly being documented, as it has been revealed that they can play an important role in modulating drug absorption, distribution, metabolism and elimination. Xenobiotic transporters are generally categorized into the ATP Binding Cassette (ABC) and solute carrier (SLC) superfamilies. The ABC transporters function as barrier

proteins extruding toxins and xenobiotics out of cells. They include P-gp, breast cancer resistance protein (BCRP), bile salt export pump (BSEP) as well as several multidrug-associated resistance proteins (MRPs). They are commonly expressed on barrier epithelia where they mediate transport across cell membranes [28]. These transporters are significant determinants of the pharmacokinetics, efficacy and toxicity of xenobiotics including phytomedicines and co-administered drugs may inactivate, inhibit or induce these transporters. Of all the transporter-mediated HDIs, P-gp is the most implicated. There are reports on the modulation of P-gp by herbs such as St. John's wort, *Vernonia amygdalina, Tapinanthus sessilifolius*, *Coptis chinensis*, *Ginkgo biloba, Piper nigrum* and *Glycorrhiza glabra* [81, 92, 164].

On the other hand, the SLC transporters mainly comprise of uptake transporters including the OATPs, organic cation transporters (OCTs), organic anion transporters (OATs), peptide transporters (PEPT) and multidrug and toxic compound extrusion transporters (MATE) [28]. These SLC transporters are known to facilitate absorption of phytomedicines and xenobiotics into the systemic circulation. Because transporters are mainly expressed in the intestinal epithelial cells and organs of elimination (kidney proximal tubules and liver hepatocytes), they are known to significantly influence the disposition of herbal remedies [173]. Fewer reports investigate the influence of SLC-mediated herb-drug interactions when compared to ABC transporters. For example, baicalin from *Radix scutellariae* showed a reduction in the plasma concentration of rosuvastatin when co-administered as a result of OATP modulation [174]. In the study, the observed decrease in the plasma concentration of rosuvastatin in the presence of baicalin was thought to be partially mediated by baicalin's induction of hepatic rosuvastatin uptake through OATP1B1

[174]. Another study reported that the pharmacokinetic disposition of salvianolic acid B was altered by rifampicin in rats due to the inhibition of Oatp-mediated influx [175]. Salvianolic acid B is one of the most bioactive components of *Salvia miltiorrhiza*, a traditional Chinese herbal medicine that is commonly used for prevention and treatment of cerebrovascular and cardiovascular disorders. Likewise, several herbs modulating OAT1, a transporter actively involved in renal active secretion have been reported. Among 63 Chinese herbal medicines examined in the study, only two exhibited significant inhibitions on hOAT1-mediated [^{3}H]-p-amino hippuric acid uptake *in vitro* as well as p-amino hippuric acid clearance and net secretion *in vivo* [176].

1.8.3 Dual enzyme- and transporter-mediated HDIs

Some phytomedicines are known to influence both transporter and cytochrome function. P-gp and CYP 3A4 both constitute a highly efficient barrier for many orally absorbed drugs with a wide overlap in their substrates [177]. *Rhodiola rosea*, a phytomedicine used in the management of depression has shown potent inhibition of both P-gp and CYP 3A4 [178]. Also, St. John's wort (*Hypericum perforatum*), one of the most widely used herbal remedies with several medicinal effects including its recognized antidepressant properties, induces both CYP 3A and the efflux transporter; P-gp [179]. In another study, quercetin and rutin which are popular herbal flavonoids induced the functions of both P-gp and CYP3A4 by decreasing the bioavailability of cyclosporin when co-administered [180]. These alterations on normal P-gp efflux and CYP activity has an impact on the pharmacokinetic disposition of CYP 3A and P-gp substrate drugs when

co-administered leading to changes, including lower efficacy and/or the emergence of toxicity [181].

1.9 Transporter substrate and inhibitor probes

1.9.1 Digoxin

Digoxin is a well-known probe drug that is used to investigate P-gp function. It was used in this study because it is selectively transported by P-gp, not by other xenobiotic transporters [182, 183]. Digoxin also has the advantage of not being extensively metabolized, allowing for the selective study of P-gp function. The elimination of digoxin is dependent on P-gp that it is routinely used as a control to investigate the influence of other drugs on P-gp activity. Digoxin also provides a clinically relevant example as it has a narrow therapeutic index. Therefore its use may make a case for other drugs with low safety margins. It is important to note that for digoxin, even a 25% increase in exposure is viewed as clinically relevant, because toxicity may occur as a result of increased drug levels [166].

1.9.2 Fexofenadine

Fexofenadine is an antihistamine that exhibits good oral bioavailability despite its efflux by P-gp as reported using various models [184, 185]. Other reports show that multiple uptake and efflux transporters play a role in its intestinal and hepatic disposition, including BSEP, OAT3, OATP1B1, OATP1B2, OATP2B1, MRP2, MRP3 and P-gp [186]. It undergoes minimal metabolism, as over 95% of an administered dose is recovered intact in urine and feces [185]. This characteristic allows for drug transport studies alone without factoring in the influence of metabolizing enzymes. Despite its modulation by several transporters, it is still of value as a probe of P-gp-mediated

transport, especially in studies investigating drug-drug interaction and disposition.

1.9.3 Rhodamine-123

Rhodamine-123 (Rho-123) is an easy-to-use, cationic fluorescent marker utilized to study the function of P-gp in both multidrug-resistant cells and normal tissues including the intestine[187]. It is commonly used because of its relatively cheap cost and since it fluoresces, it can be detected easily. Since the expression and function of P-gp in some cells are the high, Rho123 accumulation is widely used as a surrogate indicator to evaluate the degree of functional inhibition of P-gp in trials of P-gp inhibitors. It was utilized in the initial phase of this study for the preliminary screening of antimalarials as P-gp inhibitors, before further experimentation with clinically relevant substrates.

1.9.4 Verapamil

Verapamil is commonly used in drug transport studies as either a substrate or inhibitor of the drug efflux pump; P-gp. In this study, it was utilized as an inhibitor of P-gp, as its potent inhibitory effect has been severally reported [188]. It undergoes extensive oxidative metabolism mediated by CYP, especially CYP3A4, with less than 5% being excreted unchanged in urine after oral administration [189]. In instances where P-gp is inhibited, there is an enhanced absorption of the drug through the enterocytes, with a resultant increase in plasma concentration. Also, a drug that is normally eliminated by P-gp in the bile or urine will accumulate in the body when P-gp is inhibited. Inhibition of P-gp by verapamil has been responsible for several clinical drug-drug interactions. For instance, the interaction between digoxin and verapamil which takes place at P-gp located in the liver and kidneys, reduces the

excretion of digoxin [190]. Therefore, the introduction of verapamil as an inhibition control in evaluating P-gp mediated drug interactions is common, and was utilized in this study.

1.9.5 Cyclosporin A

Cyclosporin has also been used in studies as a potent inhibitor of P-gp [191]. It has been shown to reduce the renal clearance when concurrently administered with digoxin [192]. In addition to verapamil, we included cyclosporin A as an inhibition control in our study utilizing the Ussing chamber. However, cyclosporin A is not selective to P-gp, as it has been shown to modulate the activity of BCRP, MRP1 and lung resistance protein (LRP) [193].

2. MATERIALS AND METHODS

2.0 Materials.

Amodiaquin (ADQ), DIG, VER, sulphadoxin (SDX), chloroquine (CQ), artemisinin (ASN), and artesunate (ART) were purchased from Sigma-Aldrich (St Louis, MO, USA). Artemether (ATM) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); lumefantrine (LUM) was obtained from May & Baker (Lagos, Nigeria); QD was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); [^{3}H]-DIG (40 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA); and rhodamine-123 (Rho-123) was purchased from Acros Organics (Somerville, NJ, USA). All other chemicals and reagents were of the highest analytical grade available. Curcumin extract (from *Curcuma longa*) and unlabeled DIG were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Verapamil (VER) was from Wako Pure Chem. Industries Ltd. (Osaka, Japan). ^{3}H-DIG (40Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA). Cyclosporin A (CysA) and Verapamil (VER) were from Wako Pure Chem. Industries Ltd. (Osaka, Japan). βNADP$^+$, Glucose-6-Phosphate (G6P) and Glucose-6-Phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd (Tokyo, Japan). Ketoconazole (KTZ), nelfinavir mesylate, nevirapine and artemisinin were obtained from Sigma (St Louis, MO, USA), ritonavir was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada), artemether was from Tokyo Chemical Industry Ltd (Tokyo, Japan), and saquinavir was obtained from Roche (Mannheim, Germany). Ethyl acetate, acetonitrile (both HPLC grade) and $MgCl_2$ were purchased from Wako (Osaka, Japan). All other chemicals and reagents used were of the highest grade and commercially available. Pooled human liver microsomes and pooled human intestinal microsomes were purchased from Celsis *In Vitro* Technologies (Baltimore,

MD, USA).

The phytomedicines investigated were bitter leaf (*Vernonia amygdalina*), neem leaves (*Azadiractha indica*), brimstone bark (*Morinda lucida*), lemon grass *(Cymbopogon citratus),* pawpaw leaves (*Carica papaya*), and African mistletoe leaves (*Tapinanthus sessilifolius*). The plant parts were collected from their natural habitat in various locations in Jos, Nigeria and were dried under a shade for several days at room temperature. The identity of the plants was confirmed at the Forestry Research Institute of Nigeria, Ibadan where voucher specimens were deposited.

2.1 PAPER I

2.1.1 Cell culture.

Immortalized human colon carcinoma Caco-2 cells (passage #17-43) were routinely cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen, Grand Island, NY, USA). Cells were maintained in an incubator at 37°C with 95% relative humidity and a 5% CO_2 atmosphere. The culture medium was changed two to three times weekly. Upon reaching near confluence (80 to 90%), the cells were detached from the culture flask by the addition of 0.05% trypsin in 0.02% EDTA. Cells were then seeded at a density of 1 x 10^5 cells/cm^2 on Transwell collagen-coated membrane inserts (6.5 mm membrane diameter, 0.4 µm pore size, 0.33 cm^2 surface area) that were pre-loaded into Transwell 24-well cluster plates (Corning, NY, USA). They were used 18 to 25 days post-seeding in order to obtain differentiated monolayers and an anticipated high expression level of transport proteins (i.e., P-gp). Monolayer integrity on the permeable membrane was assessed by transepithelial electrical resistance (TEER), which was tested using a Millicell

ERS-2 Volt-Ohm meter (Millipore, Billerica, MA, USA). Only monolayers with TEER values above 200 Ωcm^2 were used in the transport experiments.

2.1.2 Transport experiments.

The transport experiments were conducted in transport medium consisting of Hank's balanced salt solution (HBSS) buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) at 37°C. Prior to the transport experiments, the monolayers were washed twice with transport medium and pre-incubated for 30 min. The test agents were dissolved in < 1% of the respective dissolving solvent. Each test agent was added to either the apical (A) or basolateral (B) side of the monolayer, with the apical side having a final volume of 100 µL, and the basolateral side having a final volume of 600 µL. The side of the monolayer that received the test agent was termed the donor compartment, while the other side was termed the recipient compartment. Samples (50 µL) were taken from either the donor or the recipient compartment at time intervals of 30, 60, 90, and 120 min, and the removed volume was replaced with a corresponding volume of pre-warmed, drug-free HBSS solution at 37°C. To examine the effects of ADQ, ART, LUM, ATM, CQ, ASN, and SDX on P-gp-mediated transport, the impact of drug addition to both the A and the B side of the monolayer was independently assessed. VER (100 µM) and QD (1 µM) were used as positive inhibitor controls.

2.1.3 Quantification of Rho-123 and DIG transport.

The transport of Rho-123 was quantified using a MTP-600 fluorescence microplate reader (Corona Electric, Ibaraki, Japan) equipped with a 490 nm excitation, 590 nm emission filter set. For [^{3}H]-DIG determination, a 50 µL sample of transport medium

from either the donor or the recipient compartment was mixed with 2 mL of Clear-sol I scintillation cocktail (Nacalai Tesque, Kyoto, Japan) and the radioactivity was measured with a LSC-6100 liquid scintillation counter (Aloka, Tokyo, Japan).

2.1.5 Quantification of FXD transport.

FXD analysis was carried out by LC/MS/MS. Samples were analyzed on a hybrid triple quadrupole linear ion trap (QTRAP) 4000 LC/MS/MS system from Applied Biosystems/MDS Sciex (Concord, Ontario, Canada) coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, USA). Prior to the analysis, 50µL of 1µg/mL Glipizide was added as the internal standard to 50µL of the buffer sample. Extraction was carried out using acetonitrile followed by vortex mixing, centrifugation for 5min at 15 000g, concentration using a centrifugal vaporizer CVE-200D (EYELA, Tokyo, Japan) and dilution in the mobile phase before the injection of 20µL into the LC/MS/MS system for analysis .For the chromatographic conditions, the mobile phase comprised 0.1% formic acid in ultra-pure H_2O:0.1% formic acid in acetonitrile (50:50) and a flow rate of 0.2ml/min. The analytical column was of 2.0mm ID x 150mm length (Shodex® ODP2 HP-2D) and guard column 2.0mm ID x 10mm (Shodex® ODP2 HPG-2A). Both were of 5µm particle size with the column temperature maintained at 40^0C. Detection was performed using an electrospray source (ESI) in positive ionization mode. Quantification was performed in MS/MS mode using the following fragmentation transition m/z of 502.1/466.2 and 446.0/321.1 for FXD and glipizide respectively. System control and data processing was done using *Analyst* software Version 1.4.2.

2.1.4 Data analysis.

The apparent permeability coefficient (Papp) was calculated as:

$$P_{app} = (dQ/dt)/C_o \times A \quad [1]$$

where dQ/dt is the rate of appearance of drug in the recipient compartment; A represents the membrane surface area of the Caco-2 monolayer (0.33 cm^2); and C_0 is the initial drug concentration in the donor compartment.

The efflux ratio (ER) was obtained as:

$$ER = P_{app\ B\text{-}A}/P_{app\ A\text{-}B} \quad [2]$$

where $P_{app\ B\text{-}A}$ and $P_{app\ A\text{-}B}$ are the mean Papps obtained for transport in the basolateral-to-apical (B-A) direction and the apical-to-basolateral (A-B) direction, respectively.

The DIN was calculated as:

$$DIN = Dose_i / K_i \quad [3]$$

where $Dose_i$ is the maximum therapeutic dose of the inhibitor, and K_i is the inhibition constant. The K_i values were generated from ER[195] and calculations made using *Microsoft Excel* software (Seattle, WA, USA).

All results are expressed as the mean ± the standard error of the mean (SEM) ($n \geq 3$ for each experiment). Values of $p < 0.05$ were deemed statistically significant using Dunnett's test.

2.2 PAPER II

2.2.1 Preparation of Extracts.

Extracts of the leaves or bark (for MLB) were prepared by decoction using 100g per 100mL distilled water. Prior to this, they were blended using an Ace homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan) then decocted in distilled water for 30 min at

40˚C before being allowed to steep for 4-6 hrs. This was followed by filtration with *Advantec* No. 2 qualitative filter paper (Toyo Roshi Kaisha Ltd, Tokyo, Japan) before being concentrated by evaporation at 40˚C *in vacuo* using an Eyela CVE-200D centrifugal vaporizer (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). They were stored at 4˚C until use. The final concentration of the respective phytomedicines used in the experiments ranged from 0.02mg/mL to 20mg/mL of the dried extract dissolved in distilled water. The respective extraction yield obtained for each herbal was 5.89, 8.24, 4.0, 4.92, 7.39 and 3.83% for LG, MLB, ML, CP, VA and NL respectively.

2.2.2 Cell culture.

Caco-2 cells (passage number 20 - 61) were routinely cultured at 37°C, 95% relative humidity and 5% CO_2 atmosphere and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum and 1% antibiotic-antimycotic from Gibco Invitrogen Corp (Grand Island, NY, USA). The culture medium was changed every 2-3 days. On reaching an acceptable confluency of 80-90%, the cells were detached from the culture flask by introducing a 0.05% trypsin-0.02%EDTA solution. Cells were used 16 - 24 days post seeding in order to obtain differentiated monolayers and an expected higher expression of transport proteins. Cells were seeded at $1x10^5$ cells/cm^2 on Transwell membrane inserts (Corning, NY, USA) and placed in Transwell 24 well clusters (6.5mm membrane diameter, 0.4µm pore size, 0.33cm^2 surface area) pre-coated with collagen. Monolayer integrity of the permeable membrane was assessed by the transepithelial electrical resistance (TEER) of the monolayers using a Millicell ERS-2 (Millipore, Billerica, MA, USA). Only cell monolayers with TEER values above

250Ωcm^2 were used in the transport experiments.

2.2.3 Transport study.

Transport experiments were conducted in Hank's balanced salt solution (HBSS) buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) at 37°C. Prior to the experiments, the monolayers were washed twice with the transport medium and pre-incubated for 20 min. To examine the effects of the extracts on P-gp mediated transport, the extracts (0.02, 0.2, 1, 2, 5, 10, and 20 mg/mL) were added to both apical and basolateral sides at equal concentrations. VER 100 µM was used as a positive inhibitor control. The apical chamber had a final volume of 100 µL while the basolateral chamber contained 600 µL. The experiments were initiated by the addition of ^{3}H-DIG to the donor compartment (apical chamber for A-B transport studies and basolateral chamber for B-A transport studies, respectively). 50 µL samples were taken from the either the donor or receiving compartment at time intervals of 30, 60, 90 and 120min; the removed volume was replaced with a corresponding volume of pre-warmed drug-free HBSS solution at 37°C.

2.2.4 Cellular viability study.

The cytotoxic effects of the phytomedicines under investigation was examined using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Caco-2 cells were seeded on standard 96-well microplates at a density of 1 x 10^4 cells/well. They were subsequently treated with VER, DIG and the extracts (0.02, 0.2, 1, 2, 5, 10, and 20 mg/mL) for 120 minutes with HBSS as the control. The cells were then incubated with MTT in phenol red-free Dulbecco's

modified eagle's medium (Sigma-Aldrich) at 37°C for 4 hours. DMSO was introduced to solubilize the cells and incubated for 10 minutes at 37°C. Absorbance was read at 570nm (reference at 630nm) using a Multiskan JX microplate reader (Thermo Scientific, Tokyo, Japan). Cell viability was calculated as a percentage of HBSS control.

2.2.5 Quantification of DIG transport.

In order to determine the amount of [^{3}H] – DIG transported, 50 µL sample was mixed with 2mL of Clear-sol I scintillation cocktail (Nacalai Tesque, Kyoto, Japan) and the radioactivity was measured with a LSC-6100 liquid scintillation counter (Aloka, Tokyo, Japan)

2.2.6 Data analysis.

The apparent permeability coefficient (Papp) used as an expression of the absorption rate constant was calculated as

$$Papp = (dQ/dt) / (C_o \times A)$$

dQ/dt represents the rate of appearance of drug in the receiver chamber, A represents the membrane surface area of Caco-2 monolayer (0.33cm^2), and C0 is the initial drug concentration on the donor side.

In order to compute the efflux ratio (ER), the bidirectional P_{app} of [^{3}H] – DIG in the presence and absence of the extracts and VER were collated. ER was calculated as

$$ER = P_{app\ B\text{-}A} / P_{app\ A\text{-}B}$$

With $P_{app\ B\text{-}A}$ and $P_{app\ A\text{-}B}$ as the mean permeability coefficient obtained for the basolateral-to-apical direction and apical-to-basolateral direction respectively.

2.2.7 Statistical analysis.

All data are expressed as mean ± standard error of mean (SEM) of at least 3 replicate determinations. Values of *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ were considered to be statistically significant using two-tailed t-test and Dunnett's analysis.

2.3 PAPER III

2.3.1 Herbs.

The aqueous herbal extracts investigated in this study were from bitter leaf (VA), pawpaw leaves (CP), and a species of mistletoe leaves (ML). The plant parts were collected from their natural habitat in various locations near Jos, Nigeria, and were dried in the shade for several days at room temperature. The identity of the plants was confirmed at the Forestry Research Institute of Nigeria, Ibadan, where voucher specimens were deposited. Leaves were blended using an Ace homogenizer (Nipponseiki Co. Ltd., Tokyo, Japan) and extracts were prepared by decoction for 30 min at 40°C using 100 g of leaves per 100 ml distilled water before being allowed to steep for 6 h. This was followed by filtration with *Advantec* No. 2 qualitative filter paper (Toyo Roshi Kaisha Ltd, Tokyo, Japan) before extracts were concentrated by evaporation at 40°C *in vacuo* using an Eyela CVE-200D centrifugal vaporizer (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Samples were stored at 4°C until use.

2.3.2 Animals.

Healthy male Sprague Dawley (SD) rats (220–300 g) obtained from Japan SLC Inc. (Shizuoka, Japan) were maintained in the housing facility in a temperature-controlled environment with a 12 h light/12 h dark cycle and

received a standard diet with water. The animals were housed in this facility for at least one week before experiments were initiated to ensure proper acclimatization. Prior to each experiment, the rats were fasted overnight but had free access to water and were randomly assigned to different groups. The study was approved by the Chiba University Animal Ethics Committee. The research adhered to the "Principles of Laboratory Animal Care" [194].

2.3.3 Transport studies across the rat ileum in Ussing chambers.

After anaesthetization with diethyl ether and laparotomy through a midline abdominal incision, the small intestine was immediately excised and immersed in ice-cold, freshly prepared standard Kreb Ringers bicarbonate buffer, KRBB [115 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4 maintained at pH 7.4] bubbled with carbogen (95% O_2, 5% CO_2). Ileum that had been cut 25 cm proximally from the caecum was used. The tissue was rinsed with ice-cold standard KRBB to remove the luminal contents and cut into 1.7 cm long segments, excluding visible Peyer's patches. The ileum segments were opened along the mesenteric border and stretched onto pins on one half of the chamber with an exposed tissue area of 0.64 cm^2 placed between two Navicyte side-by-side chambers (Harvard Apparatus, Holliston, MA) clamped together. Each compartment was filled with 5 ml of KRBB buffer supplemented with 10 mM D-glucose or 10 mM mannitol on the serosal and mucosal side of the ileal tissue, respectively. The tissue was kept at 37°C during the experiments using a circulating water bath attached to a heat block. The incubation buffer inside the diffusion chambers was oxygenated and circulated by bubbling with carbogen

through a gas manifold. After pre-incubation for 40–50 min with the test inhibitor agent diluted in KRBB (final concentrations of 200 μM VER; 20 μM CysA; 10 mg/ml of each of the herbal extracts), digoxin was added to a final concentration of 10 μM (7 nmol of ^{3}H-digoxin/mol of unlabeled digoxin) to the incubation buffer on the mucosal side of the chamber. At time intervals of 15, 30, 60, 90, 105, and 120 min after the introduction of digoxin, 1 ml aliquots were withdrawn from the serosal chamber and were replaced with the appropriate volume of drug-free buffer to maintain a constant volume in the chamber. The amount of permeated digoxin was assayed using liquid scintillation counting. The amount of ^{3}H-digoxin transported was quantified by mixing the 1 ml sample with 2 ml of Clear-sol I scintillation cocktail (Nacalai Tesque, Kyoto, Japan) and radioactivity was measured with a LSC-6100 liquid scintillation counter (Aloka, Tokyo, Japan). Ileal tissue integrity and viability were observed throughout each study by measuring the transepithelial electrical resistance (TEER), using a Millicell ERS-2 (Millipore, Billerica, MA).

2.3.4 *In vivo* pharmacokinetic studies.

The rats were randomly assigned into five different groups each containing 5–9 rats. One group served as the control and these rats were pre-treated with 0.5 % sodium carboxymethylcellulose 2 h before receiving 25.6 μmol/kg digoxin by oral gavage (0.17 μmol of ^{3}H-digoxin/mol of unlabeled digoxin) in 0.2 ml of vehicle. For the groups pre-treated with herbal extracts, the rats were administered a dose of 90 mg/kg (suspended in the vehicle) by oral gavage 2 h prior to receiving digoxin. The VER control group received 25 mg/kg p.o VER 2 h before receiving digoxin. A heating lamp was used to maintain blood temperature, increase circulation, and

improve blood collection. Blood samples were taken from the saphenous vein by venipuncture and transferred into heparinized tubes at 15, 30, 45, 60, 90, 120, and 180 min after oral administration of 25.6 µmol/kg digoxin. Blood samples were stored at –20°C before ^{3}H activity was quantified using liquid scintillation after mixing blood with the scintillation cocktail as described above. The rats were sacrificed with an anesthetic overdose on completion of each experiment.

2.3.5 Data treatment and statistical analysis.

The apparent permeability coefficient (Papp) was calculated as: Papp = (dQ/dt) / (C x A). dQ/dt represents the rate of appearance of digoxin in the receiver chamber, A represents the exposed membrane surface area of the ileal tissue (0.64 cm^2), and C is the initial digoxin concentration on the donor side.

All data are expressed as mean ± standard error of mean (SEM) of at least three replicates for Ussing experiments and 5–9 replicates for *in vivo* experiments. Statistical significance was assessed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA) considering values of *p<0.05, **p<0.01, ***p<0.001 to be significant using one-way analysis of variance with Dunnett's post-test for multiple comparisons.

A series of pharmacokinetic parameters were determined based on the individual plasma concentration-time data. Peak digoxin concentrations (C_{max}; in ng/ml) were taken directly from the data. The area under the plasma concentration-time curve (AUC; in ng/ml/h) of digoxin for 0–3 h was calculated by the linear trapezoidal rule and extrapolated to infinity using standard procedures to obtain $AUC_{0-\infty}$. The elimination rate constant ($k_{el;}$ h^{-1}) was estimated by log-linear regression of the

terminal portion of the plasma concentration–time curve and elimination half-life ($T_{1/2}$; h) was calculated as $0.693/k_{el}$.

2.4 PAPER IV

2.4.1 Incubations with Human Liver/Intestinal Microsomes (HLM/HIM). Several preliminary studies were carried out to determine incubation conditions that maximized artemisinin metabolism, ensured linearity with respect to microsomal protein concentration and incubation time as well as confirmed suitability of the extraction process. Briefly, a typical protein mixture (1ml/reaction) contained 0.1 mg protein/mL HLM/HIM, artemisinin, the NADPH-generating system ($MgCl_2$ 5 mM, G6P 5 mM and G6PDH 1 IU/mL in reaction buffer) and 100 mM potassium phosphate buffer containing 100 mM EDTA (pH 7.4). Each sample was pre-incubated at 37 °C for 5 min. The reaction was initiated by the addition of $NADP^+$ (0.5 mM) then incubated at 37 °C for 2 or 5min for HLM and HIM respectively. After incubation, 530 µL of a mixture of ice-cold 1M $NaHCO_3$ and 100mM Na_2CO_3 (to pH 11) was added to each sample on ice to stop the reaction followed by vortex mixing. 170 µL of 10 µg/ml artemether was introduced as the internal standard. Extraction was carried out using ethyl acetate followed by shaking on a horizontal shaker for 20 min. The samples were then centrifuged for 20 min at 5000rpm at room temperature. The organic layer was dried under a gentle stream of N_2 gas. Each sample was diluted to 100uL of the mobile phase before injection of 20 µL in the LC/MS/MS system.

2.4.2 Investigation of CYP3A4 contribution to the metabolism of artemisinin.

To assess the contribution of CYP3A4 to the metabolism of ASN, incubation studies involving chemical inhibition (using KTZ) was carried out. Briefly, for the chemical inhibition studies, KTZ was utilized as a chemical inhibitor on CYP3A4-mediated metabolism of artemisinin. Artemisinin (50 μM) was incubated with 0.1 mg of microsomal protein/mL (HIM/HLM) to a final volume of 1000 μL. The mixtures containing enzymes, potassium phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (5 mM), G6P (5 mM), G6PDH (1U/mL) and artemisinin with or without KTZ (0.1 and 1 μM) were pre-incubated at 37 ^oC for 5 minutes. The reaction was initiated by the addition of $NADP^+$ (final concentration: 0.5 mM) and incubated at 37 ^oC for 2 or 5 min. After incubation, the reaction was terminated as above and further treated for incubations with human liver/intestinal microsomes. In order to determine the degree of inhibition, metabolism of artemisinin in the absence of KTZ (control) was compared with that in the presence of KTZ and expressed as percentage remaining with respect to control. The values of % of control were plotted in comparison with the KTZ concentrations.

2.4.3 Inhibition Studies

In vitro inhibition of the biotransformation of artemisinin was investigated using SQV, RTV, NLF and NVP in HLMs. All incubations were conducted under linear rate conditions. ASN (50 μM) was incubated with 0.1 mg of microsomal protein/mL HLM to a final volume of 1000 μL. The mixtures containing enzymes, potassium phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (5 mM), G6P (5 mM), G6PDH (1U/mL) and artemisinin with or without RTV (0, 0.1, 1, 10, 20, 50 and 100 μM), NLF (0.1, 1,

5, 10, 30, 50 and 100 µM), NVP (0.1, 1. 10, 30, 100 and 300 µM) or SQV (0, 0.3, 1, 3, 10, 30 and 100 µM) were pre-incubated at 37 ^{o}C for 5 minutes. The reaction was initiated by the addition of NADP+ (final concentration; 0.5 mM) and incubated at 37 ^{o}C for 2 min. After incubation, the reaction was terminated and further treated following the same procedure as that described for the incubations with human liver/intestinal microsomes.

2.4.4 Quantification of Artemisinin by LC/MS/MS analysis.

In order to determine the amount of artemisinin remaining after the metabolism studies, samples were analyzed on a hybrid triple quadrupole linear ion trap (QTRAP) 4000 LC/MS/MS system from Applied Biosystems /MDS Sciex (Concord, Ontario, Canada) coupled to an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, USA). Each sample was diluted to 100uL of the mobile phase before the injection of 20 µL in the LC/MS/MS system. For the chromatographic conditions, the mobile phase comprised CH_3CN: CH_3COONH_4 +0.1% HCOOH (85:15) at a flow rate of 0.2ml/min. The analytical column was of 2.0mm ID x 150mm length (Shodex® ODP2 HP-2D, Showa Denko KK, Japan) and guard column 2.0mm ID x 10mm (Shodex® ODP2 HPG-2A, Showa Denko KK, Japan). Both were of 5µm particle size with the column temperature maintained at 40 ^{0}C. Detection was performed using an electrospray source (ESI) in positive ionization mode. Quantification was performed in MS/MS mode using the following fragmentation transition m/z of 283.2/151.2 and 316.3/163.2 for artemisinin and artemether respectively. System control and data processing was done using *Analyst* software Version 1.4.2(Applied Biosystems, Foster City, CA, USA).

2.4.5 Data Analysis and Statistics.

All data is expressed as mean ± standard error of mean (SEM). Kinetic parameters (apparent K_m and V_{max}) were calculated by nonlinear regression by directly fitting the data into Michaelis-Menten equation and expressed as mean ± SEM. Data analysis was carried out using GraphPad Prism Version 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was analyzed using two-tailed Students t-test as well as ANOVA followed by Dunnetts test. Values of $^*p < 0.05$ were considered to be statistically significant.

3. RESULTS

3.1 PAPER I

3.1.1 Transport and inhibition of Rho-123 across Caco-2 cell monolayers.

The B-A transport of Rho-123 (5 μM), a typical P-gp substrate, was first investigated at 120 min in the presence or absence of drugs (Figure 10) VER (100 μM) and QD (1 μM) were employed as positive controls for P-gp inhibition and significantly decreased Rho-123 transport across the Caco-2 cell monolayer. The effect of seven antimalarials (ART, ASN, ATM, ADQ, CQ, LUM, and SDX, each employed at a concentration of 1 mM) on Rho-123 transport was next examined. Among these antimalarials, ART and ADQ showed the most significant inhibitory effects. LUM also exhibited some inhibition of Rho-123 transport, although its actions were less pronounced than those of ART and ADQ. ART, ADQ, and LUM were therefore used in the subsequent experiments.

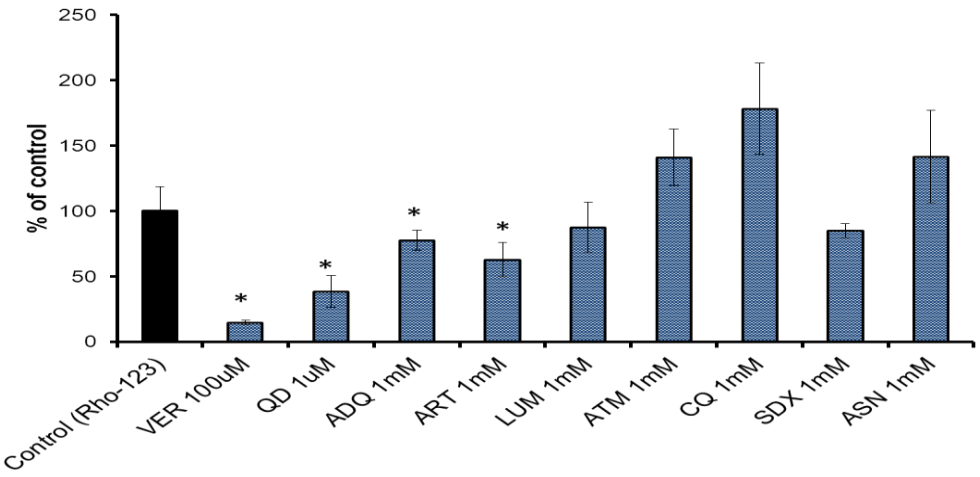

Figure 10. Preliminary screening for the inhibition of P-glycoprotein
Each column represents the mean ± the standard error of the mean (SEM) ($n \geq 3$). *Significant difference, $p < 0.05$ versus control (transport in the absence of drugs).

3.1.2 Transepithelial transport of DIG across Caco-2 cell monolayers.

The bi-directional transport of [^{3}H]-DIG was then examined. In the body, DIG is primarily transported in the B-A direction across the intestinal barrier epithelium by P-gp. Figure 11A illustrates the basic transport characteristics of DIG across the model Caco-2 cell monolayer in the presence or absence of VER (100 μM), the positive inhibition control. As shown in the figure, transport rate of DIG in the B-A direction was greater than that in the A-B direction by over ten-fold. These data are consistent with high expression of P-gp by the Caco-2 cell monolayer and with P-gp-mediated transport as the predominant means of DIG conveyance. The introduction of VER significantly decreased transport of DIG in the B-A direction ($p < 0.05$), and only slightly increased transport in the A-B direction.

3.1.3 Effect of antimalarials on transepithelial transport of DIG and FXD.

The effect of LUM, ADQ, and ART (100 μM and 1 mM) on P-gp-mediated DIG transport was next investigated. Figure 11B shows the bi-directional transport of DIG across the Caco-2 cell monolayer in the presence or absence of LUM (100 μM and 1 mM). In the B-A direction, significant 33 and 38% decreases in the transport rate of DIG were observed for LUM at concentrations of 100 μM and 1 mM, respectively, which is suggestive of P-gp inhibition ($p < 0.05$). However, transport was only minimally affected by LUM in the A-B direction. Figure 11C demonstrates that the transport of DIG in the B-A direction was also significantly impaired by ADQ. B-A transport of DIG was reduced by 38 and 77% in the presence of ADQ at concentrations of 100 μM and 1 mM, respectively ($p < 0.05$). As for LUM, this result is indicative of P-gp inhibition. Here, ADQ (1 mM) showed more potent inhibition than the positive inhibition control, VER (100 μM). In the A-B direction, ADQ (1

mM) significantly stimulated DIG transport by 25%. An increase in the A-B transport of DIG was also seen with the co-administration of VER (100 μM). ART was the last antimalarial to be investigated. Figure 11D shows significant decreases in DIG transport in the B-A direction by 36 and 56% in the presence of ART at concentrations of 100 μM and 1 mM, respectively ($p < 0.05$). No significant impact was observed on A-B transport. Therefore, LUM, ADQ, and ART all inhibited B-A transport of DIG, with 1 mM ADQ exhibiting the most potent inhibition. These results confirm the P-gp inhibitory effect of the antimalarials that was initially observed in the Rho-123 transport assay. Table 4 shows the Papps of DIG in the presence or absence of LUM, ADQ, ART, and VER in the A-B and B-A directions, calculated based on the data shown in Figure 11 (A-D). DIG alone showed an ER of 10.8, further confirming it as a P-gp substrate. Significant effects were observed for ART, ADQ, LUM (1mM) and VER in the B-A transport of DIG while ADQ (1 mM) and VER significantly increased its A-B transport.

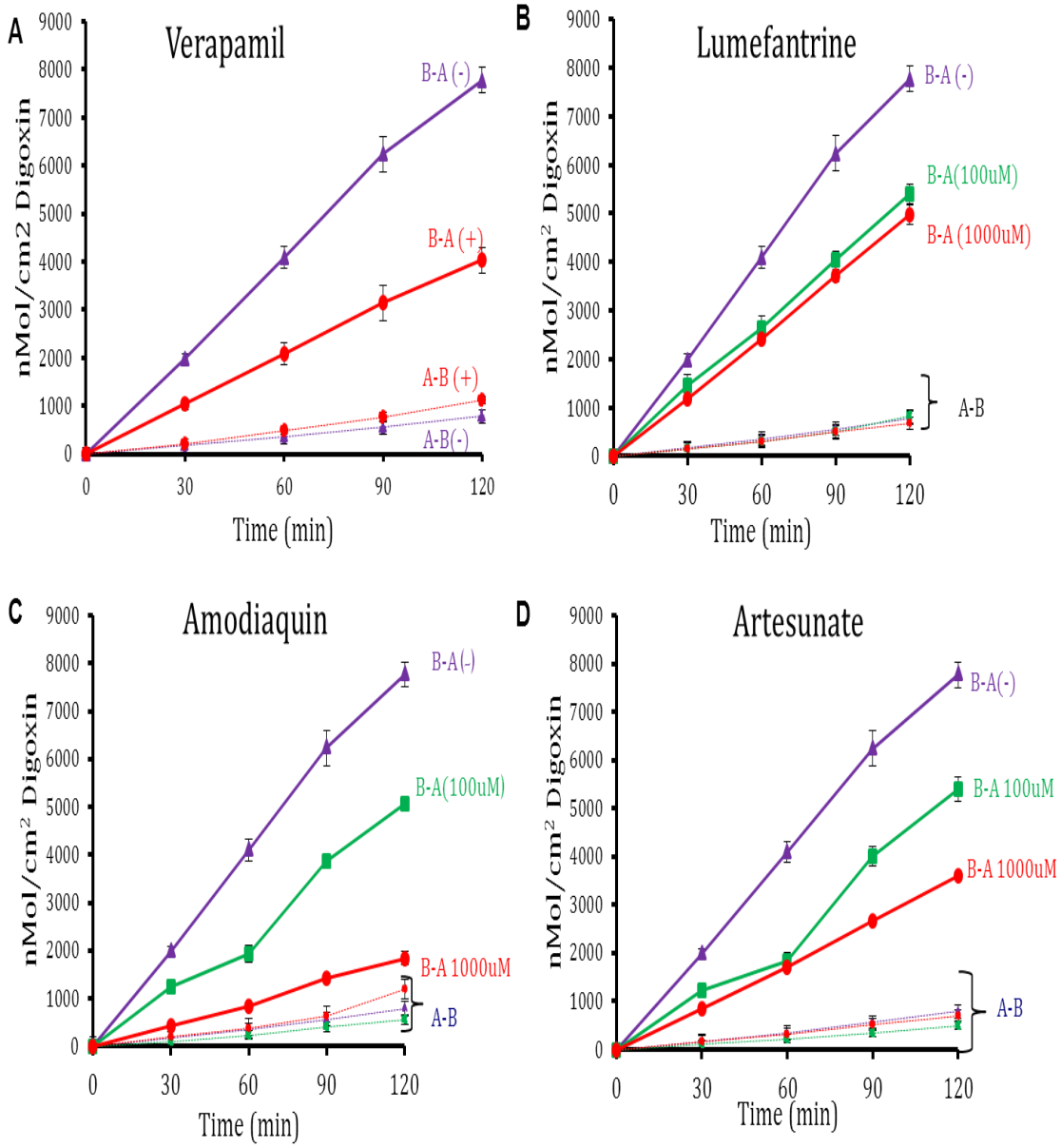

Figure 11. Digoxin (DIG) and antimalarial drug kinetics.

DIG transport across Caco-2 cell monolayer was examined as a function of time. Apical-to-basolateral (A-B) transport and basolateral-to-apical (B-A) transport of DIG was investigated in the presence or absence of A, Verapamil B, lumefantrine (LUM, 100 µM and 1 mM); C, amodiaquin (ADQ, 100 µM and 1 mM); and D, artesunate (ART, 100 µM and 1 mM). Each data point represents the mean ± SEM of at least four determinations. Solid lines represent basolateral-to-apical (B-A) transport, and dashed lines represent apical-to-basolateral (A-B) transport.

Table 4. Permeability coefficient (Papp) and efflux ratio (ER) of digoxin

	P_{app}*10^{-6} cm/s A-B	P_{app} *10^{-6} cm/s B-A	ER
DIG alone	3.14 ± 0.04	33.8 ± 1.63	10.8
DIG + 100 µM ART	2.00 ± 0.22	20.98 ± 0.24*	10.5
DIG + 1 mM ART	2.84 ± 0.22	14.85 ± 0.41*	5.2
DIG + 100 µM ADQ	2.15 ± 0.07	20.35 ± 0.81*	9.5
DIG + 1 mM ADQ	4.03 ± 0.06*	7.60 ± 0.55*	1.9
DIG + 100 µM LUM	3.01 ± 0.08	22.81 ± 1.39	7.6
DIG + 1 mM LUM	2.74 ± 0.06	20.67 ± 0.76*	7.5
DIG + 100 µM VER	4.36 ± 0.13*	17.30 ± 0.50*	4.0

The apparent permeability coefficient (Papp) and efflux ratio (ER) values of digoxin (DIG) in combination with artesunate (ART), amodiaquin (ADQ), and lumefantrine (LUM) at the indicated concentrations in the apical-to-basolateral (A-B) and basolateral-to-apical (B-A) direction across a Caco-2 cell monolayer. *Significant difference, $p < 0.05$ versus control.

The effect of 100µM and 1000µM ART, ADQ and LUM on FXD transport in Caco-2 cell monolayers was examined. Figure 12A shows FXD transport with or without the introduction of 100µM VER, the positive inhibition control. VER reduced FXD transport (B-A) by 55% and increased A-B transport by 36% as expected for the interaction between P-gp substrates and inhibitors. FXD transport in the presence and absence of 100µM and 1000µM LUM is represented in Figure 12B. However, there was non-significant increase of 2% in B-A direction by 1000µM LUM suggestive of non-inhibitory effect on P-gp of this amount. However in the A-B direction, increases of 10% and 13% for 100µM and 1000µM were observed. Figure 12C shows the bidirectional transport of FXD in the presence or absence of

100μM and 1000μM ADQ. In the B-A direction, 100μM ADQ reduced FXD transport by 39% while 1000μM ADQ increased its transport by 104%. Increase of 15% and 5% were observed for 100μM and 1000μM ADQ respectively in the A-B direction. In Figure 12D, the B-A transport of FXD in the presence of ART decreased by 26 % of control for 100μM. However at 1000μM, there was an 80% significant increase in FXD transport. Transport in the A-B direction showed increases of 17% and 6% for 100μM and 1000μM ART respectively. From these, VER, the positive control showed the most inhibition to FXD transport. 100μM LUM, ART and ADQ also showed inhibition to FXD transport. However, all the examined anti-malarials at 1000μM seemed to increase FXD transport. In the A-B direction, there was an increase in FXD transport for all the anti-malarials at both concentrations. This is suggestive of the interaction of these anti-malarials with P-gp and possibly other transporters (Table 5).

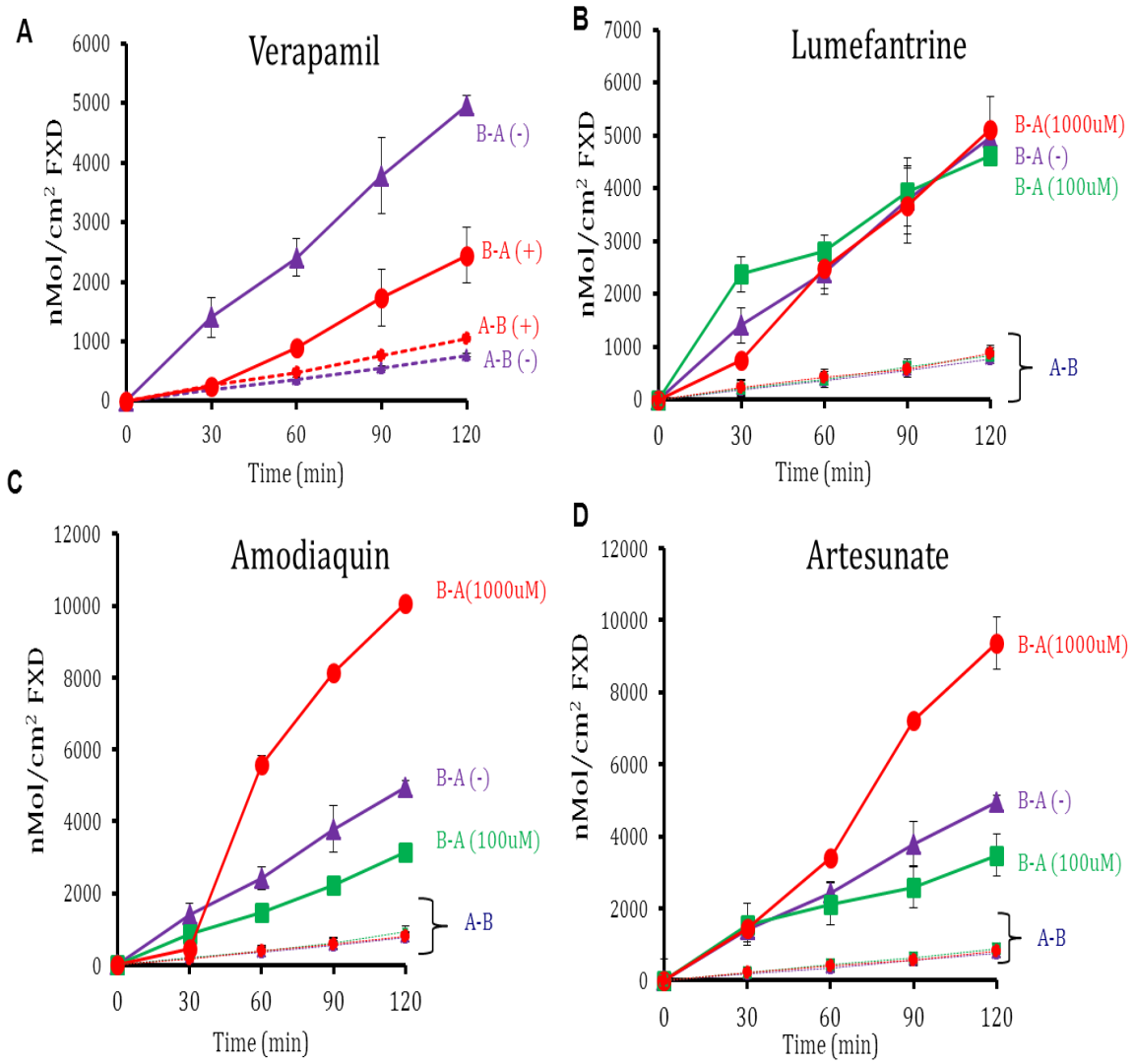

Figure 12. Fexofenadine (FXD) and antimalarial drug kinetics.

FXD transport across Caco-2 cell monolayer was examined as a function of time. Apical-to-basolateral (A-B) transport and basolateral-to-apical (B-A) transport of FXD was investigated in the presence or absence of A, Verapamil B, lumefantrine (LUM, 100 µM and 1 mM); C, amodiaquin (ADQ, 100 µM and 1 mM); and D, artesunate (ART, 100 µM and 1 mM). Each data point represents the mean ± SEM of at least four determinations. Solid lines represent basolateral-to-apical (B-A) transport, and dashed lines represent apical-to-basolateral (A-B) transport.

Table 5. Permeability coefficient and efflux ratio on co-administration with FXD

	P_{app}*10^{-6}cm/s (A-B)	P_{app}*10^{-6}cm/s (B-A)	Efflux ratio
FXD alone	3.14 ± 0.27	21.13 ± 2.47	6.7
+100μM ART	3.66 ± 0.42	16.42 ± 1.33*	4.5
+1000μM ART	3.36 ± 0.39	36.12 ± 1.88	10.7
+100μM ADQ	3.57 ± 0.13	12.95 ± 0.82*	3.6
+1000μM ADQ	3.28 ± 0.16	40.81 ± 1.13	12.4
+100μM LUM	3.43 ± 0.11	23.09 ± 3.33	6.7
+1000μM LUM	3.54 ± 0.24	20.27 ± 3.17	5.7
+100μM VER	4.26 ± 0.20*	8.94 ± 1.81*	2.1

The apparent permeability coefficient (Papp) and efflux ratio (ER) values of fexofenadine (FXD) in combination with artesunate (ART), amodiaquin (ADQ), and lumefantrine (LUM) at the indicated concentrations in the apical-to-basolateral (A-B) and basolateral-to-apical (B-A) direction across a Caco-2 cell monolayer.

*Significant difference, $p < 0.05$ versus control.

For FXD transport, there was a decrease in efflux ratio for the anti-malarials and VER except for 1000μM ART and 1000μM ADQ. The changes in the efflux ratio of DIG and FXD are illustrated in Figure 13.

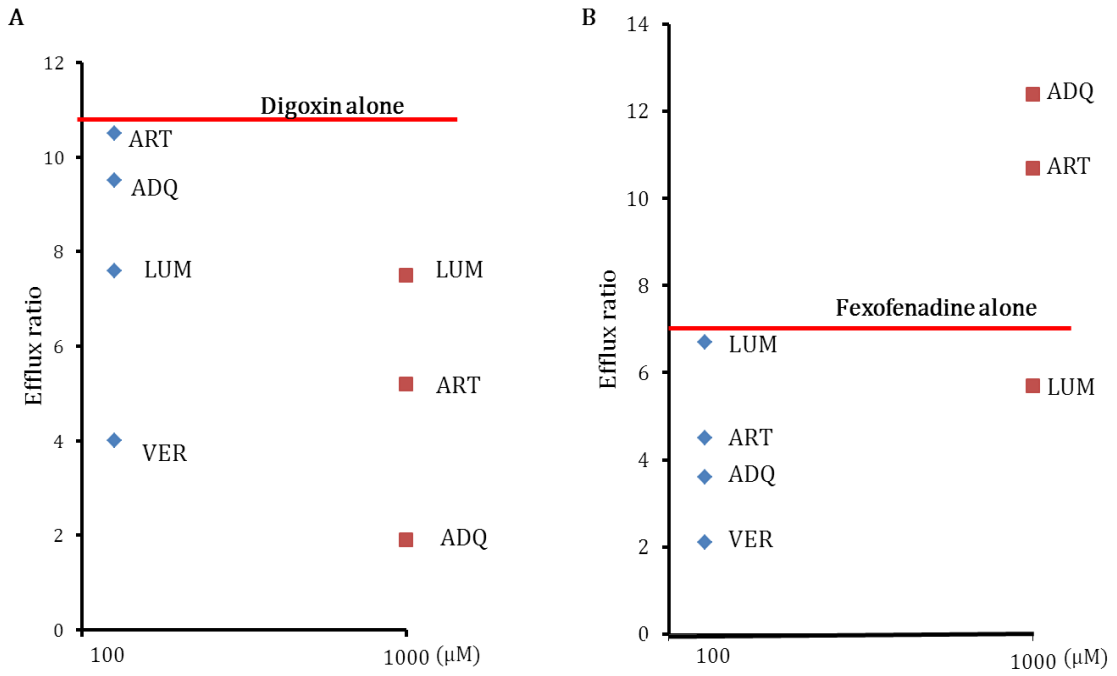

Figure 13. Efflux ratio values of digoxin and fexofenadine

The efflux ratio values of DIG and FXD alone and on co-administration with the antimalarials; lumefantrine (LUM), artesunate (ART) and amodiaquin (ADQ).

Subsequently, the DINs for ADQ, ART, and LUM on co-administration with DIG were calculated. As indicated in Figure 14, The DINs for ADQ, ART, and LUM were 46.7, 12.6, and 0.7, respectively, indicating the greatest risk of DDIs for ADQ.

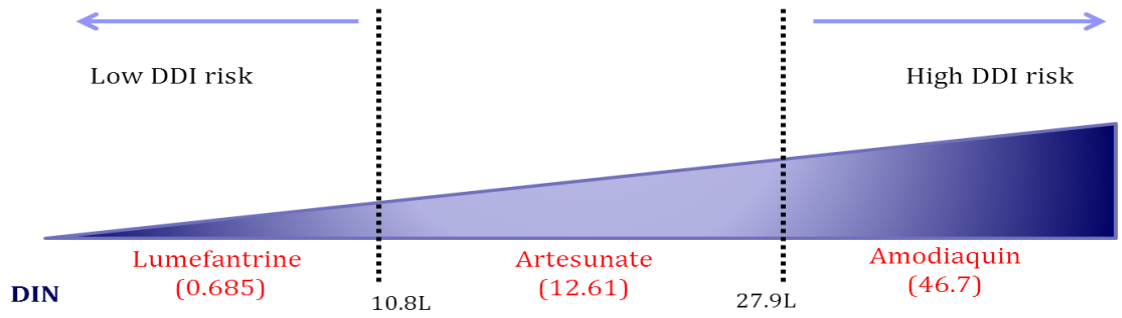

Figure 14. Drug interaction Numbers with Digoxin

The computed Drug Interaction Numbers (DIN) of the antimalarials on co-administration with Digoxin

3.2 PAPER II

To examine the transport and inhibition of [^{3}H] – DIG in Caco-2 cell monolayers, the Caco-2 cell monolayer system was validated by examining the bi-directional transport of [^{3}H] – DIG, a typical P-gp substrate. As shown in Figure 15, transport of DIG in the B-A direction was much greater than transport in the A-B direction by about seven-fold. This data confirms good expression of P-gp in the Caco-2 cell monolayers utilized as well as predominant P-gp mediated transport. P-gp inhibition was investigated by assessing bidirectional transport in the absence or presence of 100 μM VER (a typical P-gp inhibitor). In the presence of VER, there was very significant decrease (73%) in the mean B-A transport while there was significant increase (46%) in the A-B transport further confirming the P-gp inhibitory effect of VER. Transepithelial bidirectional transport of DIG was time-dependent. B-A transport studies were initially carried out for seven phytomedicines extracts at concentration range of 0.02 - 20 mg/mL and the extent of inhibition to P-gp mediated DIG transport was assessed. As illustrated in Figure 16, statistically significant inhibition was observed at concentrations of 1 - 20 mg/mL for VA and CP (with inhibition to DIG transport of 59 - 73% and 31 - 58% respectively) as well as 0.02 - 20 mg/mL for ML (with inhibition to DIG transport of 45 - 91%). CUR also showed statistically significant difference at 0.02 - 2 mg/mL. Subsequently, the A-B transport of CP, VA, and ML was further investigated. The effect of extracts of ML, CP and VA on the bidirectional transport of DIG is illustrated in Figure 17 (A and B). Increases were observed in DIG mediated A-B P-gp transport. Here, typical P-gp inhibitor; VER also enhanced the apical-to-basal transport of DIG in Caco-2 cells, suggesting that P-gp mediates the efflux transport

of DIG in the apical membranes of Caco-2 cells. In addition, as illustrated in Table 6, the ratio of B-A transport to A-B transport of CP, VA, ML and the inhibition control VER strongly suppressed the net secretory transport of DIG as evidenced by the decrease in ER when compared to the control (DIG).

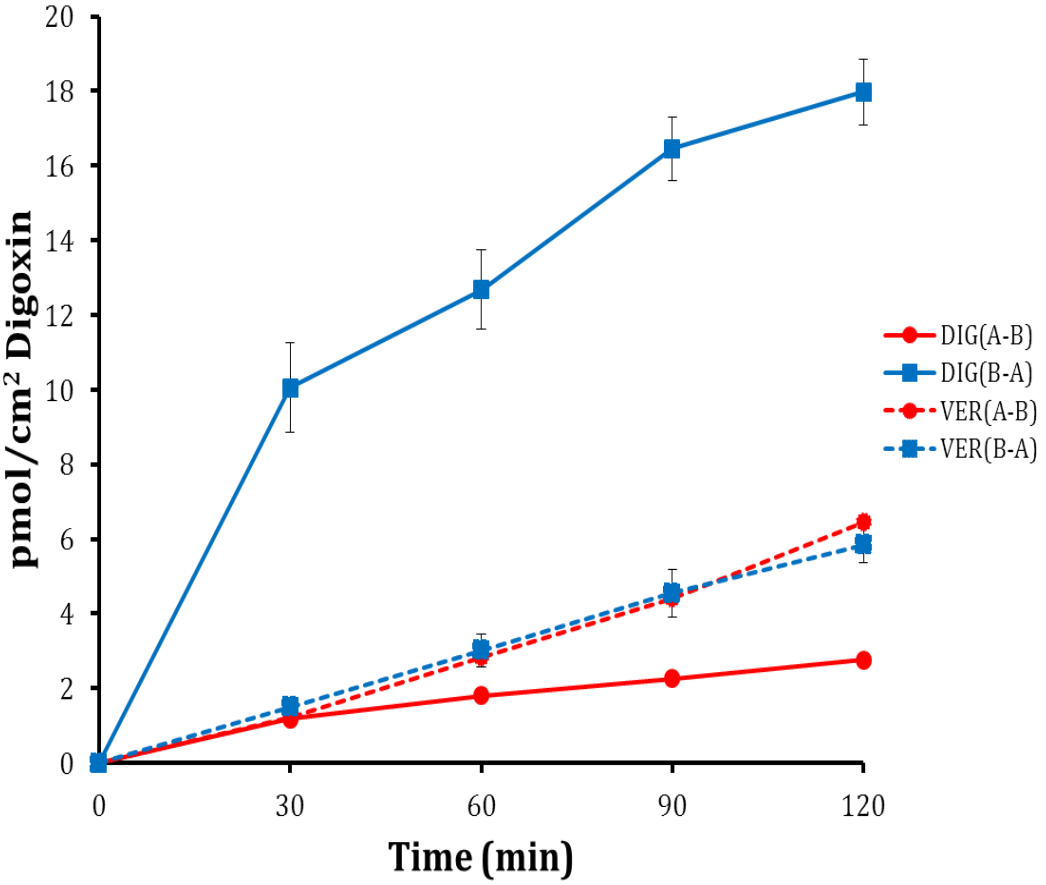

Figure 15. Digoxin transport kinetics

The bi-directional transport DIG in the absence and presence of 100 µM verapamil, VER across Caco-2 cell monolayers was determined against time. DIG transport in the basolateral to apical side was about seven-fold higher than transport in the apical-to-basolateral . Solid lines represent passage in the absence of verapamil, dashed lines represent passage in the presence of verapamil. Each data point represents the mean of 4-7 replicate assays (±SEM).

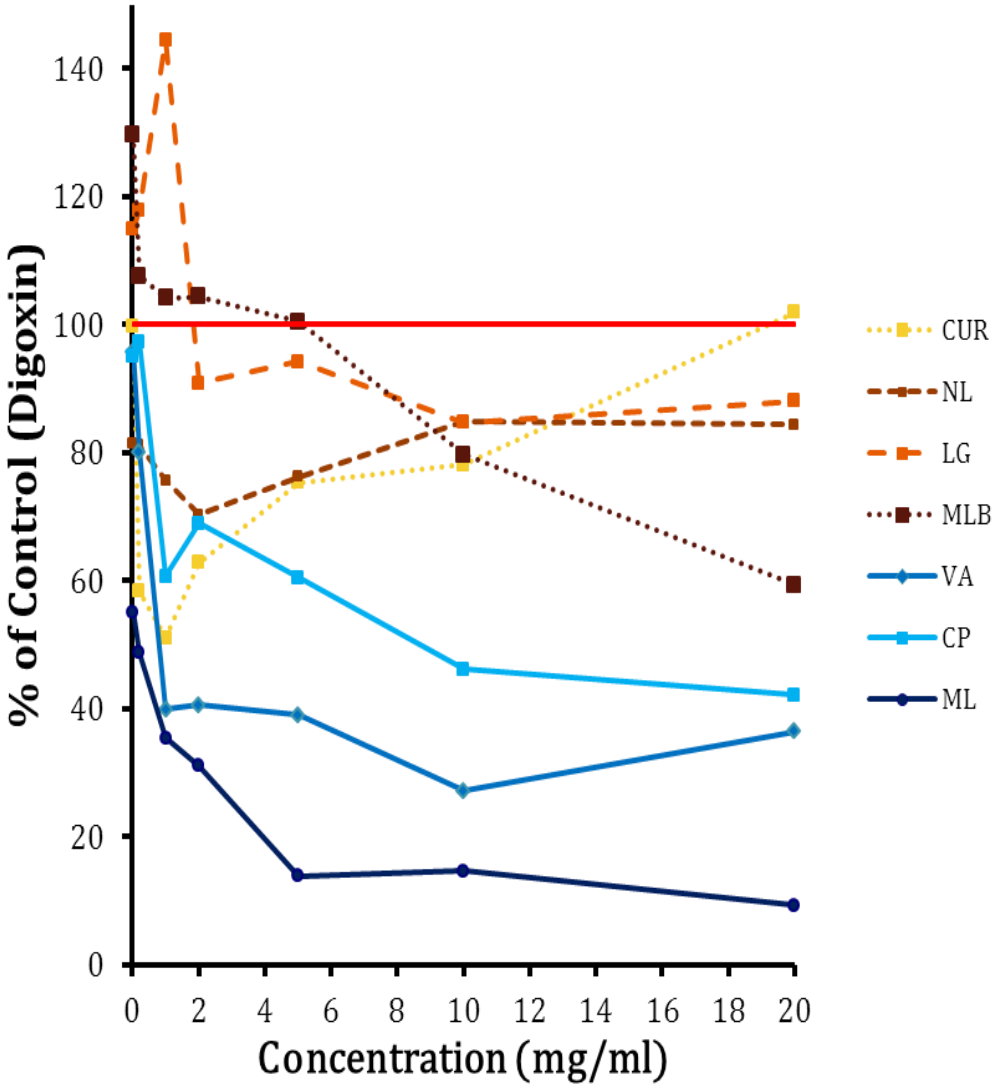

Figure 16. Basolateral-to-apical transport on co-administration with Digoxin (Dig)

Basolateral-to-apical transport of Digoxin (Solid red line) across Caco-2 cell monolayers expressed as a percentage of DIG control in the presence of 0.02mg/mL – 20 mg/mL of the respective phytomedicines; CUR, *Curcuma longa*; NL, *Azadiractha indica*; LG, *Cymbopogon citratus; MLB, Morinda lucida;* VA,*Vernonia amygdalina*; CP,*Carica papaya* and ML, Mistletoe leaves. All data are expressed as mean ±SEM of 3-4 replicates. Values of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered to be statistically significantly different from DIG control

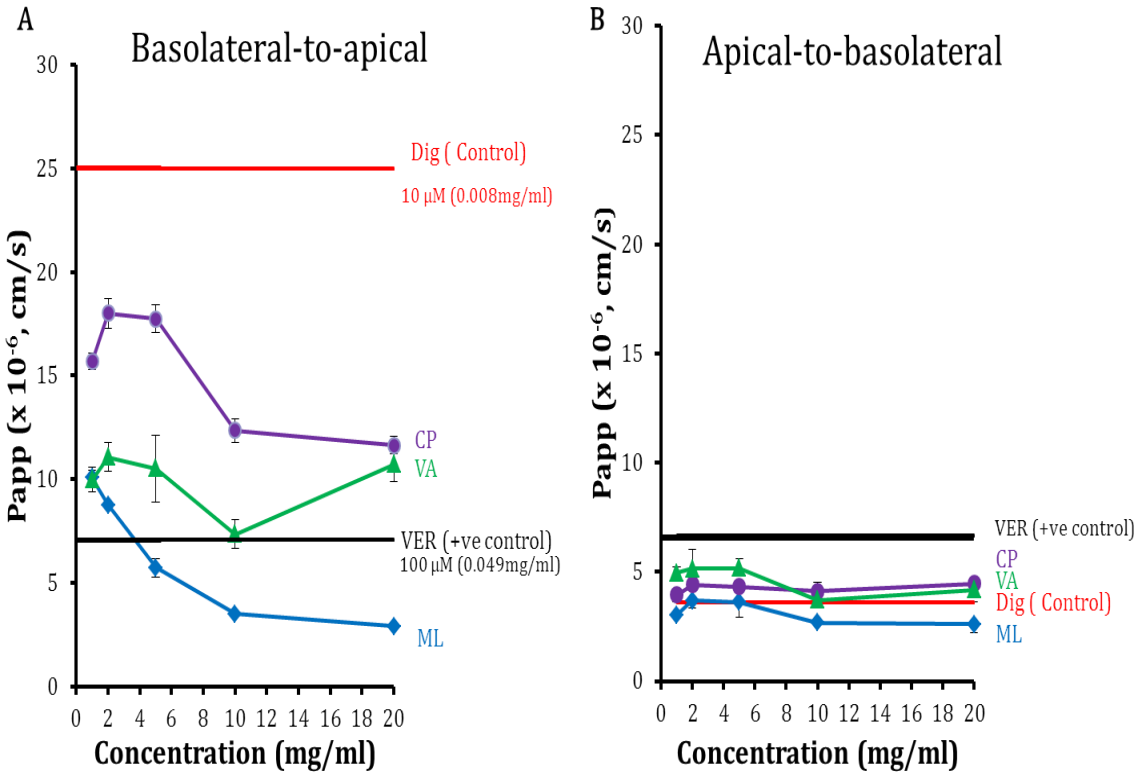

Figure 17. Bidirectional transport on co-administration with Digoxin (Dig) Bidirectional transport A) Secretory transport B) Absorptive transport of DIG; Digoxin across Caco-2 cell monolayers in the presence of 0.02mg/mL – 20 mg/mL of ML; Mistletoe leaves, CP; *Carica papaya*, VA, *Vernonia amygdalina* – the phytomedicines that initially showed significant inhibition to basolateral-to-apical transport. [A], basolateral-to-apical passage; (B), apical-to-basolateral passage. All data are expressed as mean ± SEM of 3-4 replicates.

Table 6. Permeability coefficient and efflux ratio on co-administration with digoxin

Test agent	P_{app}*10^{-6} cm/s		ER
	A-B	B-A	
DIG alone	3.60 ± 0.02	25.01 ± 1.50	6.9
DIG + ML 1 mg/mL	3.03 ± 0.06	10.07 ± 0.35**	3.3
DIG + ML 2 mg/mL	3.69 ± 0.36	8.75 ± 0.16**	2.4
DIG + ML 5 mg/mL	3.61 ± 0.69	5.72 ± 0.43***	1.6
DIG + ML 10 mg/mL	2.69 ± 0.11	3.49 ± 0.13***	1.3
DIG + ML 20 mg/mL	2.61 ± 0.39	2.90 ± 0.14***	1.1
DIG + CP 1 mg/mL	3.96 ± 0.22	15.69 ± 0.38*	4.0
DIG + CP 2 mg/mL	4.43 ± 0.09*	18.01 ± 0.71*	4.1
DIG + CP 5 mg/mL	4.33 ± 0.05*	17.74 ± 0.68*	4.1
DIG + CP 10 mg/mL	4.11 ± 0.4	12.35 ± 0.58**	3.0
DIG + CP 20 mg/mL	4.47 ± 0.03*	11.64 ± 0.41**	2.6
DIG + VA 1 mg/mL	4.96 ± 0.29**	9.98 ± 0.59**	2.0
DIG + VA 2 mg/mL	5.15 ± 0.89*	11.07 ± 0.71**	2.1
DIG + VA 5 mg/mL	5.18 ± 0.41**	10.51 ± 1.61**	2.0
DIG + VA 10 mg/mL	3.72 ± 0.16	7.35 ± 0.68**	2.0
DIG + VA 20 mg/mL	4.19 ± 0.08*	10.72 ± 0.83**	2.6
DIG + 100 µM VER	6.69 ± 0.25***	7.07 ± 0.99***	1.1

Apparent permeability coefficient (Papp) and efflux ratio (ER) of digoxin (DIG) in combination with leaf extracts of *Tapinanthus sessilifolius* (ML), *Carica papaya* (CP), *Vernonia amygdalina* (VA), and Verapamil (VER) at the indicated concentrations in the apical-to-basolateral (A-B) and basolateral-to-apical (B-A) direction across a Caco-2 cell monolayer.
Significant difference *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus DIG control.

In order to confirm that the observed inhibitory effects of the extracts were not due to their cytotoxicity, *in vitro* cytotoxicity studies were carried out with HBSS as the

control medium. The MTT test was conducted to assess the concentration-dependent cytotoxic effects of ML, CP, VA, VER and 10 µM DIG. Figure 18 shows a statistically significant increase in cell viability for 5 and 10 mg/mL ML and 1 and 5 mg/mL VA when compared with the HBSS control. No cytotoxic reduction in cell viability was significant. DIG and VER did not significantly alter cell viability (data not shown). From this data, none of the phytomedicines was found to be cytotoxic to the cells at the concentrations examined.

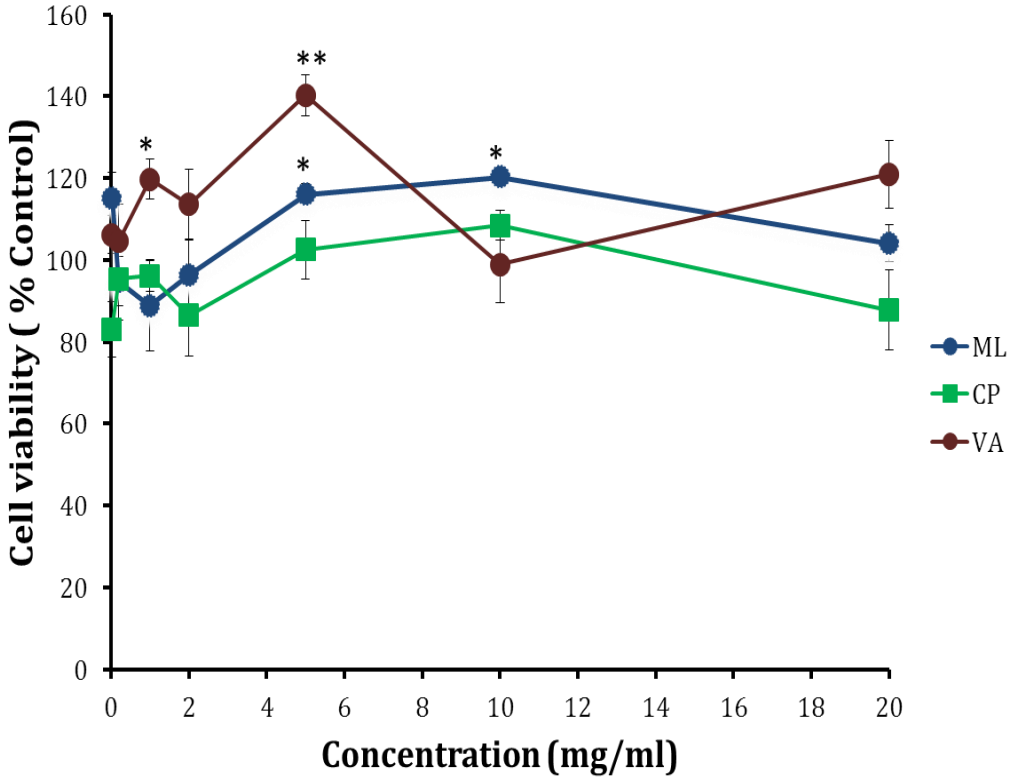

Figure 18. Cell viability of the investigated herbal extracts

Cell viability (percentage of HBSS control) for 0.02mg/mL -20mg/mL of the phytomedicines (ML, Mistletoe leaves ;CP,*Carica papaya*; VA,*Vernonia amygdalina*). All data are expressed as mean ± SEM of 3-4 replicates. Values of * $p < 0.05$ and ** $p < 0.01$ were considered to be statistically significantly different from the HBSS

control

3.3 PAPER III

3.3.1 Tissue integrity and viability.

The electrophysiological parameter TEER of the ileal segments was monitored throughout the experiments to assess the maintenance of tissue viability and integrity. The values were measured before and during the course of each permeation experiment. The TEER ranged from 55 to 75 $\Omega.cm^2$ prior to the introduction of the test agents. Any tissue segment that showed a deviation of ±15 % from this range was excluded from the experiments. The TEER values were stable with no significant variation following introduction of the test agents. Tissue viability in the Ussing chamber decreases over time; therefore, sampling times did not exceed 120 min.

3.3.2 Mucosal-to-serosal transport across the rat ileal tissue.

The time-course of the percentage of digoxin transported in the absorptive direction (mucosal-to-serosal) in the presence or absence of the test agents and the control inhibitors (VER and CysA) is illustrated in Figure 19. Digoxin was used as the model P-gp substrate as it is clinically relevant. The mean apparent permeability values obtained 120 min after the introduction of digoxin are shown in Figure 20 in comparison to the control (digoxin alone). The increase in Papp was highest following treatment with the positive control inhibitor, VER, where Papp was significantly 56 % higher than in the control. In comparison to the control, Papp was significantly higher, by 54.5, 43.3, 16.2 and 7.8 % following treatment with CP, VA, CysA, and ML, respectively.

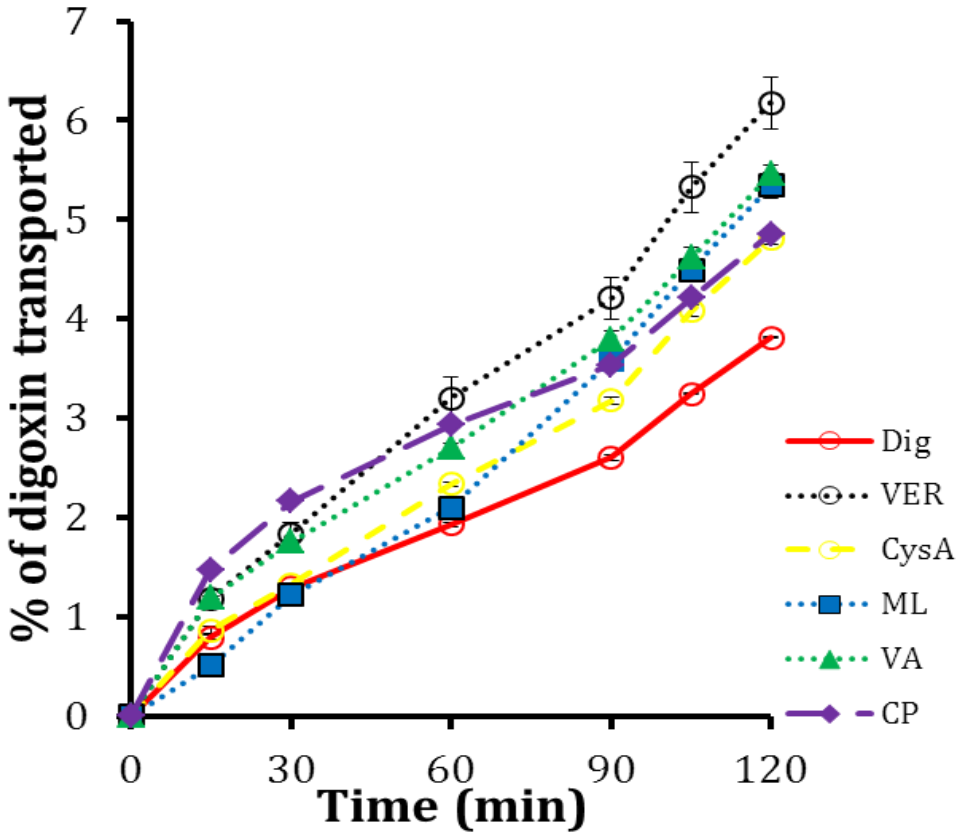

Figure 19. Time course of the absorptive transport of digoxin (Dig)

Time course of the percentage of digoxin (Dig) transported in the absorptive (mucosal-to-serosal) direction with or without verapamil (VER), cyclosporin A (CysA) and the herbal extracts; *Vernonia amygdalina* (VA), *Carica papaya* (CP) and *Tapinanthus sessilifolius* (ML). The intestinal permeability of the herbal extracts as well as the inhibitor controls was determined using the rat ileum segment in an *ex vivo* assay utilizing the Ussing chamber. Each value represents mean ± SEM (n = 4).

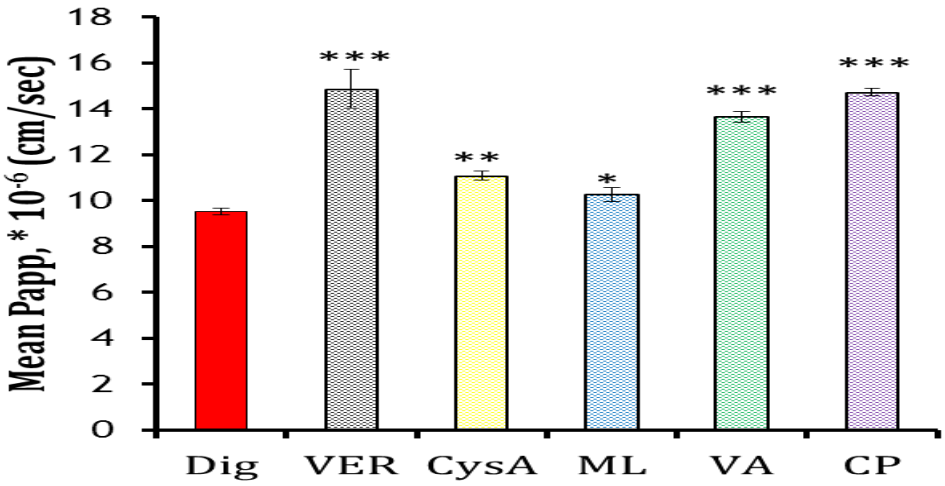

Figure 20. Mean apparent permeability coefficients for digoxin (Dig)
Mean apparent permeability coefficients obtained for digoxin (Dig) in the absorptive direction (mucosal-to-serosal) at 120 min in the presence and absence of verapamil (VER), cyclosporin A (CysA), extracts of *Vernonia amygdalina* (VA), *Carica papaya* (CP) and *Tapinanthus sessilifolius* (ML). Each value represents mean ± SEM (n = 4). Significant differences of *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ were determined using one-way *analysis* of variance *with Dunnett's* post-test for multiple comparisons.

3.3.3 *In vivo* pharmacokinetic study.

To determine whether the inhibition of P-gp observed *in vitro* (in Caco-2 cells) and *ex vivo* (in Ussing chambers) also occurs *in vivo*, pharmacokinetic parameters of digoxin with or without co-administration of the herbal extracts were determined in male SD rats. The mean (±SEM) plasma digoxin concentration versus time profiles after a single dose of oral digoxin was administered alone or was co-administered with ML, CP, VA, or VER are illustrated in Figure 21 (A-D). The pharmacokinetic parameters are summarized in Table 7.

ML administration non-significantly increased the $AUC_{(0-3)}$ and $AUC_{(0-\infty)}$ of digoxin by 1.2- and 1.4-fold, respectively, compared to the control group (Figure 21A). C_{max}

was 1.1-fold higher in the ML-treated group than in the control group. $T_{1/2}$ increased from 2.0 h to 2.7 h following ML administration. Figure 21B depicts the influence of CP on digoxin pharmacokinetics. Co-administration of CP non-significantly increased the $AUC_{(0-3)}$ and $AUC_{(0-\infty)}$ of digoxin by 1.1- and 1.3-fold, respectively, compared to the control group. C_{max} was 1.2-fold higher in the CP-treated group than in the control group. $T_{1/2}$ increased from 2.0 to 2.7 h following CP administration. As demonstrated in Figure 21C, the effects of VA on digoxin pharmacokinetics were detected within the first hour following digoxin administration. Co-administration of VA increased the $AUC_{(0-3)}$ and $AUC_{(0-\infty)}$ of digoxin by 1.9- and 2.1-fold, respectively, compared to the control group. C_{max} was 1.7-fold higher in the VA-treated group than in the control group. VA administration did not significantly affect clearance or $T_{1/2}$.

VER inhibits P-gp and thereby reduces digoxin transport. VER was used as a positive control in this study. Figure 21D illustrates the impact of VER on the pharmacokinetics of digoxin. As expected following administration of a P-gp inhibitor, VER significantly increased both the $AUC_{(0-3)}$ and $AUC_{(0-\infty)}$ of digoxin by 1.5-fold compared to the control. VER administration did not significantly affect $T_{1/2}$, although there was a non-significant reduction in the clearance compared to the control group. C_{max} was significantly higher (1.3-fold) in the VER-treated group than in the control group. The absorption rate constant (K_a) increased 1.2-, 1.3-, and 1.4-fold following administration of ML, CP, and VA, respectively, compared to the control group. Therefore, of the herbs studied, VA exerted its inhibitory effect on digoxin transport at the shortest time after administration.

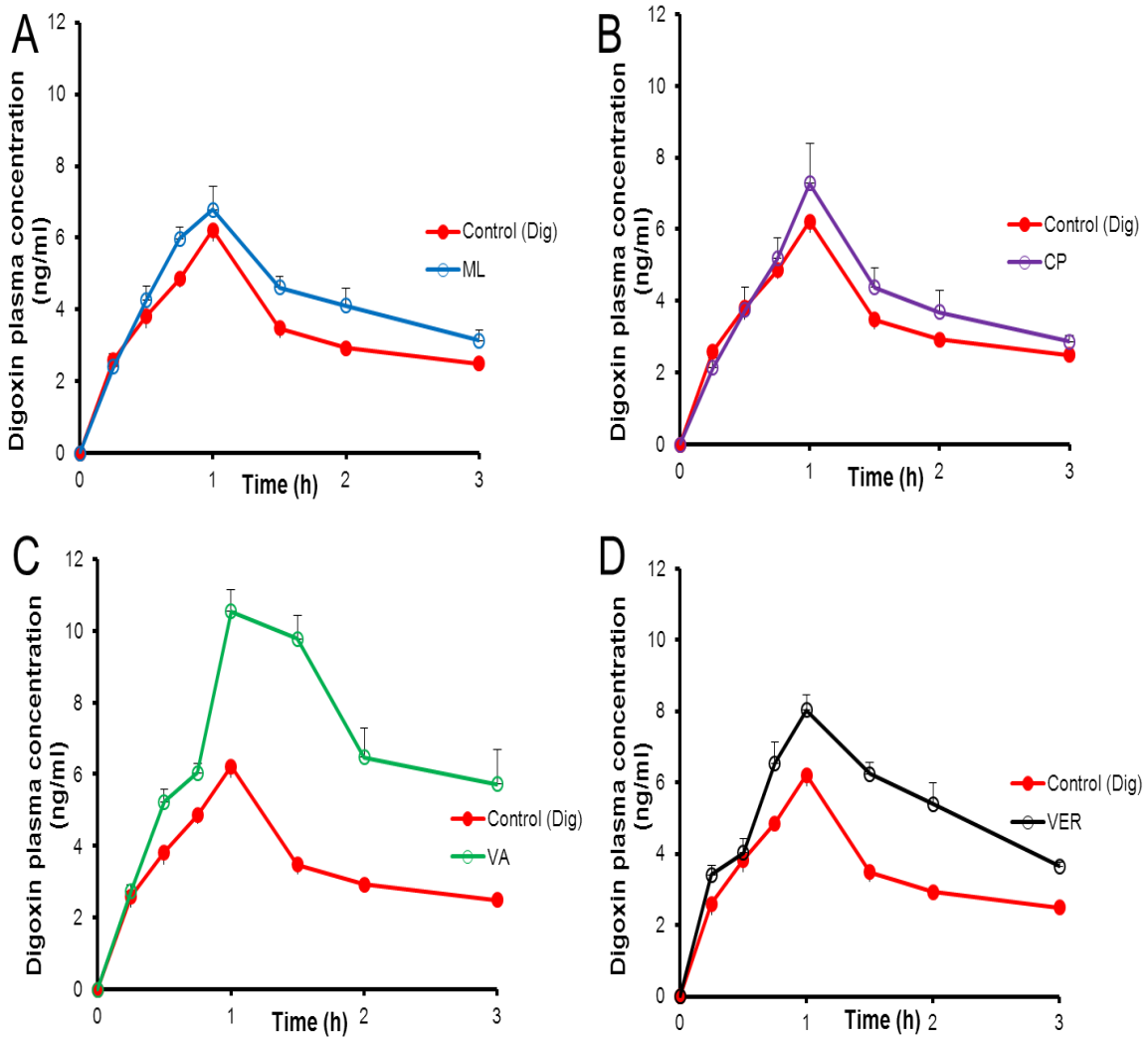

Figure 21. Plasma concentration-time curves

Mean plasma concentration-time curves in Sprague Dawley rats after orally administered digoxin (25.6umol/kg); *closed circles* when co-administered with (A) *Tapinanthus sessilifolius,* ML (B) *Carica papaya,* CP (C) *Vernonia amygdalina,* VA (D) Verapamil, VER; *open circles*. The pre-treated groups were orally administered a dose of 90mg/kg for ML, CP and VA or 25mg/kg for VER. They were administered 2 h prior to digoxin dosing. Error bars represent SEM, (n=5-9/group)

Table 7. Pharmacokinetic parameters of digoxin

Pharmacokinetic parameter	DIG	+ ML	+ CP	+VA	+VER
AUC_{0-3} (ng/ml/h)	10.34 ± 0.71	12.66 ± 1.18	11.88 ± 1.63	20.01 ± 3.66*	15.49 ± 2.27*
$AUC_{0-\infty}$ (ng/ml/h)	17.69 ± 2.25	24.65 ± 4.65	23.04 ± 3.38	36.53 ± 8.37*	25.78 ± 5.01*
CL (L/kg/h)	3.66 ± 0.11	2.94 ± 0.08	3.45 ± 0.11	3.64 ± 0.09	3.01 ± 0.03
C_{max} (ng/ml)	6.21 ± 0.3	6.78 ± 0.7	7.28 ± 1.1	10.55 ± 1.9*	8.03 ± 0.9*
K_a (h^{-1})	1.24 ± 0.16	1.45 ± 0.23	1.58 ± 0.20	1.68 ± 0.08	1.23 ± 0.10
$T_{1/2}$, el(h)	2.04 ± 0.11	2.65 ± 0.08	2.70 ±0.11	1.99 ± 0.11	1.95 ± 0.03

Pharmacokinetic parameters of digoxin after oral treatment with and without ML, CP, VA (90mg/kg) or VER (25mg/kg) in Sprague Dawley rats. (AUC, Area under the concentration-time curve; C_{max}, peak concentration; K_a, absorption rate constant; $T_{1/2}$, elimination half-life; CL, total clearance). Each value represents the mean ± SEM of 5-9 rats. *statistical significant difference compared to the control rats, $p < 0.05$

3.4 PAPER IV

3.4.1 Enzyme kinetics of artemisinin metabolism in HLM

Figure 22 denotes the main metabolic pathway for artemisinin. Artemisinin (10 -1000μM) was incubated in HLM for 2min at 37°C and the metabolic rate-concentration profile is shown in Figure 23. The kinetic parameters V_{max} and K_m were obtained as 9.54 ± 3.52 mmol/min/mg microsomal protein and 922 ± 582 μM respectively.

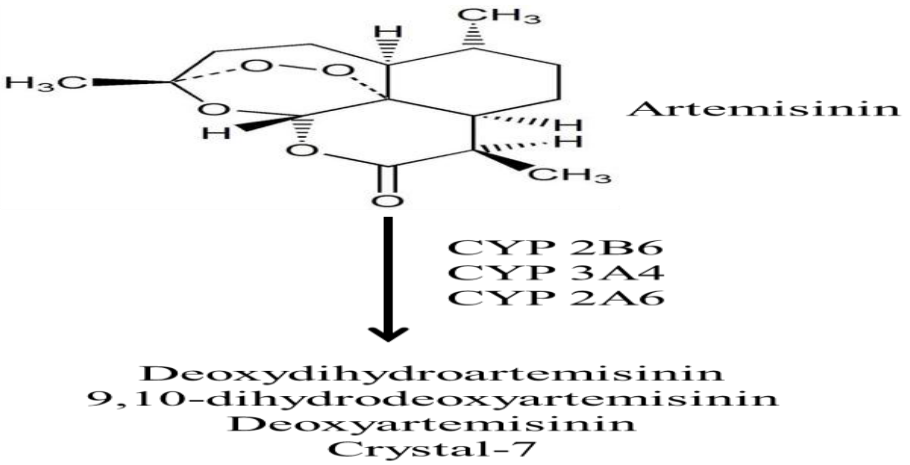

Figure 22. Scheme for the oxidative metabolism of artemisinin.
The major pathways of artemisinin hydroxylation and deoxylation are illustrated

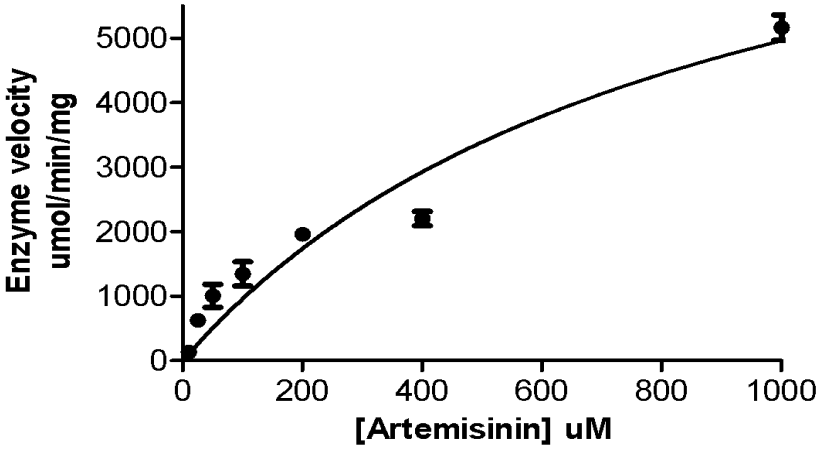

Figure 23. Artemisinin kinetics in human liver microsomes.
Kinetics for the disappearance rate of artemisinin in human liver microsomes (HLM). An increasing concentration of artemisinin (10 – 1000 μM was incubated in HLM (0.1 mg/ml) and an NADPH-generating system for 2 min at 37 ^{o}C. The figure shows curves for the rate of disappearance of artemisinin versus artemisinin concentration where data were fitted into the Michaelis-Menten equation. Each data point represents the mean of triplicate incubations.

3.4.2 Inhibition of artemisinin's metabolism by antiretrovirals.

The effect of various antiretroviral agents (RTV, SQV, NLF and NVP) on the rate of

disappearance of artemisinin was investigated (Figure 24). As shown, RTV (0.1 to 100 μM) showed significant inhibition of the metabolism of artemisinin. SQV (0.3 to 100 μM) reduced the metabolism of artemisinin to 23.7 – 37.8 % of control, but significant inhibition was not observed. NVP (0.1 to 300 μM) showed potent inhibition to artemisinin's metabolism at 0.1, 1, 100 and 300 μM.

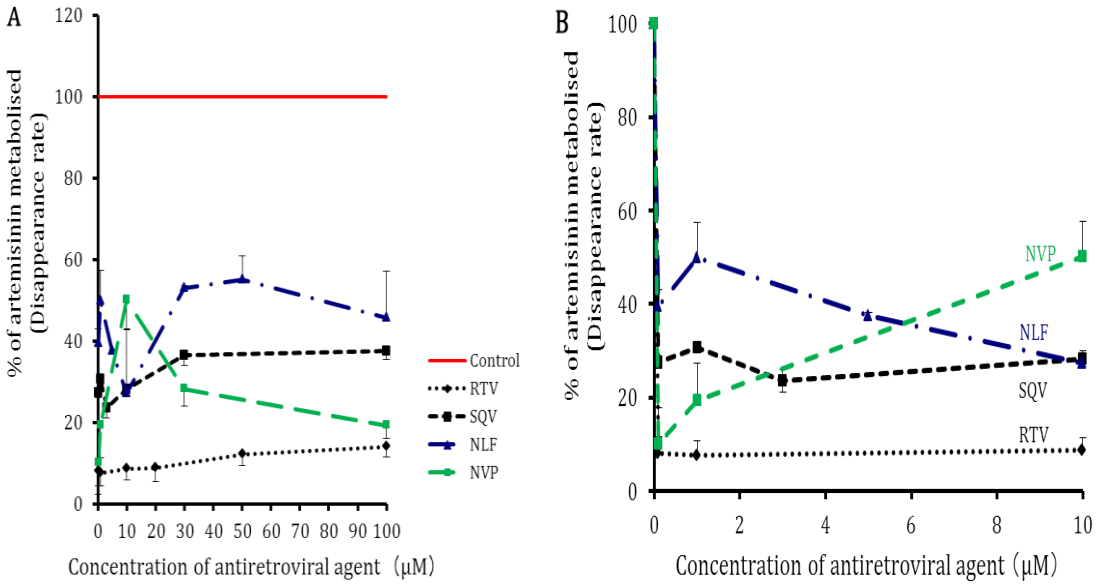

Figure 24. HIV antiretroviral influence on the metabolism of artemisinin
Effect of the antiretrovirals- Saquinavir (SQV), Ritonavir (RTV), Nevirapine (NVP) and Nelfinavir (NLF) on the rate of metabolism of artemisinin expressed as a percentage of the control. Panel B illustrates their influence at concentrations < 10μM

3.4.3 Contribution of CYP3A4 to the metabolism of artemisinin in HIM.

CYP2B6 and 3A4 are known to be the main enzymes responsible for the metabolism of artemisinin. In the study, the contribution of CYP3A4 was examined using ketoconazole (KTZ), a potent CYP3A-selective inhibitor. In order to ascertain

the contribution of intestinal CYP3A enzymes to artemisinin's metabolism, the percentage of artemisinin remaining unmetabolized after incubation with KTZ (0, 0.1 and 1 μM) in HIM was examined. Figure 25 illustrates the percentage of artemisinin unmetabolized after a 10-min co-incubation period. The presence of 0.1 and 1 μM KTZ, contributed ~6-49 % respectively to the intestinal metabolism of artemisinin, in comparison to that obtained in the absence of the inhibitor. At concentrations of 1 μM or less, CYP3A is the main enzyme inhibited. Also, 80 % of artemisinin was metabolized in the absence of KTZ. In humans, KTZ plasma concentration ranges from 1.4 to 4.5μM [195].

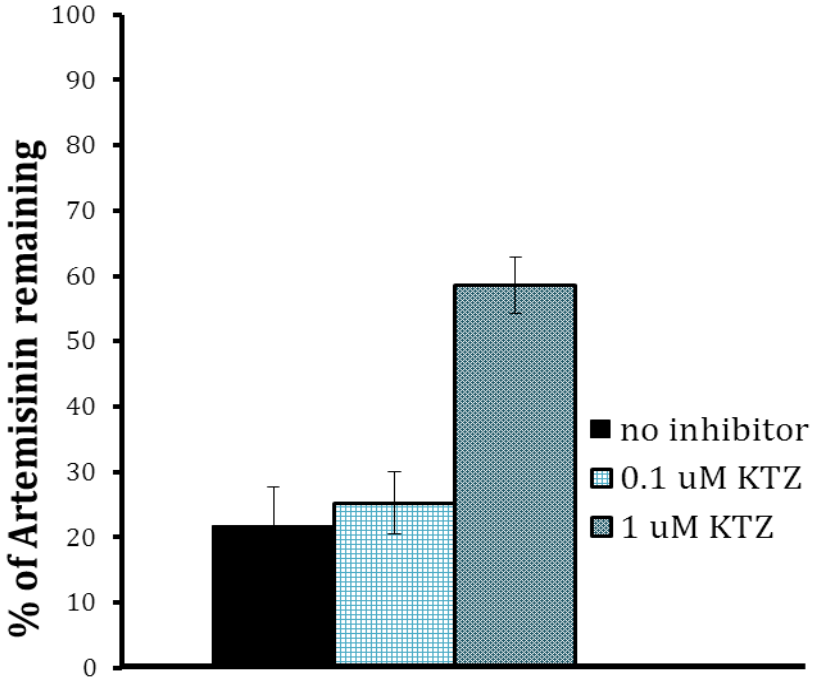

Figure 25. CYP3A contribution to the intestinal metabolism of artemisinin. Plot estimating the relative contribution of CYP3A to the intestinal metabolism of artemisinin in human intestinal microsomes (HIM). 0, 0.1 and 1μM ketoconazole was incubated in HIM (0.1 mg/ml) and an NADPH-generating system for 10 min at 37 °C. The figure shows the percentage of the disappearance rate of artemisinin. Each data point represents the mean of triplicate incubations.

4. DISCUSSION

4.1 PAPER I

The inhibition and induction of transporters that mediate the uptake and efflux of xenobiotics are important mechanisms underlying DDIs. The P-gp transporter is known to modulate intestinal absorption of its substrates. The expression of P-gp on the apical membrane of mucosal cells may play an important role in extruding orally administered drugs into the intestinal lumen, resulting in lower bioavailability of pharmaceutical agents. Therefore, the potential of intestinal P-gp-mediated DDIs of antimalarials with P-gp substrates was examined in this study. The test concentrations of the antimalarials (100 µM and 1 mM) were chosen to simulate theoretical drug concentrations in the gut, which were determined in the present study to be 5.16 mM for ADQ, 2.08 mM for ART, and 3.63 mM for LUM. These theoretical concentrations were calculated by dividing the maximum therapeutic dose by 250 mL (assuming complete dissolution in a glass of water)[196]. VER, a widely used P-gp inhibitor, was employed as a positive inhibitor control at a concentration of 100 µM.

A preliminary screening was first carried out using seven antimalarial drugs in a Rho-123 transport assay to investigate their potential as P-gp inhibitors or non-inhibitors. Of the seven antimalarials initially screened, ART, ADQ, and LUM showed the most efficacious blockade of Rho-123 transport in the B-A direction across a Caco-2 cell monolayer. This observation confirms previous reports that demonstrated an inhibitory effect of ADQ against P-gp-mediated transport in Caco-2 cells, as well as an inhibitory effect of ART in K562/adr and GLC4/adr resistant cell lines [197, 198].

Next, the impact of ART, ADQ, and LUM on DIG transport was evaluated. In this

study, all three antimalarials inhibited DIG transport at concentrations of both 100 μM and 1 mM. ADQ and ART (1 mM) exhibited potent inhibition that was comparable with that of VER (100 μM). Under the conditions of this study, LUM was shown to be a relatively weak P-gp inhibitor, even at a concentration of 1 mM. This was further exemplified by the similar ERs obtained with LUM at 100 μM and 1 mM (7.6 and 7.5, respectively).

FXD (the active metabolite of terfenadine) is commonly used as a P-gp probe agent with minimal hepatic and enteric biotransformation. Due to the increase in the global incidence of allergic diseases, there is an increased potential for drug interactions involving FXD. Evidence to support the role of human organic anion transporting polypeptide (OATP) drug uptake transporters and the drug efflux transporter, P-glycoprotein (P-gp) in the cellular uptake and excretion of FXD has been reported [185]. Some recent studies have implicated MRP2 and MRP3 in FXD transport [199-201]. Although a review of clinical trials indicates that grapefruit juice, orange juice and apple juice can reduce oral bioavailability of FXD with a potential of reducing pharmacodynamic effects of FXD, the clinical importance of the interaction is not clearly established [202]. Elevated FXD concentrations in volunteers co-administered FXD together with erythromycin or ketoconazole has been well described. These results and other recent evidence show that these drug interactions occur through non-CYP-mediated mechanisms [203]. In this study, at anti-malarial concentration of 100μM, LUM exhibited non-significant inhibitory effect on FXD transport. Both ADQ and ART inhibited FXD transport when co-administered. However at 1000μM anti-malarial concentration, ART and ADQ enhanced FXD transport. Its mechanism is unclear however a plausible mechanism

may be due to the contribution of multiple transporters including P-gp, OATP, MRP2 and MRP3 mediation [204]. A similar increase in basolateral-to-apical FXD transport was reported when co-administered with 10µM erythromycin as well as 1µM and 10µM Rifamycin possibly due to the inhibition of both OATP and P-gp. However inhibition of OATP and P-gp caused a reduction in apical-to-basolateral transport of FXD [205]. Estrone-3-sulphate also decreased the apical uptake of FXD [204]. Inhibition of efflux transporters including MRP3 may result in increase in basolateral-to-apical transport with minimal effects on apical-to-basolateral transport. An explanation of the contribution of uptake and efflux transporters on FXD in Caco-2 cell monolayers is illustrated in Figure 26.

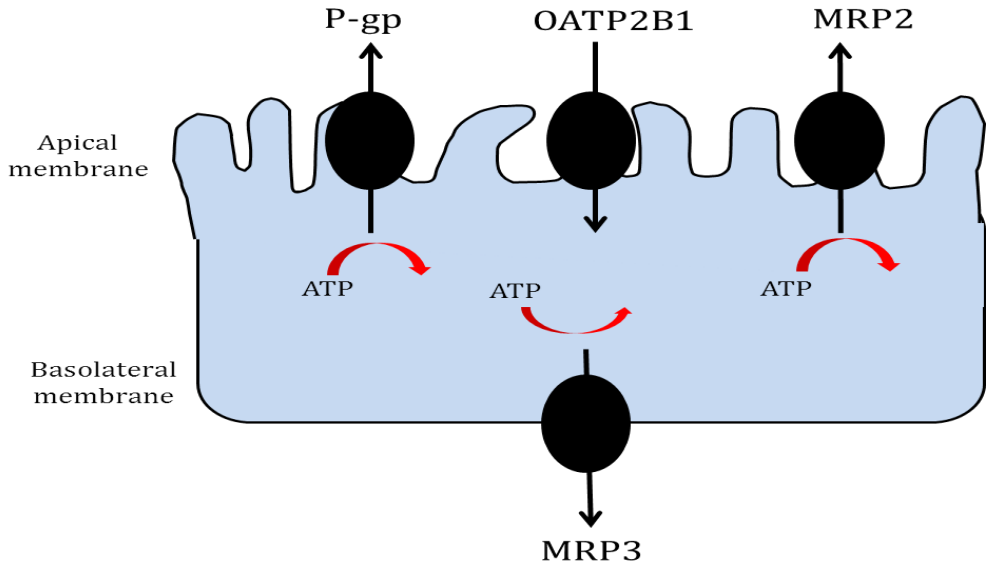

Figure 26. Transporters modulated by fexofenadine in Caco-2 cells

In FXD transport, MRP3 plays an important role in its basolateral-to-apical transport while P-gp and OATP are vital for its apical-to-basolateral transport. Due to the contribution of various transporters to FXD, an inhibition of uptake transport on the apical membrane or an inhibition of the efflux transporter on the basolateral

membrane will result in a decrease in the $P_{app\ A\text{-}B}$ as well as an increase in $P_{app\ B\text{-}A}$ as observed with some concentrations of the antimalarials. However, the involvement of P-gp in drug interactions might be difficult to prove in humans due to the overlapping substrate specificity of inducers and inhibitors of CYP3A4 and P-gp and also because some drug interactions involve CYP3A4 enzymes, P-gp as well as other transporters.

Tachibana *et al.*[126] described the DIN as an index for predicting DDI potential (Eq. 3, section 2.1.4). It was reported that P-gp inhibitors with a DIN below 10.8 have a low risk of interacting with P-gp substrates, while those with a DIN above 27.9 present a high risk. Therefore, the DINs for the potential interaction of the antimalarials with DIG were calculated. From these determinations, the DIN for ADQ (46.7) suggests a high potential for DDIs involving ADQ. The calculated DIN for ART was 12.6, suggestive of moderate DDI risk. LUM had a DIN of 0.7, suggestive of low DDI risk. This implies that, although all three antimalarials inhibited P-gp-mediated DIG transport under the study conditions, there may be a higher risk of clinically relevant DDIs with ADQ and ART than with LUM. Since a higher DIN tends to correlate with a higher AUC ratio [126], it may cause toxicity when drugs with narrow therapeutic indices are involved. In addition, there is a likely increase in bioavailability when P-gp substrate drugs are co-administered with ADQ or ART. DIN as an index for predicting DDI potential is similar to FDA's draft guidance. For P-gp interactions, the guidance recommends an evaluation for potential clinical DDIs when $[I_2]/ IC_{50}$ is greater than or equal to 10 [206]. Here, $[I_2]$ is the theoretical maximum gastrointestinal concentration. ADQ besides being used as an effective antimalarial especially in chloroquine-resistant malaria is also known to possess

potent anti-inflammatory and antipyretic properties. There is therefore the potential of several DDIs occurring. In addition, ART (which has a moderate DDI risk from the computed DIN) belongs to the class of artemisinin-based antimalarials, which is WHO's recommended first line therapy when used in combination with other antimalarials. It is therefore widely used and may pose a higher intestinal DDI potential when orally administered due to the higher exposure levels in the intestine.

In clinical settings, P-gp has been implicated in the disposition of some human immunodeficiency virus-1 (HIV-1) protease inhibitors (e.g., amprenavir, indinavir, nelfinavir, and saquinavir) [207, 208]. Because malaria and HIV share a wide overlap in their socio-economic and geographical areas of occurrence, many locales with a high malaria burden also have high a HIV burden. Thus, co-administration of antimalarials with antiretroviral drugs is common. Therefore, there might be an increased likelihood of DDIs when HIV protease inhibitors are co-administered with LUM, ART, and ADQ, which may in turn require dose adjustments of the antimalarial or the antiretroviral agent, or both. In addition, colchicine, a drug used to treat gout and a well-known P-gp substrate, may be co-administered with antimalarials such as CQ, which can also be used to treat gout. Colchicine is notorious for its narrow therapeutic index, and hence DDIs with antimalarials may result in an increased risk of colchicine-induced toxicity. This potentially clinically relevant DDI may also require dose adjustments [209].

Previous studies have shown that some antimalarial drugs exhibit low absorption after oral administration, leading to their classification as P-gp substrates or inhibitors [210-212]. Current malaria treatment guidelines recommend combination

therapy, preferably ART-based [213] Thus, ART (as well as ADQ and LUM) are clinically used in combination with additional antimalarial drugs and non-antimalarial drugs that, as described above, may be P-gp substrates. This could result in unexpectedly high therapeutic outcomes for the co-administered drug, again necessitating dose adjustments to maximize therapeutic efficacy. Although several clinical reports detail treatment failure and antimalarial drug resistance, these reports have mainly emphasized mutations that mediate resistance in malarial parasites [214-216]. However, the current study brings new light to the fact that altered therapeutic outcomes might be due to DDIs between these antimalarials and other drugs that interact with P-gp. Poor oral bioavailability (as is the case with most P-gp substrates) with a resultant wide range in blood drug levels will favor the emergence of resistance because doses are mainly chosen based on therapeutic ratios. Co-administration of antimalarial P-gp substrate drugs with antimalarials that are P-gp inhibitors may improve oral bioavailability of the former and thereby reduce doses that are needed to clear infection [217]. This is expected to reduce the cost of therapy, as well as the emergence and spread of resistance.

4.2 PAPER II

In recent years, there has been a global increase in the demand for plant derived products. However there are only few reports on potential of these phytomedicines interacting with transport proteins. In this study the potential of phytomedicines commonly used for malaria treatment to encounter a p-glycoprotein mediated intestinal interaction was examined as it is known that many patients on conventional malaria therapy alter their diets, include supplements or

phytomedicines with an aim of achieving better therapeutic outcomes [93]. Here, whole herbal extracts were used rather than isolated constituents. This is because most herbal remedies are taken whole as a mixture of constituents and not just as isolated components [218, 219]. Also, there is an uncertainty over the particular component that may be implicated in a drug interaction. For the selection of test doses, the test concentrations of 0.02, 0.2, 1, 2, 5, 10, and 20 mg/mL of the extracts were used to simulate theoretical intestinal concentration. This was obtained by dividing the highest recommended daily doses in literature by 250mL (anticipating complete dissolution of the dose in 250mL of water) [196]. Since P-gp inhibitors when orally administered are known to cause high levels of exposure in the intestine and may cause DDIs in the intestine rather than in the liver, higher doses of 1mg - 20mg/mL are more likely to be the relevant intestinal concentration range of these phytomedicines while lower doses may relate to the plasma concentration.

From the initial B-A studies, CUR, VA, CP, and ML at almost all the concentrations investigated exhibited significant inhibition of ^{3}H-DIG mediated P-gp transport with ML being the most potent inhibitor. Transport of DIG in the A-B direction was enhanced by VA, CP and ML as well as the P-gp inhibitor VER. This increase further suggests their P-gp inhibitory role. On the other hand NL, LG and MLB did not significantly inhibit B-A ^{3}H-DIG mediated P-gp transport.

ML showed significant concentration-dependent inhibition to P-gp-mediated transport of DIG at 0.02 - 20 mg/ml. Another specie of mistletoe- *Viscum album* has previously been suggested to be an inhibitor at concentrations below 0.022mg/mL [220]. It is also very well known for its use in cancer therapy and epilepsy [221]. In addition to some antimalarials which are P-gp substrates (quinine, chloroquine,

quinidine), several orally administered anticancer agents (etoposide, tamoxifen, topotecan, everolimus, imatinib, lapatinib, nilotinib) are also P-gp substrates. Hence, there may be a higher likelihood of drug interactions on co-administration of ML with some of these conventional antimalarial or chemotherapeutic agents especially as some of them have narrow therapeutic indices. These interactions are expected to affect therapeutic outcomes which may be either beneficial or detrimental to the patient.

Although there are well documented reports on the ethnopharmacological use of VA for malaria [222)] and for its potent hypoglycaemic effects in diabetes mellitus [223)], this is the first known report on its interaction with P-gp. In this study VA significantly ($p < 0.01$) inhibited P-gp at 1 - 20 mg/mL. There were increases in mean A-B transport at all concentrations examined. This further suggests the P-gp blockade effect of VA. Conventional antidiabetic and lipid lowering drugs that are P-gp substrates and which may be concomitantly used with VA include glyburide, sitagliptin, saxagliptin and glibenclamide. An interesting observation is VA's extensive use as a vegetable in diets in various regions in Africa. These factors are expected to cause drug-phytomedicine and drug-food interactions which previously might have been noticed but the underlying mechanism uninvestigated.

There are several reports on the ethnomedicinal uses of CP leaves. Evidence for the use of its decoctions as an antimalarial, cancer therapeutic, antihelminthic, antioxidant abounds in literature [82, 224)]. This suggests the possibility of toxicity when CP leaves and conventional P-gp substrate drugs are co-administered. In this study, curcumin at test concentrations of 0.02mg/mL - 2mg/mL showed significant inhibition to P-gp mediated DIG transport. However at 20mg/mL, there was a

non-significant increase in B-A DIG transport. Its A-B transport was not investigated as earlier reports on the P-gp inhibitory effect of curcumin in multidrug resistant human cervical carcinoma cell line KB VI, L-MDR1 and LLC-PK1 have been documented previously [225-227]. Also, findings from the MTT assay showed that VA exhibited increases in cell viability in comparison with HBSS control which was significant at 1mg/mL ($p < 0.05$) and 5mg/mL ($p < 0.01$) suggestive of its possible cytoprotective effect. Its hepatoprotective effect has been previously reported [228]. ML at 5 and 10mg/mL also showed significant ($p < 0.05$) increase in cell viability. In this study however, these observations are not expected to significantly affect cell functionality.

4.3 PAPER III

The efflux transporter P-gp is highly expressed in the intestinal tract where it extrudes drugs, toxins, and xenobiotics. The pharmacokinetic changes arising from concomitant administration of drugs with P-gp modulators may cause beneficial or detrimental drug interactions. Therefore, drugs and herbs that interfere with P-gp can be clinically important in modifying the bioavailability, pharmacokinetic disposition, and safety profiles of substrate drugs.

The herbs CP, VA, and ML, are popular ethnomedicines with clinical significance that are widely used in certain countries and continents. VA and ML are commonly used in tropical Africa. Other species of Vernonia are medicinally used in the Americas and parts of Asia. Although CP originated from Central America and its fruit is mainly consumed there, several studies report that its leaves are used as a herbal medicine in vast regions of the world. These herbs function as

antihelminthics and are used to treat cancer, malaria, diabetes mellitus, fevers, allergic and gastrointestinal disorders [224, 229-231]. Multi-center clinical trials of VA have been initiated [222].

We initially examined the influence of seven herbs on the bidirectional transport of digoxin using a Caco-2 cell monolayer model [81]. The transport of digoxin in the apical-to-basolateral direction was significantly enhanced by VA, CP, and ML, as well as the P-gp inhibitor VER. Furthermore, the basolateral-to-apical transport of digoxin was significantly inhibited by these herbs. These results suggested that ML, CP, and VA inhibit P-gp. A cell viability assay indicated that these *in vitro* effects were not due to cytotoxicity. The present study was designed to further investigate these findings by examining the effects of VP, VA, and ML on the modulation of P-gp in *ex vivo* and *in vivo* models using digoxin as a clinically relevant substrate.

The present study used crude herbal extracts, rather than single components isolated from the herbs, because patients generally take whole herbal products, usually containing several constituents. Consequently, crude extracts may contain additional components that can affect P-gp, besides their pharmacologically active components. Studies on drug disposition are complicated by the overlapping tissue distributions of CYP3A4 and P-gp, and the broad spectrum of their interacting substrates. Therefore, digoxin was chosen as the substrate in this study because it selectively interacts with P-gp but negligibly with CYP3A4 [232].

In the Ussing chamber study, CP significantly increased the mean apparent permeability ($p<0.001$), comparable with VER. However, the AUC did not significantly increase following oral administration of CP in rats. Ussing chambers containing rat intestinal segments provide a simple method to investigate

permeability, which highly correlates with absorption properties in humans [134, 233]. However, despite the merits of Ussing chambers, some drawbacks have been reported. For example, Ussing chambers may underestimate drug transport because the drug needs to cross the entire intestinal wall, which might be rate-limiting, whereas this is not necessary *in vivo* [134]. Furthermore, only rat ileum was used in Ussing chambers in this study and regional differences in intestinal absorption were not investigated. Also, in the rat model, tissue viability is not a limitation, as may be the case in an Ussing system. These factors may underlie the difference in the effects of CP observed in the two systems. Importantly, although the mucosal architecture is intact in the Ussing system, it may not behave exactly as the intact gut does [234]. Although medicinal herbs are efficacious in the treatment of various ailments, the lack of standard prescribed dosages is a major concern [235]. This may lead to the consumption of large quantities of herbs, resulting in their bioaccumulation and complications in humans. However, non-standardized doses of up to 6 cups (2 cups thrice) of aqueous extracts (approximately 20–40 g of plant material) are consumed daily by humans. We converted the estimated human dose to the equivalent dose in rats according to body surface area. Therefore, 90 mg/kg of all the studied herbs were administered to rats in this study [236].

The effects of these herbs on the function of P-gp *in vivo* were investigated using male SD rats. Oral administration of VA enhanced the AUC and C_{max} of digoxin. The $T_{1/2}$ of digoxin was similar to that previously reported [237, 238]. $T_{1/2}$ did not significantly change when VA or VER were co-administered, which suggests that they increased the intestinal absorption of digoxin but did not affect its systemic elimination. The herbs affected digoxin transport within one hour of administration.

This suggests that digoxin absorption may be enhanced due to inhibition of intestinal P-gp. This is in agreement with the findings of the Ussing chamber study. No significant change in the total clearance of digoxin on co-administration with the herbs was observed. Organ blood flow, extraction ratio, and transporters influence the clearance and disposition of drugs [104]. The *in vivo* study suggests that VA increases digoxin absorption more potently than VER; co-administration of VA or VER increased $AUC_{(0-\infty)}$ 2.1- and 1.5-fold, respectively, compared to the control. This effect of VA is of particular importance because in addition to its potent medicinal value, it is also a common vegetable in the African diet. There is no known study reporting that VA may modulate the pharmacokinetics of drugs that are P-gp substrates. Hence, this finding may be of particular clinical relevance as it indicates a food-drug interaction may also occur. Digoxin is a substrate of P-gp, and this interaction results in the extrusion of digoxin from enterocytes throughout the intestinal mucosa into the gut lumen. Consequently, the P-gp inhibitor VER reduced the intestinal secretion of digoxin. VA inhibited the secretion of digoxin more than VER. It is thought that these herbs and P-gp compete for binding to digoxin and this underlies the changes in digoxin disposition observed following its co-administration with herbs.

Extracts of VA have very high concentrations of flavonoids [239]. There is increasing evidence that flavonoids can inhibit P-gp. Flavonoids obtained from the diet or supplements can alter P-gp levels and thereby affect the oral availability of drugs that are transported by P-gp [240, 241]. The flavonoids kaempferide, quercetin, apigenin, naringenin, genistein, and rutin are reported to affect P-gp activity through various mechanisms including direct interaction with the vicinal

ATP-binding sites, the steroid-binding site, or the substrate-binding domains, or via a heterotropic allosteric mechanism [242]. Similarly, the *in vivo* absorption of nitrofurantoin is enhanced following administration of the flavonoid chrysin due to modulation of transporter function [243]. Several other flavonoids including silymarin, morin, biochanin A, and phloretin modulate P-gp [244, 245]. Therefore, the high level of flavonoids in the herbal extracts may be responsible for the inhibition of P-gp. The bioactive phytochemicals in VA include flavonoids (luteolin, luteolin-7-O-β-glucuronoside, and luteolin-7-O-β-glucoside), bitter sesquiterpene lactones, tannins, and steroid glucosides [246, 247]. Because of the common backbone flavone structure of luteolin as illustrated in Figure 27 and other flavonoids which have shown P-gp inhibition, the observed effect may be partly contributed by luteolin. CP is rich in carpaine, pseudocarpaine, dehydrocarpaine I and II, choline and carposide alongside flavonoids (quercetin and kaempferol) that are found in the leaves [248, 249]. Hydrolysable tannins, terpenes, saponins, and flavonoids are abundant in mistletoe leaves [250].

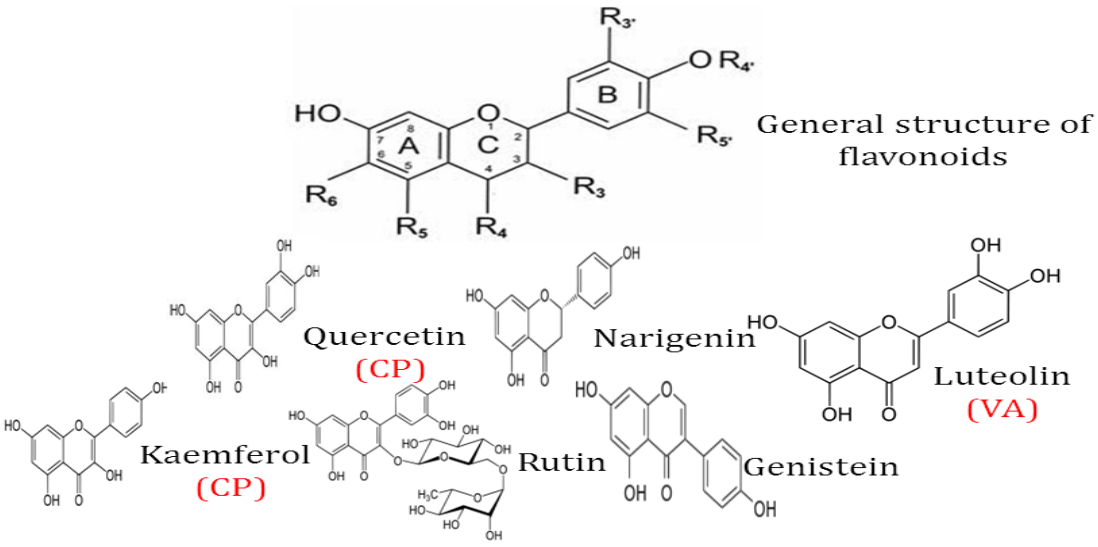

Figure 27. Flavonoids as inhibitors of P-glycoprotein

ML is a species of mistletoe. Other mistletoe species including *Viscum album* have been reported to potently inhibit P-gp [220]. In our previous research using Caco-2 cells, treatment with 2-20 mg/ml ML significantly inhibited P-gp, with similar potency to treatment with 100 µM VER. The reason why ML did not inhibit P-gp as potently in the present study may be because the expression of drug transporters and permeability varies between Caco-2 cells and the human intestine. C_{max}, AUC_{0-3}, and $AUC_{(0-\infty)}$ of digoxin were increased in the presence of VA in comparison to the control. In addition, the systemic clearance of digoxin was reduced following treatment with ML and VER; however, this was not statistically significant. These results are consistent with a study investigating the HDI potential of curcumin when co-administered with celiprolol and midazolam [251]. A similar study reported that co-administration of VER decreases the clearance of digoxin [252].

Inhibition of P-gp by VA increased the digoxin plasma concentration after a single oral dose, which is in agreement with the previous *in vitro* results. This effect was observed within the first hour after administration, suggesting that VA mainly affects the absorption of digoxin. The findings suggest that VA could have beneficial roles, especially as adjuvants in cancer chemotherapy and diabetes treatment. Complementary and alternative forms of treatment are utilized by many patients in addition to well-established conventional treatments for several medical ailments. Thus, it is important to investigate the likelihood of changes in drug disposition when conventional and alternative medicines are co-administered. The majority of anticancer agents are P-gp substrates that many cancers are resistant to; therefore co-administration with VA may increase the oral

bioavailability of these agents or promote MDR reversal. The hypoglycaemic agents, glyburide, saxagliptin, sitagliptin, and linagliptin have some P-gp substrate activity [253, 254]. The potential risk of HDIs occurring with VA is thought to be high since it is commonly used to treat diabetes mellitus alongside conventional medicines. Caution may need to be exercised when drugs with narrow therapeutic indices are co-administered, such as digoxin, as this may result in toxic responses. When herbs are used as adjuvants in cancer therapy they can provide support to patients being treated with conventional medicines by alleviating side-effects and improving self-defense and quality of life [241, 255]. P-gp inhibitors, including tariquidar, GF120918, and biricodar, are used as adjuvants in cancer therapy [256-258]. Doses adjustment may be necessary in clinical situations and caution is recommended. Our findings indicate that HDIs need to be investigated further to obtain a more detailed understanding of their impact in human subjects and to ensure appropriate clinical intervention when they are co-administered with conventional P-gp substrate drugs. Also as illustrated in Table 8, significant *in vitro* inhibition of P-gp may not translate to significant inhibition in animal models

Table 8. Summary of herb-drug interactions using different study models.

Model	Dose	CUR	LG	NL	MLB	CP	ML	VA
In vitro (Caco-2 cells)	0.2-20mg/ml	+	-	-	-	+++	+++	+++
Ex vivo (Ussing chamber)	10mg/ml	n.d	n.d	n.d	n.d	+++	+++	+++
In vivo PK (Rats)	90mg/kg BW	n.d	n.d	n.d	n.d	+	+	+++

Key: -, Non inhibition +, non significant inhibition +++, significant inhibition
n.d, not determined]

4.4 PAPER IV

Artemisinin is mainly metabolized by the CYP system in the liver with less than 1% being excreted unchanged in the urine [259]. It is also known to induce the *in vivo* enzymatic activity of CYP2C19 and -2B6. Therefore, it is both a substrate and an inducer to CYP2B6, inducing its own metabolism in both patients and healthy subjects [260]. It is also postulated to induce the expression of CYP by activating pregnane X receptor (PXR) as well as constitutive androstane receptor (CAR). Figure 28 illustrates the modulation of CYP by the various drugs investigated.

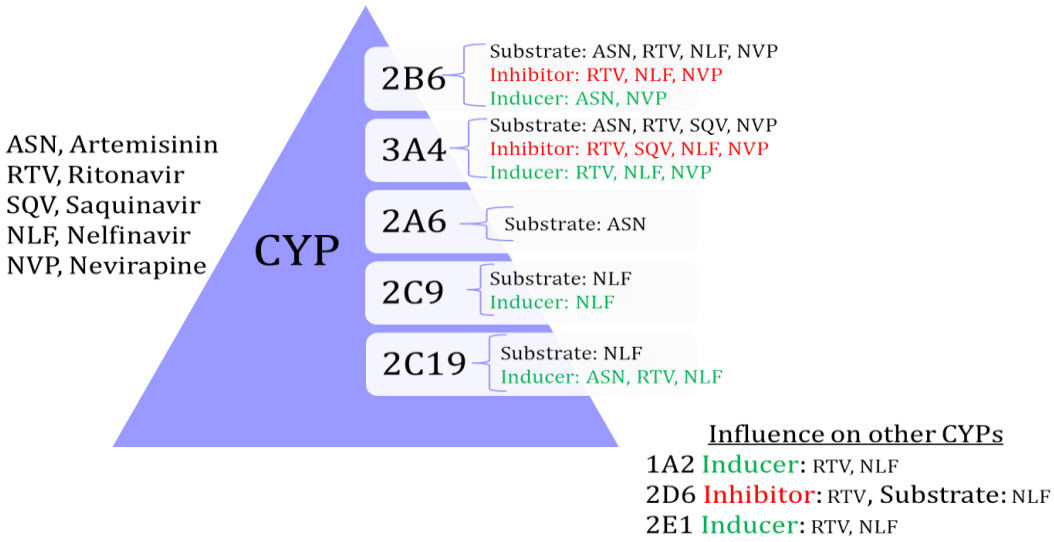

Figure 28 CYP modulation by artemisinin and the investigated antiretrovirals

The V_{max} and K_m obtained in this study are higher than a previous report which utilized rat microsomal proteins [261]. However, this study reports values obtained from the overall disappearance of artemisinin, rather than that for metabolite formation. In addition, this study utilized Michaelis-Menten kinetics rather than first order kinetics which has been reported to be a better measure for the hepatic microsomal metabolism of ASN [262]. In this study, KTZ at concentrations of 0.1 – 10

μM were used in order to examine the contribution of CYP3A to the metabolism of artemisinin. It is known that high concentrations of >10 μM inhibit not only CYP3A but also other CYPs [263, 264]. A previous report had shown that in individual HLMs, orphenadrine (a CYP2B6 inhibitor) inhibited the metabolism of artemisinin by 45-75 %. In addition, 4 μM KTZ inhibited artemisinin's metabolism by 46 % in pHLM [265].

The HIV protease inhibitors are known to have differential effects (inhibition, inactivation or induction) on CYP isozymes with RTV and SQV showing inhibition to CYP3A4 and -2C9 [266]. The NNRTI, NVP inhibits CYP3A4, -2C9, and -2C19. Also, the CYP2B6 inhibitory effect of NLF and RTV has been reported [267]. Although not investigated in this study, this ability of artemisinin to induce its own metabolism through inducing the xenosensors PXR and CAR may be of clinical significance especially as the HIV antiretrovirals are substrates of various CYPs. This could alter the pharmacokinetic disposition of both the antiretrovirals and artemisinin itself. In comparison to other CYP3A inhibitors, RTV and SQV are potent inhibitors of CYP3A as demonstrated in HLM for the inhibition of midazolam-1-hydroxylation and the 6β-hydroxylation of testosterone. NVP has shown a Ki of 31 μM to CYP3A4 in HLM [268]. Furthermore, RTV has been shown to be a potent CYP2B6 inhibitor with a Ki of 5 μM to bupropion hydroxylation [269]. Daily doses of RTV (600mg twice daily) and NVP (200mg twice daily) produces a maximal steady state plasma concentration of 11 ± 4 μg/ml and 4.5 μg/ml respectively [270, 271]. These values correspond to 15.2 ± 5.5 μM and 8.8-19.9 μM which falls into the range of concentrations examined.

RTV is known to be a potent inhibitor of CYP3A using nifedipine oxidation (IC50 =

0.07 μM) and terfenadine hydroxylation (IC50 = 0.14 μM) as metabolic probes [272]. However in this study, only the effects on CYP3A, -2B6 and -2A6 are of relevance as these are the main CYP isoforms involved in artemisinin's metabolism. The findings show that RTV exhibited very potent inhibition to the metabolism of artemisinin with 73-84 % of artemisinin remaining unmetabolized on co-incubation. This is thought to be as a result of the simultaneous blockade of CYP3A4 and -2B6 which are the main pathways for artemisinin metabolism.

NVP has been shown to be a CYP3A inhibitor with an IC_{50} of 106 and 250 μM using triazolam hydroxylation with or without pre-incubation, respectively [273]. Wen *et al.* [268], reported a K*i* value of 31 μM to CYP3A4 in HLM which further buttresses the significance of the NVP concentrations examined. Similar to the protease inhibitor RTV, NVP a NNRTI exhibited potent inhibition to the metabolism of artemisinin. However, 10 μM NVP showed a large increase in the amount metabolized. However, it should be noted that apart from being a potent CYP3A inhibitor and substrate; nevirapine has also been reported to be a potent inducer of CYP3A and -2B6 in both in humans [274-276]. Therefore, in clinical situations, its induction effect may be significant. Although it is known that transcriptional and post-transcriptional regulation cannot be evaluated by the use of microsomal assays, the enhancement of CYP activity has been observed [277]. An enhancement of CYP2B6 and -3A activity may be responsible for the effect observed on co-incubation at 10 and 30 μM. This is because artemisinin is mainly metabolized by CYP2B6, an increase in its metabolism via inducing CYP2B6 and -3A4 by NVP will result in a lower amount of artemisinin remaining. We are of the view this partial inhibition of the metabolism of ASN may be due to a net enhancement of

CYP2B6 and -3A4 rather than enzyme inhibition. At the other concentrations, it is thought that net inhibition/ inactivation of CYP3A4 plays the major role.

NLF and SQV mainly showed non-significant effect on the metabolism of artemisinin. Apart from being a CYP3A4 and -2B6 inhibitor [267], NLF also induces CYP2B6, -3A4, -2C9 and -2C19. It is also a substrate of CYP3A4 and -2C19. There is also a report on its -1A2 and -2E1 potential [278-281]. The net contribution of these CYP isoforms did not seem to significantly affect the *in vitro* metabolism of artemisinin in this study. The insignificant inhibitory effect on artemisinin's metabolism by NLF is thought to be as a result of net enhancement of CYP2B6. SQV on the other hand inhibits CYP3A4, with minimal effects on other CYPs. In addition, saquinavir weakly binds PXR and activate its target genes [282]. However, under the conditions of this study, the likelihood of encountering a DDI on co-administration of either SQV or NLF with artemisinin is low.

5. CONCLUSION

5.1 PAPER I

In conclusion, the current findings show that ART, ADQ, and LUM decreased P-gp-mediated transport across Caco-2 cell monolayers. Although DIG was used as a representative P-gp substrate in this study, there may be a potential for DDIs between certain antimalarials and P-gp substrates in general. These interactions may be of clinical relevance given the high incidence of co-administration of P-gp substrate drugs with antimalarials. Thus, the present study is anticipated to provide useful information that will lead to more effective treatment policies for malaria. However, further *in vivo* investigations are recommended to confirm the *in vitro* findings.

5.2 PAPER II

The results from this study may provide better understanding to the effect of phytomedicines intervention on concomitant conventional drug administration. In conclusion, the present study has shown that CP, VA and ML are transport inhibitors of P-gp in Caco-2 cells and particular caution should be observed when they are ingested with other P-gp substrate drugs as they are expected to enhance the absorptive transport of P-gp substrate drugs. Because dose standardization is usually a problem with herbal remedies, the need for well controlled trials, improved regulation and global harmonization in the use of phytomedicines is of paramount importance to ensure their optimal safety and efficacy. Although this *in vitro* investigation gives valuable information, further *in vivo* studies may be required to verify the clinical relevance since *in vivo* investigations of possible interactions may be more complicated and may bring into play the effect of enzymes, pH, etc. that are not considered in an *in vitro* study.

5.3 PAPER III

In conclusion, the observed changes in the pharmacokinetics of digoxin and the absorption profile from the Ussing chamber study indicate that VA co-administration appears to pose a more clinically significant risk for P-gp mediated HDIs than either CP or ML. The series of investigations spanning *in vitro* to *ex vivo* to *in vivo* studies in rats confirm the P-gp inhibitory effect of VA. Importantly, the study has shown that significant *in vitro* P-gp inhibition may not translate to inhibition in animal models. Our findings point to the need for further assessment of HDIs in order to obtain a more detailed understanding of their impact in human subjects and to ensure appropriate clinical intervention when they are co-administered with conventional P-gp substrate drugs.

5.4 PAPER IV

Our *in vitro* study has shown the potent inhibitory effect of RTV and NVP on the main metabolic pathways of artemisinin at clinically significant concentrations. In view of this, DDIs should be considered on co-administration with artemisinin. The antiretroviral concentrations utilized in this study are within the usual plasma concentration range, suggestive of the potential of clinically relevant DDIs occurring on co-administration of especially RTV or NVP with artemisinin

Nowadays, a mechanistic approach to evaluating DDIs can be achieved by extrapolating findings from clinical studies to other drug classes metabolized by the same isoforms. Taken together, *in vitro* data from this study is strongly suggestive of the influence of RTV and NVP on CYP2B6 and -3A4 mediated metabolism of artemisinin.

5.5 GENERAL CONCLUDING REMARKS

Herbal medicines are currently in high demand, and their popularity is steadily increasing. Because of their perceived effectiveness, fewer side effects, and relatively low cost they are being used in the management of numerous medical conditions. However, they are capable of affecting the pharmacokinetics and pharmacodynamics of co-administered conventional drugs. These interactions are particularly of clinically relevance when metabolizing enzymes and xenobiotic transporters, which are responsible for fate of many drugs, are induced or inhibited. The effect of HDIs could be of benefit or deleterious to the patient. The use of herbal medicines is increasing, possibly due to their widespread promotion in the media as well as unsubstantiated health care claims. Although herbal medicines are beneficial, despite popular belief, they are not completely harmless and HDIs may occur on concomitant use with conventional drugs, but many possibly go unnoticed due to various factors. It is safer to view them as unrefined pharmaceuticals, capable of producing physiologic change, for better or worse.

Most herbal remedies, unlike conventional drugs comprise a complex mixture of chemical constituents. In order to better understand the detection and handling of HDIs, an expansion on knowledge on phytochemicals in herbal medicines is essential. Presently in most cases, complete characterization of the bioactive compounds is not well defined, information on toxicity and adverse effects are insufficient. It is expected that standardization, including chemical fingerprinting would become potent tools for quality control of herbal medicines. However, because most of the available HDI information is based on individual case reports, animal studies and *in vitro* data, extensive research is required to confirm and

assess the clinical significance of these potential interactions. HDIs may be under-reported and appropriate pharmacovigilance requires the collective responsibility of the patient, the health practitioner and researchers.

In this review, both pharmacokinetic (drug metabolizing enzymes and drug transporter systems) and pharmacodynamic mechanisms have been considered to play a role in these interactions. There are reports on the inductive role of PXR as a nuclear receptor, however it is of importance to research into other receptors; constitutive androstane receptor (CAR), and vitamin D-binding receptor (VDR), as these may also play an integral role in the mechanism of inductive processes involved in HDIs. Numerous conventional medicines have been developed from plants and because of the demerits of some conventional drugs, there is an active search for new drug development from plants. Future perspectives for the application of HDIs are in new drug development, use of herbal medicines as adjuvants to conventional drugs. They may also be used for their additive or synergistic effect when co-administered with modern medicine. This may require a decreased dose of the conventional drug, and possibly result to reduction in the manifestation of side effects. In particular, patients taking drugs with a narrow therapeutic index should be discouraged from using herbal products because of the ease of toxicity or ineffectiveness.

In conclusion, HDIs certainly occur and may have serious consequences. However because they are under-researched, present knowledge is incomplete. It is worth mentioning that the further research and controlled clinical studies are needed to clarify and determine the underlying mechanisms for these altered drug effects.

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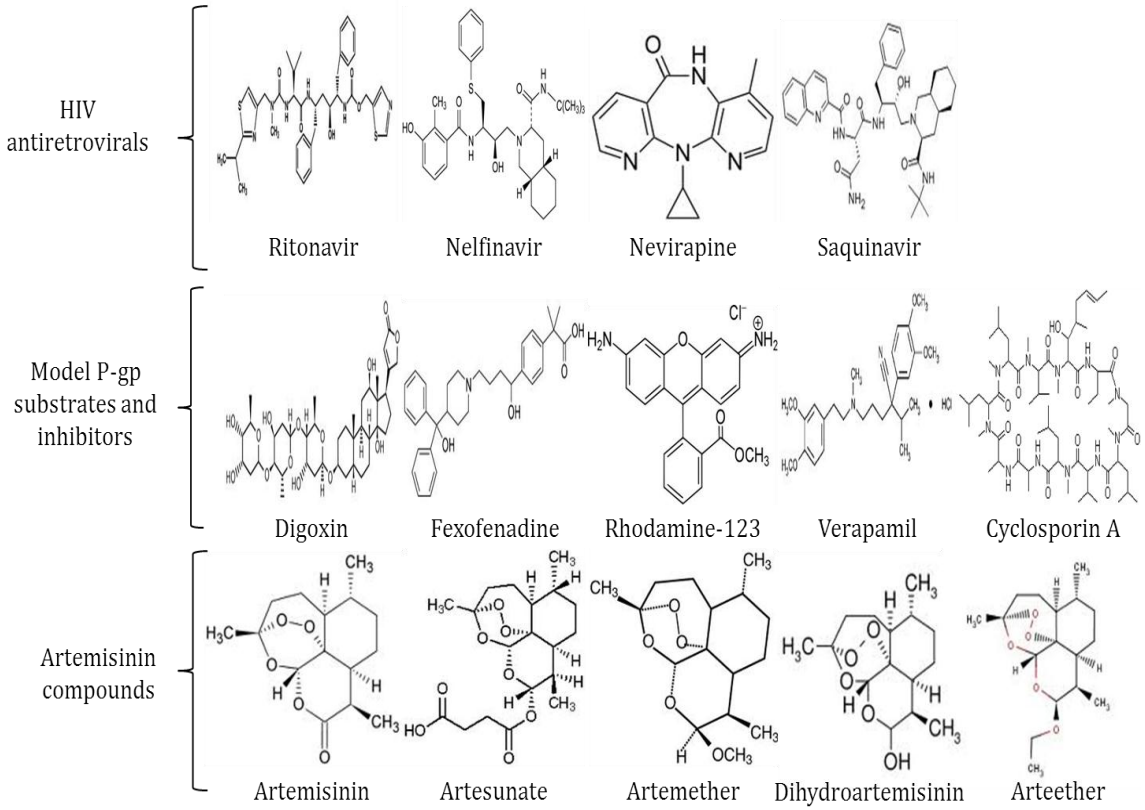
APPENDIX



Chemical structures of the HIV antiretrovirals, P-glycoprotein substrates and inhibitors utilized in the study. The structure of artemisinin and its derivatives are shown.

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