THE PXR/CAR NUCLEAR RECEPTOR SYSTEM AND ANTIMALARIA CHEMOTHERAPY

DER EINFLUSS VON ERZNEIMITTELN ZUR THERAPIE DER MALARIA AUF DIE KERNREZEPTOREN PXR UND CAR

DISSERTATION

DER FAKULTÄT FÜR CHEMIE UND PHARMAZIE
DER EBERHARD KARLS UNIVERSITÄT TÜBINGEN

ZUR ERLANGUNG DES GRADES EINES DOKTORS
DER NATURWISSENSCHAFTEN

2010

VORGELEGT VON

RITA PIEDADE

Tag der mündlichen Prüfung:	23 Juli 2010
Dekan:	Prof. Dr. Lars Wesemann
1. Berichterstatter:	Prof. Dr. Matthias Schwab
2. Berichterstatter:	Prof. Dr. Michael Schwarz

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

a.a - aminoacids

ACT- Artemisinin combination therapy

ARE – Arteether

ARM - Artemether

ART – Artemisinin

AS - Artesunate

CAR – Constitutive androstane receptor

C.A.R.L.A. – Coactivator-dependent receptor ligand assay

CI –Confidence interval

CITCO - 6-(4-chlorophenyl)imidazo [2,1-b]thiazole-5-carbaldehyde O-(3,4 dichlorobenzyl)oxime

COMT – Catecholamine-O-methyltransferase

CQ - Chloroquine

CYP – Cytochrome P450

DBD – DNA binding domain

DCC – Dextran-coated charcoal

DEAQ – N-Desethylamodiaquine

DEPC - DiethylenePyrocarbonate

DHA – Dihydro-artemisinin

DHFR – Dihydrofolate reductase

DHPS – Dihydropteroate synthase

DME – Drug metabolizing enzymes

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic acid

DR – Direct Repeat

EC_{50} - Half maximal effective concentration

ER – Everted Repeat

FBS - Fetal Bovine Serum

F.I. – Fold Induction

Fw - Forward

G6PD - Glucose-6-phosphate dehydrogenase

GST – Glutathione S-transferase

HRE – Hormone response element

IR – Inverted Repeat

LBD – Ligand binding domain

LB - Lysogeny broth

MAF – Minor allele frequency

MALDI-TOF-MS - Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

MDR – Multidrug-restance

MOPS - 3-(N-morpholino) propanesulfonic acid)

MRP – Multidrug resistance-associated protein

NADPH - Nicotinamide adenine dinucleotide phosphate

NAT – Arylamine N-acetyltransferase

NR – Nuclear receptor

ORF – Open reading frame

PAR – Pregnane activated receptor

PBS - Phosphate-buffered saline

PCR – Polymerase chain reaction

P-gp – P-glycoprotein

PXR – Pregnane X receptor

RID - Nuclear receptor interaction *domain*

RNA - Ribonucleic acid

RT-PCR - Reverse-transcription PCR

Rv - Reverse

RXR - Retinoid X receptor

SE - Southeast

SNP – Single nucleotide polymorphism

SOC - Super Optimal Broth

SRC1 - Steroid receptor coactivator 1

Std. – Standard

SULT – Sulfotransferases

SXR – Steroid and xenobiotic receptor

TfB – Transformation buffer

TPMT – Thiopurine S-methyltransferase

UGT – UDP glucoronosyltransferase

USA – United States of America

WHO – World health organization

XR – Xenobiotic Receptor

ABSTRACT

Malaria is one of the oldest diseases know to mankind, still having devastating consequences; killing 1 million people/year. The efforts to control this disease have been focused on the control of its transmission and, most importantly, its clinical management by effective chemotherapy. Concerning the latter, several antimalarials have been developed in the last decades. They can be subdivided into four structural classes: aminoquinolines, antifolates, artemisinin derived compounds and naphtoquinones. Although reasonably well studied in pharmacokinetics aspects, very few data is available concerning their effects in the gene expression regulation system. This is particularly relevant concerning the xenosensors PXR and CAR.

In a first part, this project aimed to elucidate the capacity of currently used antimalarials and their main known metabolites to activate the PXR/CAR system. For this purpose, mammalian two hybrid and gene reporter assays, together with induction studies in primary human hepatocytes were performed.

For PXR and CAR we could observe activation, by compounds from the artemisinin family (artemisinin, arteether, artemeether, deoxy-arteether and deoxy-artemisinin) both *in vitro* and in primary human hepatocytes, corroborated by the induction of prototypical target genes. A repression by artesunate of key targets genes, CYP3A4 and CYP2B6 was observed by this compound in primary hepatocytes, confirming the observed inhibition in vitro of the induction of both PXR and CAR.

Further activation of PXR by the aminoquinolines, lumefantrine, carboxymefloquine, amodiaquine, DEAQ and chloroquine, was observed in the *in vitro* system. Amodiaquine also induced CAR in the *in vitro* system. From the aforementioned compounds only carboxymefloquine, consistently induced the induction of *CYP2B6* in the hepatocytes, indicating a promoter specific activity. Both amodiaquine and DEAQ showed a repression of CYP3A4 and CYP2B6, indicating that although they may induce the interaction of PXR with its co-activators, they may not be able to induce the release of co-repressors.

The present data is of particular interest regarding the pivotal role of artemisinins and aminoquinolines in the current worldwide WHO supported artemisinin combination therapies (ACT), the first line treatment currently recommended. Future evidence based developments

of this therapy can benefit from this data on the high probability of induction of this two gene expression systems by ACT components, and its drug-drug interactions.

The second part of this project focused on the study of the genetic basis of variability of PXR dependent induction of key CYP450 upon exposure to artemisinins. The project was based on a previously study in 75 Vietnamese subject (Asimus *et al.*, 2007), where metabolic ratios for a number of pivotal PXR/CAR inducible CYPs (e.g. CYP3A4, CYP2C19) were determined upon artemisinin compounds exposure. For this purpose the promoter region, exons and exon-intron boundaries of the PXR gene were re-sequenced for every subject. This approach allowed the identification of 32 SNPs, six of them described for the first time. Overall, we could observed an increased prevalence of the minor allele for the polymorphisms 252 /275 A>G and 10331 A>G, 10483 T>C in the group of higher inducers. Further analysis of these SNPs in primary human hepatocytes showed a higher CYP3A4 mRNA induction in the carriers of the wt/wt genotype. This is an indication that different distribution of the allele in the high and low inducers group is not influencing directly the expression of CYP3A4.

Taken together we could confirm some of the data published concerning phenotypic changes induced by PXR SNPs. However no clear results were obtained for the newly identified SNPs due to a reduced number of samples, except for the SNP F420Y.

ZUSAMMENFASSUNG

Malaria ist eine der ältesten Krankheiten der Menschheit, mit immer noch verheerenden Folgen; jedes Jahr sterben an ihr weltweit 1 Million Menschen. Die Anstrengungen zur Bekämpfung der Krankheit konzentrieren sich zum einen auf die Unterbindung ihrer Übertragung und zum anderen auf eine effektive Arzneimitteltherapie. Zahlreiche Malariatherapeutika wurden in den letzten Jahrzehnten entwickelt. Sie werden in vier strukturelle Klassen unterteilt: Aminochinoline, Antifolate, Artemisinin-Derivate und Naphtochinones. Während pharmakokinetische Aspekte der Malariatherapie vergleichsweise gut untersucht sind, ist nur wenig darüber bekannt, inwieweit diese Arzneimittel die Regulation der Genexpression arzneimittelabbauender Enzyme und von Arzneimitteltransportern beeinflußen. Dies ist insbesondere hinsichtlich der Aktivierung der Fremdstoffsensoren PXR und CAR, welche die Induktion dieser Enzyme und Transporter vermitteln, von Interesse.

In ersten Teil dieser Arbeit wurde daher untersucht, ob die zurzeit eingesetzten Antimalariatherapeutika und ihre wichtigsten Metabolite die Fremdstoffsensoren PXR und CAR aktivieren. Zu diesem Zweck wurden „mammalian two-hybrid“ und Reportergen-Studien durchgeführt. Desweiteren wurde untersucht, ob diese Substanzen die Expression arzneimittelabbauender Enzyme und Transporter in primären, menschlichen Hepatozyten induzieren.

Für die meisten Substanzen aus der Artemisinin-Familie (Artemisinin, Arteether, Artemether, Deoxyarteether und Deoxyartemisinin) konnte eine Aktivierung von PXR und CAR *in vitro* beobachtet werden. Diese Befunde wurden durch die Induktion prototypischer Zielgene von PXR und CAR in mit diesen Substanzen behandelten primären Hepatozyten bestätigt. Im Gegensatz hierzu reprimiert Artesunat die Expression wichtiger Zielgene, wie CYP3A4 und CYP2B6, in primären Hepatozten, welches die *in vitro* beobachtete Hemmung der Aktivierung von PXR und CAR bestätigt.

Weiterhin aktivierten die Aminochinoline Lumefantrin, Carboximefloquin, Amodiaquin, DEAQ und Chloroquin PXR im *in vitro* System. Amodiaquin aktivierte zusätzlich CAR. Von den genannten Substanzen induzierte nur Carboxymefloquin die Expression von CYP2B6 in entsprechend behandelten primären Hepatozyten, was auf eine Promotor-spezifische Aktivität des Carboxymefloquin-aktivierten PXR hinweist. Amodiaquin und DEAQ reprimierten in

primären Hepatozyten die Expression von CYP3A4 und CYP2B6. Dies läßt vermuten, dass diese beiden Substanzen, obwohl sie die Interaktion von PXR mit seinen Ko-Aktivatoren induzieren, nicht die an PXR gebundenen Ko-Repressoren freisetzen können.

Diese Ergebnisse sind, angesichts der zentralen Rolle der Artemisinine und Aminochinoline in der zurzeit von der WHO zur Behandlung der Malaria hauptsächlich empfohlenen Artemisinin-Kombinationstherapie (ACT), von besonderem Interesse. Für die zukünftige Weiterentwicklung der ACT könnten die hier gewonnenen Ergebnisse zur Aktivierung von PXR und CAR und eventuell darausfolgende Arzneimittelinteraktionen von Bedeutung sein.

Im zweiten Teil dieser Arbeit wurde versucht, genetische Ursachen der ausgeprägten interindividuellen Variabilität der PXR-abhängigen Induktion wichtiger Zytochrom-P450-Enzyme durch Artemsinine zu ermitteln. Die Untersuchung basiert auf einer kürzlich durchgeführten klinischen Studie an 75 Vietnamesen (Asimus et al., 2007), in der die metabolischen Aktivitäten einiger wichtiger PXR/CAR-induzierbarer Zytochrom-P450-Enzyme (z.B. CYP3A4, CYP2C19) nach Behandlung mit Artemisininen bestimmt wurden. Zu diesem Zweck wurden die Promotor-Region, alle Exons und Exon-Intron-Übergänge des PXR-Gens in allen an der klinischen Studie teilnehmenden Individuen vollständig sequenziert. Dieser experimentelle Ansatz ermöglichte die Identifizierung von 32 genetischen Polymorphismen, von denen sechs hier zum ersten Mal beschrieben werden. Die PXR-Polymorphismen 252/275 A> G, 10331 A> G und 10483 T> C waren mit einer stärkeren Induktion der CYP3A4 Aktivität assoziiert. Genau der gegenteilige Effekt wurde jedoch bei der Untersuchung der Induktion der CYP3A4 Expression durch Artemisinin in primären menschlichen Hepatozyten beobachtet. Dies weist daraufhin, dass diese PXR-Polymorphismen die Expression von CYP3A4 wohl nicht direkt beeinflußen.

Zusammenfassend, konnten in diesem zweiten Teil der Arbeit einige bereits publizierte Assoziationen von PXR-Polymorphismen mit phänotypischen Veränderungen bestätigt werden. Hinsichtlich einer Assoziation mit dem Ausmaß der Induktion, wurden für die neu identifizierten Polymorphismen jedoch keine eindeutigen Ergebnisse erzielt, was vermutlich auf eine zu kleine Probenzahl zurückzuführen ist. Lediglich der Polymorphismus, der zu der neuen PXR Proteinvariante F420Y führt, ist eindeutig mit einer sehr schwachen Induktion verbunden.

1. INTRODUCTION

1.1. MALARIA

Malaria has devastating consequences: it strikes over 250 million people worldwide and kills near 1 million people each year, many of whom being children under 5 years of age (Eastman and Fidock, 2009). This disease is transmitted in 109 countries spread throughout Africa, Asia, Oceania, and Latin America, with special incidence in Sub-Saharan Africa, where more than 90% of the cases occur (Sachs, 2005; WHO, 2009).

Although one of the oldest diseases known to Mankind, descriptions of this disease are found in ancient Roman, Chinese, Indian and Egyptian manuscripts, it was only in the 19th century that the mosquito *Anopheles* was identified as the vector of this disease. This discovery was described by Ronald Ross. At the same time the protozoans from the *genus Plasmodium* responsible for the disease were identified by Charles Louis Alphonse Laveran (Cox, 2010). These two breakthroughs were distinguished by the earning of two physiology or medicine Nobel prizes, respectively in, 1902 and 1907.

Presently five species of *Plasmodium* are known to infect the human species, namely, *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium falciparum,* and the recently identified *Plasmodium knowlesis* (White, 2008).

For millennia human societies have been trying to control this disease. The efforts in the last 60 years have been strongly focused on two strategies – vector control, trying to reduce the populations of host mosquitoes (and hence the transmission of the disease) and effective chemotherapy. At both fronts, the development of drug resistance to insecticides by the vector and against antimalarial drugs by the parasite, have curtailed these efforts (Ollomo *et al.*, 2009).

1.1.1. *PLASMODIUM* LIFE CYCLE

Plasmodium parasites have an intricated life cycle, comprising two main stages: an exogenous sexual phase in the mosquito (Sporogony) and an endogenous asexual phase in the human host (Schizogony) (Fig.1). These stages are similar in all species, varying mainly in the duration of the Schizogony phase.

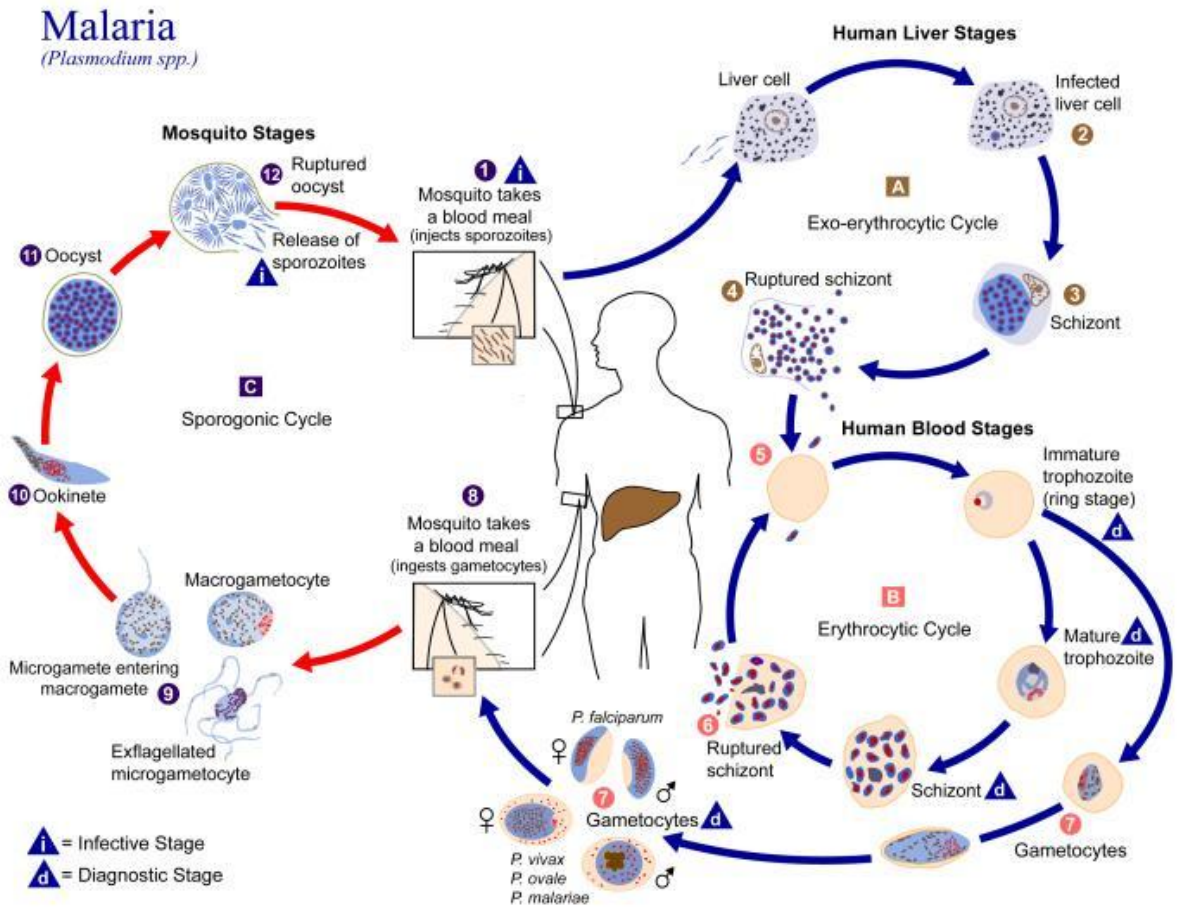


Figure 1: *Plasmodium* life cycle. The life cycle comprehends one sexual stage in the mosquito (C), and two stages in the human host, a liver stage also known as exo-erythrocytic cycle (A) and a blood stage, the erythrocytic cycle (B) (http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Malaria_il.htm, accessed 01 April 2010).

The infection starts by the injection of the human host with sporozoites together with the saliva of a feeding mosquito (Fig.1). This sporozoites migrate in the blood stream to the liver, were the parasite will undergo a first asexual replication. In the liver cells, the parasite will

pass through different forms culminating with the production of merozoites (invading forms) that are released to the blood stream where they will invade erythrocytes. In these cells the merozoites will develop to immature trophozoites, progressing to mature trophozoites or gametocytes. This maturation is accompanied by an active metabolism including the ingestion of host cytoplasm and proteolysis of hemoglobin into amino acids. The erythrocyte phase will end with the rupture of the cell, and the release of new merozoites that can invade new erythrocytes. In a separate development program a small fraction of this intra-erythocytic cycling population will become gametocytes. These sexual forms are able to continue the life cycle of the parasite (now including a recombination step through meiosis) inside the mosquito gut, upon their ingestion by the vector. The aforementioned rupture of red blood cells is the cellular event responsible for the intermittent fever symptoms of the pathology. For *P.malariae* the periodicity is 72h of the cycle (and hence the fever periods), for *P.falciparum, P.ovale* and *P.vivax* is a 48h cycle, while *P.knowlesi* presents a shorter 24h cycle (Singh *et al.*, 2004).

In the mosquito the female and male gametocytes, ingested during the mosquito blood meal, will form a zygote in the insects mid gut. The zygote will then suffer meiosis and develop into the ookinete. This form of the parasite will then migrate from the blood meal to the cells of the intestinal wall of the mosquito forming an oocyst. This oocyst will undergo several rounds of asexual replication, during a period of 10-14 days resulting in the production of thousands of sporozoites that will migrate to the mosquito's salivary gland waiting for the next blood meal of the insect, and hence, to be transmitted to a new vertebrate host (Aly *et al.*, 2009).

1.1.3. MALARIA TREATMENT

Quinine and artemisinin – the chemical precursors of the present antimalarials

Chemotherapy is the main strategy for the control of malaria, particularly in the perspective that a fully effective malaria vaccine for the uncomplicated forms of the disease is most likely decades ahead for the endemic region populations.

The first malaria chemotherapy known to Western populations emerged in the XVII century. An extract from the bark of the chinchona trees from Peru, used by the local Peruvian Indians populations for the treatment of fevers came to the attention of the Jesuit priests operating in

these regions. This powder was brought to Europe by this congregation, gaining its common name- Jesuit Bark. It was only in 1820 that the French chemists Joseph Pelletier and Jean Biename Caventou identified quinine as the active compound in the chinchona bark powder (Schlitzer, 2007).

Although unknown by the Europeans, the Chinese medicinal herb *Artemisia annua* is most likely the oldest documented antimalarial chemotherapy. The anti-fever properties of teas prepared with this type of plants were first described in the fourth century A.D. by Ge Hong. The information on these documents was taken to full advantage in the XX century as a guideline for the discovery of the artemisinin class of sesquiterpenes by the Chinese academy of militar sciences during the Vietnam War (Li and Wu, 2003).

Both artemisinin and quinine led to the production of several new synthetic or semi-synthetic antimalarials, forming the basis of the majority of the presently available antimalarial drugs (Cui and Su, 2009).

The currently available antimalarials can be subdivided in four classes: aminoquinolines, antifolates, artemisinin derived drugs and most recently, the napthoquinones (e.g. atovaquone).

The success of these drugs depends mainly from on the interactions between the drug and the parasite and on interactions between the antimalarials and the human host. This thesis will focus on these last interactions, with particular focus on possible drug interactions between the antimalarials with other used drugs. The characteristics of currently available antimalarials will be described in the next chapter.

1.1.3.1. QUINOLINES

This group of antimalarials comprises compounds derived from the quinine structure. It may be subdivided in 4-aminoquinolines (e.g. chloroquine and amodiaquine), 8-aminoquinolines (Primaquine) and aminoquinoline alcohols (mefloquine, lumefantrine, halofantrine and quinine). Their structures are shown in figure 2.

Quinine

Quinine is a second line antimalarial used normally in combination with an antibiotic to treat uncomplicated *P. falciparum* malaria. It may also be used as first line for the treatment of pregnant women particularly in the first trimester, travelers returning to non-endemic countries and for the treatment of severe malaria. A 7 day treatment regimen starting with an initial dose of 20 mg/kg of body weight followed by a maintenance dosage of 10mg/kg body weight each 8 h is necessary to achieve full recovery (WHO, 2010). Recently, quinine is also emerging as a second line of choice in several national malaria control programmes (http://malaria.who.int/treatmentpolicies.html).

This drug acts mainly in the mature trophozoite stage of the parasite development. It may also kill the sexual stages of *P. vivax, P.malariae* and *P.ovale*. Its antimalarial action it thought to be related with the inhibition of the parasite haem detoxification in the food vacuole (Famin and Ginsburg, 2002). In the human host, quinine is metabolised to its primary active metabolite, 3-hydroxyquinine, by CYP3A4/5. The formation of its minor metabolites (10S)-11-dihydroxy-dihydroquinine, 2L-quininone and (10R)-11-dihydroy-dihydroquinine is dependent on CYP3A4 and CYP2C9 activity, respectively (Kerb *et al.,* 2009).

Despite being one of oldest antimalarials available, quinine, is one of the few with only very rare cases of clinical resistance. The first reliable clinical reports of quinine resistance occurred 100 years ago in Brazil, among non-immune immigrants involved in railway constructions in the West of the country (Neiva, 1910). Since then, only sporadically resistance to this compound has been reported in South-East Asia and Western Oceania (WHO, 2006). This may be due mainly to the short half life of the compound, not allowing a window of low dose selection, and to the fact that quinine has been only used as a second/third line antimalarial.

Chloroquine

The original synthesis of chloroquine was performed in 1934 by the hands of H. Andersag and co-workers at the I.G. Farbenindustrie AG laboratories under the name of Resochin®. At the time it was determined as too toxic for human use and ignored until the World War II. At

this time, the United States launched a national program for the discovery of new antimalarials. A substance coded SN-7619 was uncovered upon large compound surveys; that revealed to be identical to the previous Resochin®. This was renamed as chloroquine (CQ). At this time clinical trials showed that CQ had a therapeutic value as an antimalarial, without any major side effects. It reached the pharmaceutical market in 1947 as a prophylaxis agent (Jensen and Mehlhorn, 2009).

Chloroquine is currently being used as a monotherapy or in combination with primaquine in the treatment of chloroquine sensitive *P.vivax, P.malariae* and *P.ovale* malaria, at a dosage of 25 mg base/kg body weight over 3 days. This drug is also used in the treatment of uncomplicated *P.falciparum* malaria in some Indian provinces and central America/Caribbean (WHO, 2010). This is the best characterized quinoline antimalarial – in terms of mechanism of action; research in this drug has set the current model that this type of compounds exert its action through the inhibition of the process of haem detoxification in the food vacuole of the parasite (Hempelmann, 2007).

In the human host, chloroquine is biotransformed to its metabolites, N-desethylchloroquine, bisdesethylchloroquine, 7-chloro-4-aminoquinoline, chloroquine side-chain N-oxide, and chloroquine di-N-oxide by the action of CYP2C8, CYP3A4/5, and to a lesser extent CYP2D6 (Kerb *et al.*, 2009).

Resistance to chloroquine has widespread, with the exception of some regions in Central America and the Caribbean, starting only a few years after its introduction in Thailand and Colombia (Young *et al.,* 1963).

Amodiaquine

Amodiaquine was patented in the 1940s by the pharmaceutical corporation Parke Davis (Burckhalter *et al.,* 1948). Used in prophylaxis it was correlated with rare but severe cases of agranulocytosis and hepatotoxicity (Meshnick and Alker, 2005). Despite the initial reported side effects, amodiaquine has been introduced in areas with high incidence of chloroquine resistance, without any major reported side effects and considerable efficacy (WHO, 2006; Ursing *et al.*, 2007; Martensson *et al.,* 2005).

Presently this drug is widely used in many regions, namely in Africa, combined with artesunate, for the treatment of uncomplicated *P.falciparum* malaria. Treatment with this drug involves a 3 day regimen of co-formulated artesunate and amodiaquine, in a daily intake of 4mg/kg artesunate and 10 mg/kg day amodiaquine (WHO, 2010). Being a member of the 4-aminoquinoline class, amodiaquine is structurally and functionally related with chloroquine. Resistance to this drug used as monotherapy was early documented. The first descriptions were published in the 1960s in Colombia (Young, 1961). These phenomena did not expand as rapidly as with CQ, possibly due to its less frequent use.

Amodiaquine is metabolised to it active metabolite, N-desethylamodiaquine mainly by CYP2C8 in the liver, and most likely by CYP1A1 and CYP1B1 in extra-hepatic tissues (Gil, 2008).

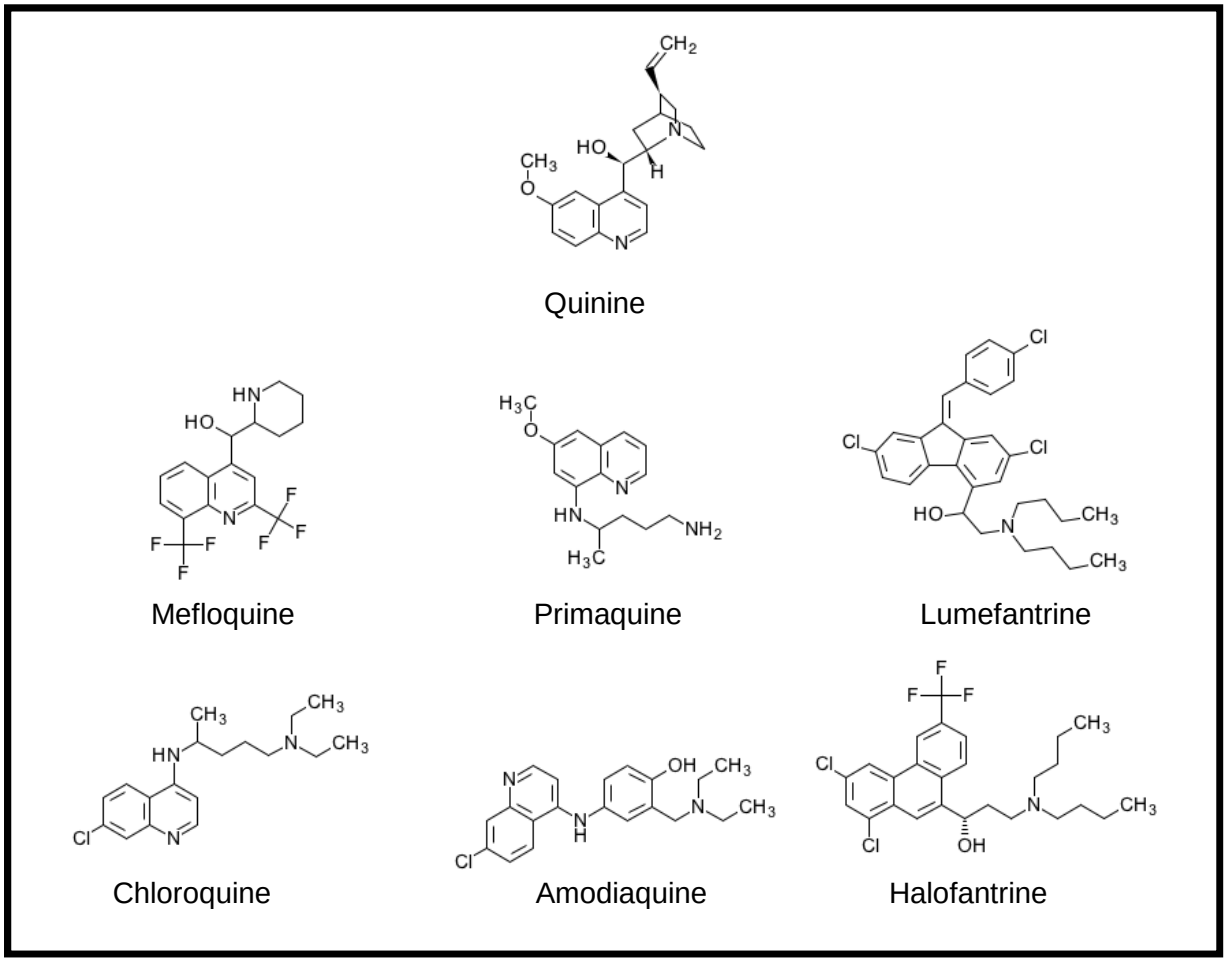


Figure 2: Structures of the quinolines.

Primaquine

Primaquine was first synthesized in the US in 1946. This 8-aminoquinoline is still the only available antimalarial that targets hypnozoites – the dormant forms of *P. vivax* in the liver. Its mechanism of action is still unknown. Although effective, this antimalarial has been associated with severe side effects, e.g. haemolytic anaemia. During the last years efforts to develop equivalently effective antimalarials not associated with these severe side effects have output two new primaquine analogues aablaquine and tafenoquine, both already in final stages of clinical trials against *P.falciparum* and *P.vivax* (Vale *et al.*, 2009).

This drug is still mainly used as a radical treatment for *P.vivax* malaria, given once a day for 14 days at a dosage ranging from 0.25-0.50 mg/kg/day (WHO, 2010).

Primaquine is primarily metabolized to carboxyprimaquine by CYP1A2, CYP3A4 and by the monoamine oxidase. This metabolite is thought to be responsible for its toxicity (Kerb *et al.*, 2009).

Mefloquine

Mefloquine is a synthetic analogue of quinine, developed in the 1970s at the Walter Reed army institute of research in the USA as a response to the chloroquine resistant malaria burden in the US troops engaged in the Vietnam War (Modell, 1968; Powell, 1972). This drug is mainly used combined with artesunate for the treatment of uncomplicated malaria. The treatment regime comprises a 3 day treatment course with a daily dose of 4 mg/kg/day artesunate and 25 mg/kg of mefloquine either split over 2 days as 15 mg/kg and 10 mg/kg or over 3 days as 8.3 mg/kg/day once a day for 3 days. It has been hypothesized that this drug acts through inhibiting the ingestion of host cell hemoglobin (WHO, 2010). It is used in prophylaxis, as well as, in the treatment of chloroquine resistant *P.falciparum*, and in combination with primaquine phosphate to treat uncomplicated P.vivax malaria (http://www.cdc.gov/malaria/pdf/treatmenttable.pdf, accessed 19 April 2010). This is one of the antimalarials with most common side effects, e.g., severe depression, anxiety, paranoia, aggression, nightmares, insomnia and seizures. It has also been reported to be associated with birth defects, among others (Alkadi, 2007).

Mefloquine is mainly metabolised by CYP3A4 to its two major inactive metabolites carboxymefloquine and hydroxymefloquine (Kerb *et al.*, 2009).

Extensive resistance has emerged in SE Asia (mainly Thailand) and in some other regions (e.g. Kenya). Due to its structural similarity with quinine, cross-resistance should be expected between the two drugs (Nelson *et al.*, 2005).

Lumefantrine

Lumefantrine (Benflumetol) is a synthetic aryl amino alcohol quinoline originally synthesized by the academy of military medical sciences in Beijing, presently only available in the market as a co-formulation with artemeether, in tablets containing 20 mg of artemeether and 120 mg lumefantrine (Premji, 2009). This combination was licensed under the name Coartem® for the developing world in 1999, by Novartis AG. It is mainly used in Africa to treat uncomplicated *P. falciparum* malaria, being presently the most prevalent first liner in the world. The present administration format comprised by 6 doses/3 days (1.7/12 mg/kg body weight of artemether and lumefantrine, respectively, per dose, given twice a day for 3 days,) is a well-tolerated and efficient treatment for uncomplicated malaria, even in regions with multi-drug resistance (Makanga *et al.*, 2006). Taking in account its structural characteristics, this antimalarial probably has a similar mechanism of action as the other amino alcohol quinolines, mefloquine and halofantrine.
In the human host this drug undergoes biotransformation mainly through CYP3A4, to form desbutyl-lumefantrine, which has a higher antiparasitic activity that the parent compound. This is the only lumefantrine metabolite characterized (Kerb *et al.*, 2009).

Halofantrine

This drug represents another arylamino-alcohol quinoline, hence structurally related to mefloquine and lumefantrine. This compound was developed in parallel with mefloquine by the Walter Reed Army Institute of Research in collaboration with SmithKline Beecham in the late 1960s. The use of this antimalarial has been mostly discontinued, due to its short half life (1-2days), slow and variable absorption and – most importantly- severe side effects; of

particular concern is its cardiotoxicity (Kitchen *et al.,* 2006). Together with mefloquine and lumefantrine, it is hypothesizes that this antimalarial acts by inhibiting the ingestion of host cell haemoglobin.

Studied performed with this antimalarial in microsomes, indicated a major role of CYP3A4 and to a minor extend CYP3A5 in the metabolism of halofantrine to *N*-debutylhalofantrine, its major metabolite (Baune *et al.,* 1999).

1.1.3.2. ANTIFOLATES

This group of antimalarials targets the *de novo* antifolate synthesis pathway in the *Plasmodium*. The antifolates can be subdivided in two groups, the inhibitors of the dihydropteroate synthase (DHPS) (class I antifolates), and the inhibitors of the dihydrofolate reductase (DHFR) (class II antifolates) (Wang *et al.,* 2004). Their structures are shown in figure 3.

Dapsone

Dapsone was synthesized for the first time in the early 20th century, as the result of a search for molecules that produced azo dyes (Fromm and Wittmann, 1906). This drug has been used as a monotherapy for malaria (*P.falciparum* and *P.vivax*)in some circumstances, but presently is only rarely used for prophylaxis in combination with other antimalarials, due to its limited efficacy and severe hemolytic side effects in individuals carrying G6PD mutations, quite common in Africa (Nzila, 2006).

Dapsone is also used in the treatment of dermatological inflammatory diseases such as dermatitis herpetiformis and in human chemotherapy (Nzila, 2006). The toxicity of this compound is thought to be initiated by the formation of hydroxylamine metabolites trough the action of CYP2E1 (Mitra *et al,.* 1995).

The inhibitors of dihydrofolate reductase (Type II antifolates), include a higher range of drugs, e.g., proguanil, chlorproguanil, pyrimethamine (Fig.3).

Proguanil

The first described antifolate antimalarial drug was proguanil. As a number of other antimalarials, it was the result of an intensive effort by the British Imperial Chemical Industries, to synthesize new antimalarials during the Second World War. The first reports on this drug are from 1945, showing its activity against avian malaria. This drug has been used as a prophylactic agent, either as a monotherapy or in combinations with chloroquine or, more recently, with atovaquone. The potency of proguanil led to the search for further active analogues, leading to the discovery of chlorproguanil and clociguanil (Nzila, 2006).

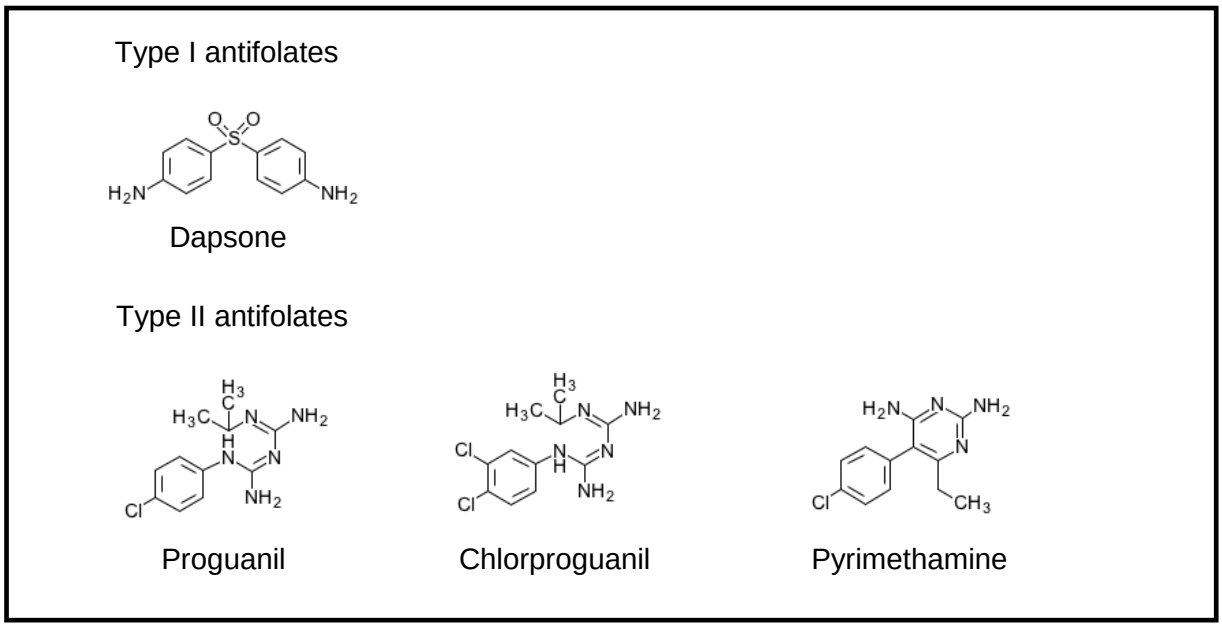


Figure 3: Chemical structures of the most commonly used type I (dapsone) and type II antifolates (proguanil, chlorproguanil and pyrimethamine).

Chlorproguanil

Chlorproguanil is generated by the chlorination of proguanil. This compound was initially recommended for prophylactic use, but due to inadequacy of the recommended dose, no prophylactic protection was acquired (Nzila, 2006).

Both proguanil and chlorproguanil are metabolised respectively to cycloguanil and chlorcycloguanil (the active metabolite), by CYP2C19 and to a lesser extent by CYP3A4. These enzymes are also involved in the inactivation of the parent compounds to 4-chlorophenylbiguanide and dichlorophenylbiguanide (Kerb *et al.*, 2009).

Pyrimethamine

The last member of the class II of antifolates is pyrimethamine, initially used in anti-cancer therapy. Due to its structural similarity to proguanil, its antimalarial activity was hypothesized and later confirmed (Falco *et al.*, 1951). This antimalarial used initially as a monotherapy is nowadays only administered in combination with sulfadoxine (section 1.1.3.2.1) (Nzila, 2006).

1.1.3.2.1. ANTIFOLATES COMBINATION THERAPY

The combination between antifolates from class I and II is synergistic; hence they are normally used in combination for the treatment of malaria. The most widely used antifolate, pyrimethamine, is usually used in combination with sulfa drugs like sulfadoxine (Fansidar®), or with dapsone (Malprin®). The short half life of dapsone decreases significantly the synergistic effect of the combination, thereby decreasing dramatically its efficiency. The other combination, Fansidar®, has been widely used in malaria endemic regions, with a higher success rate than Malprin ® (Nzila, 2006).

Another combination is proguanil/dapsone. This has been tested in treatment and prophylaxis of malaria. Recently, a new combination of these two drugs with artesunate has also been tested (Nzila, 2006). Both combinations are presently not widely used.

Chlorproguanil, a derivative of proguanil, is also available in combination with atovaquone; this fixed-dose combination has been reporter as highly effective in *P.falciparum* malaria prophylaxis and treatment (Nicosia *et al.*, 2008).

1.1.3.3. ARTEMISININ AND DERIVATIVES

Artemisinin, the active principal of the Chinese medicinal herb *Artemisia annua* and its derivates (dihydroartemisinin, arteether, artemeether and artesunate) are the group of compounds with the highest parasite reduction ratio (~10000/ erythrocyte cycle) and low toxicity (White, 1997). They have the broadest parasite stage specificity, being effective in most intra-erythrocytic cycle stages, due to a pleiotropic action in the parasite believed to be associated to the collapse of the characteristic endoperoxide functional bridge and consequent generation of oxygen radicals (Ter Kuile *et al.,* 2003; Skinner *et al.,* 1996). The resulting alkylating molecules lead to the disruption of the parasite essential functions, enzymes and membrane integrity (Meshnick, 2002).

Artemisinin is metabolized to four inactive compounds (deoxyartemisinin, deoxydihydroartemisinin, 9, 10-dihydrodeoxyartemisin and the so called "crystal 7") due to the action of mainly CYP2B6, or in cases where the levels of CYP2B6 are low, by CYP3A4. The derivatives artesunate, artemeether and arteether are metabolized to the active compound dihydroartemisinin (DHA) by CYP2A6, CYP3A4/5 and CYP3A4 (with a minor contribution of CYP2B6 and CYP3A5), respectively (Kerb *et al.,* 2009).

With the rise and global spread of *P. falciparum* resistant to the mainstay antimalarials chloroquine and pyrimethamine, a new paradigm rose in the last ten years – the use of these well tolerated and highly efficacious antimalarials in combination strategies with long half life drugs. These combination therapies will be further discussed in the next chapter. (www.rollbackmalaria.org/cmc_upload/0/000/015/364/RBMInfosheet_9.htm, accessed 04 February 2010).

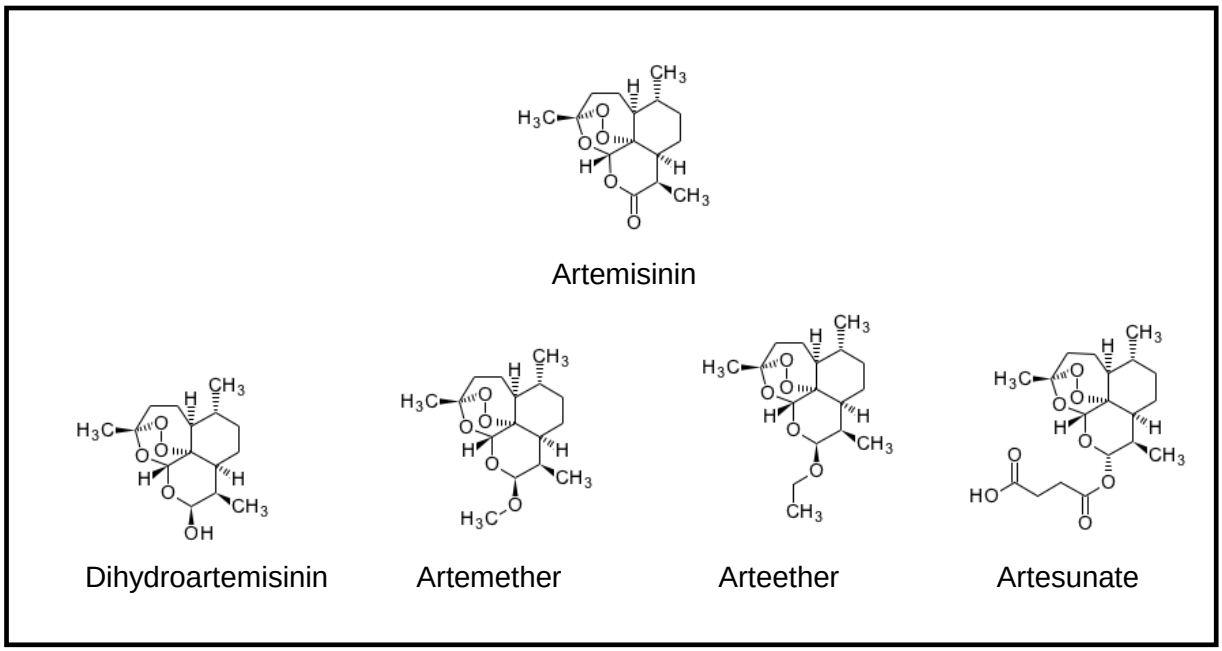


Figure 4: Chemical structures of artemisinin and its derivatives.

1.1.3.3.1. ARTEMISININ COMBINATION THERAPY (ACT)

Although possessing the highest known antimalarial effect, the use of artemisinin and its derivatives as a monotherapy is usually associated with frequent recrudescence of the infection, mainly due to the extreme short half life of these compounds (Alin *et al.*, 1995). To reduce recrudescence and prevent or slow the development of resistance, WHO has been recommending the use of artemisinin combined with another effective more slowly eliminated antimalarial (White, 1999). This strategy includes another important logistic point – the therapy can be completed in three days, not in the minimum of seven days postulated for artemisinin monotherapies.

The rationale of this type of therapy is based on 4 characteristics: 1. The fast acting artemisinin will reduce dramatically the parasite load in the first hours of treatment; 2. The slowly eliminated combination partner will only face a decreased amount of parasites; 3. Since the mechanism of action of both combination partners is different, the slowly eliminated partner, will target the parasites that might have been selected by the artemisinin partner; 4. These drugs frequently show a pharmacodynamic synergism between the

combination partners, being another advantage of this type of drugs (Mariga *et al.*, 2005; Gil and Gil Berlung, 2007).

The currently deployed ACTs include: artemether-lumefantrine (Coartem®, Novartis, Basel), artesunate-amodiaquine (Coarsucam® or Northeorp®, Sanofi-Aventis, Paris) artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and DHA-piperaquine (WHO, 2009). As with most co-administrations, these combination therapy strategies, although effective, also increase the possibility of drug-drug interaction. Little is known about these potential interactions.

1.1.3.4. NAPTHOQUINONES

Atovaquone

Atovaquone is a 2-hydroxinaphtoquinone (Fig. 5), indentified in the 1940s, but only certified as an antimalarial in 1997 in combination with proguanil, available under the commercial name Malarone®. This compound is as an analogue of coenzyme Q (ubiquinone), acting by interfering with the organelle membrane potential of the malaria parasite. Drug resistance to this compound appeared very rapidly upon its implementation as a monotherapy; however, its combination with proguanil retarded this resistance. This combination has been implemented as prophylaxis for travelers to South East Asia (Hyde, 2007).

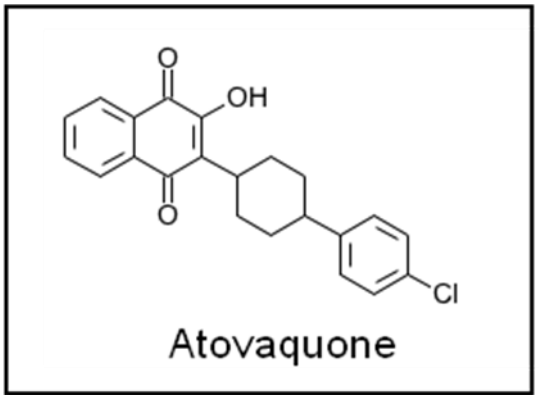


Figure 5: Chemical structure of atovaquone.

1.2. DRUG METABOLISM

The majority of the xenobiotics that enter the body undergo several biotransformations, in order to be transformed from a lipophilic compound to a more hydrophilic compound, easily excreted, to the bile or urine. Therapeutic drugs correspond to one of the most important xenobiotic exposures in the modern times being the main general focus of this thesis.

Drug metabolism occurs mainly in the liver, but tissues like the gastrointestinal tract, kidneys, skin and lungs may also be involved in this process. This process starts with the entry of the drug in the cells that may happen by diffusion or by the action of specific uptake transporters (phase 0), followed by two independent phases: Phase I, or functionalization process, involves reactions of hydrolysis, reduction and oxidation; Phase II, or conjugation phase, involves conjugation of the drug molecule with a hydrophilic endogenous substrate. As a general concept, phase I precedes phase II, but this is not strict, as they may occur independently from each other. The biotransformation process will end with the transport of the hydrophilic compounds to the outside of the cell (phase III), also this transport phase may occur independently of the previously described metabolic phases.

1.2.1. PHASE I

In this first stage of drug metabolism, the enzymes involved are mainly from the cytochrome P450 family (CYP) and to a certain extent flavin monooxigenases. The P450's and flavin moonoxigenases are both anchored in the membrane of the endoplasmatic reticulum of the cells. The similarity between these enzymes is reflected by on their main chemical reaction, as they both use NADPH + oxygen to oxidize their substrates (Kenakin, 2009).

1.2.1.1. CYTOCHROME P450S

The first descriptions of cytochrome P450s occurred in the 1950s; First by Axelrod and Brodie *et al.*, who identified an enzymatic system in the endoplasmic reticulum, which was able to oxidize xenobiotics (Axelrod, 1955; Brodie *et al.*, 1955). Later in the same decade Garfinkel and Klingenberg detected a carbon monoxide binding pigment with an absorption

maximum at 450nm (Garfinkel, 1958; Klingenberg 1958). This was demonstrated to be a hemoprotein of the b-type class in 1964 by Omura and Sato, and named cytochrome P450 due to its characteristic absorption at 450nm (Omura and Sato, 1964 a and b). At this time it was thought only one cytochrome P450 existed; further studies identified the different enzymes that belong to this family (reviewed in Josephy, 1997).

Due to the large number of human P450s, a specific nomenclature had to be established, essentially based in the degree of homology between the proteins amino acid sequence. Enzymes sharing ≥ 40% a.a. identity are classified in the same family. To share the same subfamily the enzymes have to share ≥ 55% a.a. identity. The first Arabic number indicates the family (CYP3), the letter indicates the subfamily (CYP3A) and another Arabic number corresponds to the individual gene (*CYP3A4*). The gene name is written in italic (*CYP3A4*) and the mRNA and protein in regular capital letters (CYP3A4). Taken this classification in consideration, presently, the 57 human P450s are organized in 18 families and 44 subfamilies (Nelson, 1999; http://drnelson.uthsc.edu/CytochromeP450.html, accessed 17 April 2010).

The major human CYP enzymes responsible for catalyzing drug biotransformation are members of families CYP1, CYP2 and CYP3. Taken together these 3 families are responsible for ~80% of the metabolism of clinical used drugs (Bertz and Granneman, 1997). The principal isoforms involved in drug metabolism include CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4/5 (Figure 6).

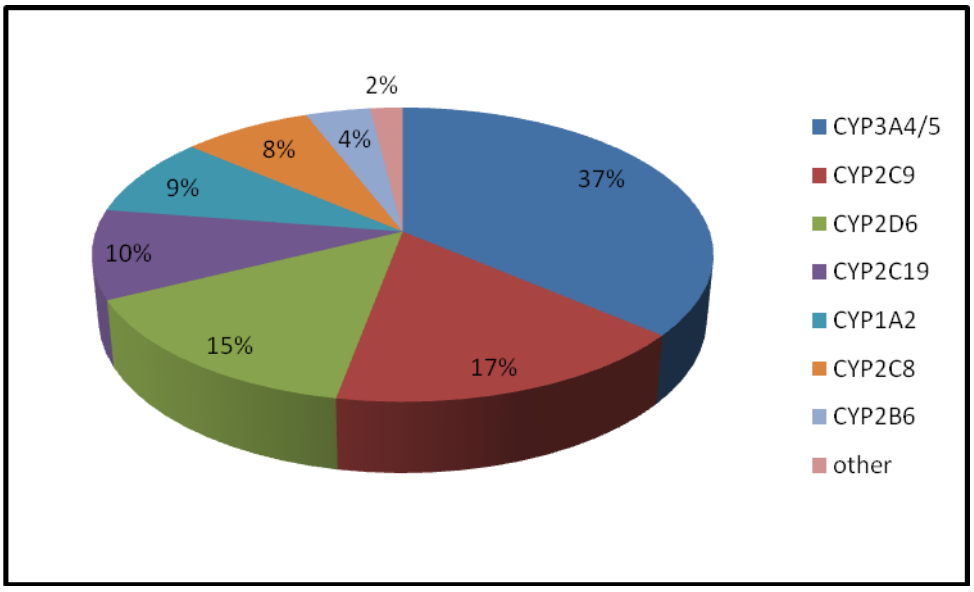


Figure 6: Relative contribution of cytochrome P450s to the metabolism of the 200 most commonly prescribed drugs in the USA, compiled from Zanger *et al.*, 2008.

The P450s of families 4-51 are involved in endogenous metabolic pathways of steroids, fatty acids, prostaglandins, etc**.** (Zanger *et al.*, 2008).

The enzymatic activity of the P450s may be influenced by several factors, both genetic, e.g., genetic variability and gene regulation and non-genetic, i.e., age, gender, hormonal status and disease. Of importance for this project is the variability of P450s due to the regulation of gene transcription, especially by nuclear receptors. This will be further discussed in chapter 1.2.4.

1.2.2. PHASE II

Phase II reactions are conjugation reactions, mainly, glucuronidations, sulfonations, methylations, acetylations. These reactions covalently link a functional group onto the target molecule to create highly polar, water soluble, metabolites that are rapidly excreted into urine (Kenakin, 2009). As stated, frequently the molecule has been previously modified through hydroxylation creating chemically favorable groups for the conjugation reactions. The major enzymes in phase II are the UDP glucuronosyltransferase (UGT), sulfotransferases (SULT), arylamine *N*-acetyltransferases (NAT), glutathione *S*-transferases (GST), thiopurine *S*-methyltransferases (TPMT) or the catecholamine-*O*-methyltransferases (COMT).

1.2.4. NUCLEAR RECEPTOR MEDIATED GENE REGULATION IN DRUG METABOLISM

Variations in the activity of DMEs can lead to altered drug levels, and consequently, to a modulation of its pharmacodynamics and risk of side effects.

The control of the expression of DME´s may occur at four different stages: Transcription, mRNA stability, translation and in a post-translational way. On the transcriptional level, these enzymes are controlled mainly by transcription factors. Of particular interest for this thesis is the gene expression control by a particular family of ligand-dependent transcriptions factors, the nuclear receptors.

The first nuclear receptor was identified in the 1960s by Jensen and collaborators, their work identified estradiol cellular activity as being mediated by a specific high-affinity receptor (Jensen, 1962). Since then nuclear receptors have been discovered and organized as part of a family comprising, 48 members in humans (table 1), that are responsible for sensing the presence of many different small molecules, including endobiotics and xenobiotics. In response to it, NRs interact with other proteins to regulate the expression of their respective target genes, controlling development, homeostasis and metabolism processes in the organism (Gronemeyer *et al.*, 2004).

Table 1: The human nuclear receptor family according to its official nomenclature and examples of ligands (adapted from Germain *et al.*, 2006 and Moore *et al.*, 2006)**.**

Official Nomenclature	***Name***	***Abbreviation***	***Ligand***
NR1A1	Thyroid hormone receptor alpha	TRα	Thyroid hormones
NR1A2	Thyroid hormone receptor beta	TRβ	Thyroid hormones
NR1B1	Retinoic acid receptor alpha	RARα	Retinoic acid
NR1B2	Retinoic acid receptor beta	RARβ	Retinoic acid
NR1B3	Retinoic acid receptor gamma	RARγ	Retinoic acid
NR1C1	Peroxisome proliferator- activated receptor alpha	PPARα	Fatty acids
NR1C2	Peroxisome proliferator- activated receptor delta	PPARδ	Fatty acids
NR1C3	Peroxisome proliferator- activated receptor gamma	PPARγ	Fatty acids
NR1D1	Reverse erb alpha	Rev-erbα	Unknown
NR1D2	Reverse erb beta	Rev-erbβ	Unknown
NR1F1	RAR-related orphan receptor alpha	RORα	Cholesterol
NR1F2	RAR-related orphan receptor beta	RORβ	Retinoic acid
NR1F3	RAR-related orphan receptor gamma	RORγ	Unknown
NR1H2	Liver X receptor alpha	LXRα	Oxysterols
NR1H3	Liver X receptor beta	LXRβ	Oxysterols
NR1H4	Farnesoid X receptor	FXR	Bile acids
NR1I1	Vitamin D receptor	VDR	Vitamin D

NR1I2	Pregnane X receptor	PXR	Xenobiotics
NR1I3	Constitutive androstane receptor	CAR	Xenobiotics
NR2A1	Hepatocyte nuclear factor 4 alpha	HNF4α	Linoleic acid
NR2A2	Hepatocyte nuclear factor 4 gamma	HNF4γ	Unknown
NR2B1	Retinoid X receptor alpha	RXRα	Retinoic acid
NR2B2	Retinoid X receptor beta	RXRβ	Retinoic acid
NR2B3	Retinoid X receptor gamma	RXRγ	Retinoic acid
NR2C1	Testis receptor 2	TR2	Unknown
NR2C2	Testis receptor 4	TR4	Unknown
NR2E1	Tailless-like receptor	TLX (TLL)	Unknown
NR2E3	Photoreceptor-specific nuclear receptor	PNR	Unknown
NR2F1	Chicken ovalbumin upstream promoter–transcription factor I	COUP-TFI	Unknown
NR2F2	Chicken ovalbumin upstream promoter–transcription factor II	COUP-TFII	Unknown
NR2F6	Chicken ovalbumin upstream promoter–transcription factor III	COUP-TFIII (EAR2)	Unknown
NR3A1	Estrogen receptor alpha	ERα	Estradiol, Estrogens
NR3A2	Estrogen receptor beta	ERβ	Estradiol, Estrogens
NR3B1	Estrogen-receptor-related receptor alpha	ERRα	Unknown
NR3B2	Estrogen-receptor-related receptor beta	ERRβ	DES
NR3B3	Estrogen-receptor-related receptor gamma	ERRγ	DES
NR3C1	Glucocorticoid receptor	GR	Cortisol
NR3C2	Mineralocorticoid receptor	MR	Aldosterone
NR3C3	Progesterone receptor	PR	Progesterone
NR3C4	Androgen receptor	AR	Testosterone
NR4A1	NGF-induced factor B	NGFI-B	Unknown
NR4A2	Nur-related factor 1	NURR1	Unknown
NR4A3	Neuron-derived orphan receptor 1	NOR1	Unknown
NR5A1	Steroidogenic factor 1	SF1	Unknown
NR5A2	Liver receptor homologous	LRH-1	Unknown

NR6A1	Germ cell nuclear factor	GCNF	Unknown
NR0B1	DSS-AHC critical region on the chromossome gene 1	DAX-1	Unknown
NR0B2	Small heterodimer partner	SHP	Unknown

The NR family can be divided in 3 distinct groups according to the nuclear receptor ligand specificity: **(a)** the endocrine receptors (e.g., androgen receptor, estrogen receptor and glucocorticoid receptor), that respond to steroid hormones; **(b)** the so-called orphan receptors, which are proteins with all the structural characteristics of a nuclear receptor, but with no ligand yet identified; **(c)** a last group, growing from the previous one representing the *adopted* orphan receptors, which had their ligands identified since their own isolation (e.g. pregnane X receptor, constitutive androstane receptor) (Sonada *et al.*, 2008).

Some of these nuclear receptors may act as a monomer, but the majority acts as dimers, either with themselves (homodimer) or with the nuclear receptor RXR (RXR-heterodimer) (Olefsky, 2001).

Although with different ligand specificities, the structure of the nuclear receptors is very similar throughout the family. It comprises 5 distinct domains: an N-terminal domain, a DNA binding domain (DBD), a hinge region, a ligand binding domain (LBD) and a C-terminal domain. (Fig.7) (Giguère, 1999).

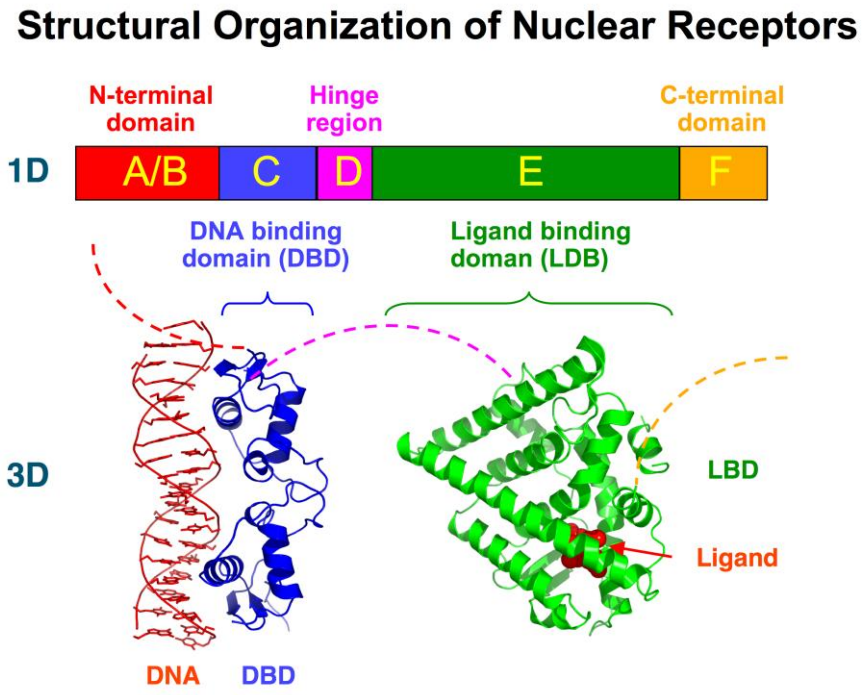


Figure 7: Structural organization of nuclear receptors.

(http://upload.wikimedia.org/wikipedia/commons/3/3e/Nuclear_Receptor_Structure.png, accessed on 21 April 2010)

The main areas responsible for the specific features of the nuclear receptors are the amino terminus, the LBD and the DBD. The amino terminus contains a transactivation domain (AF-1) that is recognized by co activators and/or other transcription factors. The LBD identifies specific ligands and directs the biologic response to this stimulus. The DBD is responsible for the binding of the nuclear receptor to specific binding sites in the promoter regions of the target genes. These binding sites or response elements are derived from the consensus motif, RGGTCA. The modification, extension or duplication (these duplications may have alternate relative orientations, e.g. direct, inverted or everted) of this sequence, creates response elements that more or less selective for a particular nuclear receptor (Gronemeyer *et al.*, 2004).

Several of these nuclear receptors exhibit specific xenobiotic binding capacity. These, so called, xenosensors are capable of regulating the transcriptional activation of several genes encoding for phase I and II drug-metabolizing enzymes, as well as transporters. The major

xenobiotic receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), will be further discussed in the next chapters.

1.2.4.1. PREGNANE X RECEPTOR (NR1I2; PXR)

The pregnane X receptor was initially identified in 1998, as being a nuclear receptor involved in the induction of cytochrome P450s, particularly CYP3As, and was named PAR, SXR and PXR in the respective publications (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Kliewer *et al.*, 1998). Further investigations identified this nuclear receptor as regulator of other key DMEs and transporter, such as, CYP1A1, 1A2, 2A6, 2B6, 2C8, 3A4, 3A5, GSTs, UGTs, SULTs, MDR1 and MRP2 (Maglich *et al.*, 2002; reviewed in Tirona and Kim, 2005). This nuclear receptor acts as a heterodimer together with RXRα, binding to response elements such as DR3, DR4, DR5, ER6, ER8 and IR0 in the promoter regions of the aforementioned genes (Lehmann *et al.*, 1998; Geick *et al.*, 2001; Goodwin *et al.*, 1999 and 2001; Kast *et al.*, 2002; Sonoda *et al.*, 2002)

PXR is mainly expressed in the liver, and represents one of the most promiscuous receptors, being activated by a large range of structurally diverse compounds, including xenobiotics and endobiotics. This is due to a ligand-binding pocket unusually large (reaching >1100 Å3) and flexible, spherical in shape and extremely hydrophobic, as twenty of the twenty eight residues in the ligand-biding pocket are hydrophobic (Watkins *et al.*, 2001). A representative list of PXR ligands is described in table 2 (Orans *et al.*, 2005; Carnahan and Redinbo, 2005).

Of central interest for this thesis is the previously described role of artemisinin, arteether and artemeether as ligands of PXR (Burk *et al.*, 2005). These data led to the follow up project described in this thesis, with the intent of identifying new ligands among the other antimalarials available on the market.

Table 2: List of some known agonists of PXR (for a more comprehensive review on PXR ligands, Meyer zu Schwabedissen and Kim, 2009).

PXR Agonists		
Artemisinin	Docetaxel	Nifedipine
Arteether	Efavirenz	Paclitaxel
Artemether	Fluvastatin	Tamoxifen
Atorvastatin	Ginko Biloba extract	Lithocolic acid
BK8644	Hyperforin	5β-pregnane-3, 20-dione
Carbamazepin	Ifosfamide	Progesterone
Clotrimazole	lovastatin	Ursodeoxycholic acid

1.2.4.1.1. PXR GENETIC STRUCTURE AND VARIABILITY

The human P*XR* gene was mapped to chromosome 3 (3q12-q13.3) and spans approximately 35kb. It consists of 9 exons, with the translation starting site located in exon 2 (Fig 8). The open reading frame (ORF) codes for a 434 amino acids protein (49.7kDa), named hPXR.1, representing the main splice variant of PXR (Sandanaraj *et al.*, 2008).

Nine other splice variants have been identified for this protein (Lamba *et al.*, 2004; Kurose *et al.*, 2005). The second most common transcript variant is hPXR.2, encoding a 393 a.a. protein lacking 41 a.a. from the ligand binding domain (Sandanaraj *et al.*, 2008). Another interesting splice variant is PAR2, it difers from PXR, by being transcribed from exon 1b, while PXR is trancribed from exon1a. PXR has a CTG translation start site in exon 2, whereas PAR2 has an ATG translation start site in exon 1b leading to an additional 39 amino acids at the amino terminal end (Lamba *et al.*, 2008).

The first polymorphisms in the coding and non-coding regions of the human *PXR* gene were described in 2001. These were associated with individual phenotype variations in basal and rifampicin inducible expression of CYP3A and MDR1/P-gp (Zhang *et al.*, 2001; Hustert *et*

al., 2001). Meanwhile, a large number of SNPs have been described in this gene, also leading to altered induction of target genes. They may affect the disposition of many currently administrated drugs, metabolised and/or transported by PXR induced proteins. Ultimately, polymorphisms in *PXR* leading to functional changes in this receptor may play a key role in the regulation of drug response. For a comprehensive review of the previously identified polymorphisms in PXR please refer to the table in annex1 and figure 8.

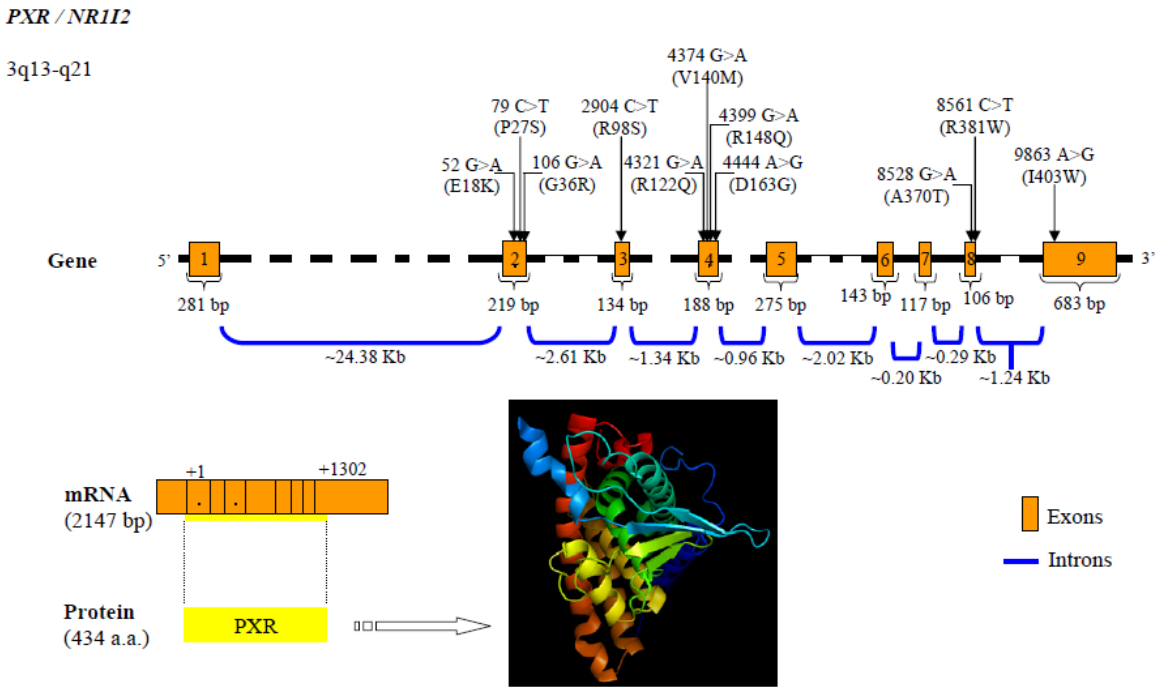


Figure 8: Genomic structure of the PXR gene and protein. In the gene structure the main coding region single nucleotide polymorphisms are annotated (Cavaco, 2008).

1.2.4.2. CONSTITUTIVE ANDROSTANE RECEPTOR (NR1I2; CAR)

Human constitutive androstane receptor (hCAR), was initially cloned by Baes and colleagues in 1994, and named MB67 (Baes *et al.*, 1994). As PXR, it functions as a xenosensor, sensing the presence of xenobiotics and in response, upregulating the expression of key DMEs, e.g., *CYP1A, SULT1A1, FMO5, CYP2B, CYP3A, CYP2C, CYP2A, UGT1A1, GSTA1, ALDH1A, MRP3, MDR1*, through the binding to DR1, DR3, DR4, DR5, ER6, and ER8 binding sites in their regulatory regions (Li and Wang 2010). The similarities between these two nuclear

receptors are visible also, when it concerns their heterodimerization partner, both form heterodimers with RXRα (di Masi et al. 2009).

CAR received its name constitutive androstane/activated receptor due to its particular characteristic of being constitutively active in immortalized cells but not in the liver in the absence of ligand. This led to its initial definition as a constitutive active receptor (Choi et al 1997). Besides highly expressed in the liver, CAR is also expressed in the intestine, and to a minor extent in human heart, skeletal muscle, brain, kidney, and lung (Baes *et al.*, 1994; Wei *et al.*, 2002; Arnold *et al.*, 2004)

1.3. AIMS

This project was motivated by the fact that only limited data was available concerning antimalarial chemotherapy and modulation of the PXR/CAR system. Induction of PXR- and/or CAR-dependent drug metabolism and transport by antimalarial drugs may result in autoinduction of metabolism and drug-drug interactions, compromising the success of antimalarial therapy. This is of particular relevance when taking in account the global importance of malaria as a major lethal infectious disease and facing the increasing need of antimalarial combination therapies

In the first part of this project we intended to elucidate the capacity of the currently used antimalarial drugs and their main metabolites to activate PXR and/or CAR, and to evaluate the drug-drug interaction potential of the antimalarials, using *in vivo* and *in vitro* assays.

In the second sub-project we intended to understand the genetical basis of variable PXR-dependent induction of key cytochrome P450s, through a molecular epidemiology approach using previously studied healthy individuals exposed to pivotal artemisinin derivatives as case studies.

2. MATERIAL AND METHODS

2.1. MATERIAL

2.1.1. ANTIMALARIC DRUGS AND METABOLITES

2.1.1.1. SELECTION OF THE COMPOUNDS TO BE TESTED

The compounds tested in this project were selected based on malaria treatment recommendations. If known and available, their major active and non-active metabolites were also included in this study. The selected compounds were used in sub-project 1 and are described in table 3.

2.1.1.2. CHARACTERISTICS OF ANTIMALARIC DRUGS AND THEIR METABOLITES

For the study of the induction capacity of the antimalarials and metabolites, stock solutions of 100mM of the compounds were prepared in Me_2SO. This solvent was used as a control in all induction assays.

The molecular weight and supplier of each compound is described in table 3. Chlorocycloguanile, pyrimethamine and sulfadoxine were kindly provided by Dr. José Pedro Gil, Department of medicine, Karolinska Institutet, Stockholm, Sweden.

Table 3: List of compounds tested in sub-project 1. For each compound molecular weight and supplier are given.

Name	*Molecular Weight*	*Supplier*
Amodiaquine	464.8	Sigma
Arteether	312.4	Dafra
Artemether	298.4	Dafra
Artemisinin	282.3	Dafra

Artesunate	384.4	Dafra
Atovaquone, free base	366.8	GlaxoSmithKline
Carboxymefloquine	309.16	TRC
Chlorocycloguanile HCl	251.7	-
Chloroquine	515.0	Sigma
Cycloguanile HCl	288.2	TRC
Dantrolene sodium salt	336.2	Sigma
Dapsone (4-aminophenyl sulfone)	248.3	TRC
Dapsone Hydroxylamine	264.3	TRC
Deoxy-arteether	296.4	TRC
Deoxy-artemisinin	266.3	TRC
Desbutyl-benflumetol	472.8	Novartis
Desethylchloroquine	291.8	TRC
Didesethylchloroquine	263.8	TRC
Halofantrine	536.9	Sigma
Isoquinine	324.4	TRC
Lumefantrine	530.0	Novartis
Mefloquine HCl	414.8	Sigma
N-desethylamodiaquine (DEAQ)	327.8	TRC
Primaquine	455.3	Sigma
Proguanile HCl	290.2	GlaxoSmithKline
Pyrimethamine	248.7	-
Pyronaridine	910.0	Avachem
Quinine	324.4	Sigma
Sulfadoxine	310.3	-

2.1.2. COMMON REAGENTS

Common reagents used in this project were obtained from the following companies: GE Healthcare (Freiburg, DE), Biozym (Hess. Oldenburg, DE), Difco (Detroit, MI, USA), Fluka

(Buchs, CH), Invitrogen (Groningen, NL), Merck (Darmstadt, DE), Roth (Karlsruhe, DE), Serva (Heidelberg, DE) and Sigma (Deisenhofen, DE).

2.1.3. ENZYMES AND PCR REAGENTS

Enzymes were obtained from the following companies, and used together with specific buffers as described by the supplier: GoTaq® Polymerase (Promega, Fitchburg, WI, USA). Reagents used for PCR reactions were obtained from the following companies: dNTPS (Promega, Fitchburg, WI, USA), Real time qPCR master mix (Eurogentec, Seraing, BE).

2.1.4. KITS

Kits were used according the manufacturer instructions and were obtained from the following companies: PureYield™ Plasmid Midiprep System (Promega, Fitchburg, WI, USA); Effectene Transfection Reagent (Qiagen, Hilden, DE); mirVana Kit (Applied Biosystems/Ambion, Austin, USA); Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, Buckinghamshire,UK) PureYield™ Plasmid Midiprep System (Promega, Fitchburg, WI, USA).

2.1.5. CELL LINES, CELL CULTURE MEDIUMS AND SUPPLEMENTS

COS1: African green monkey kidney fibroblast-like cell line (American type culture collection, Manassas USA).

HeLa: Human cervical carcinoma cell line (American type culture collection, Manassas USA).

All cell culture media, supplements and fetal calf serum were obtained from Invitrogen (Groningen, NL).

Two additional compounds were used as controls in the mammalian two hybrid and gene reporter assays, namely, 6-(4-chlorophenyl)imidazo [2,1-b]thiazole-5-carbaldehyde O-(3,4-

dichlorobenzyl)oxime (CITCO) obtained from Biomol (Plymouth, PA), and Rifampicin (Sigma, Deisenhofen, DE)).

2.1.6. PLASMIDS

Specific plasmids were used for the mammalian two hybrids gene reporter assay and C.A.R.L.A. Their characteristics and origin are described in table 4.

Table 4: Plasmids used in the mammalian two hybrid assay, reporter gene assay and C.A.R.L.A.

Name	*Description*	**Reference**
pGL3-G5	Luciferase reporter plasmid used in mammalian two hybrid assays	Arnold *et al.,* 2004
pCMVß	ß-Galactosidase expression vector	Clontech
pUC18	Fill up plasmid for transfections	Invitrogen
pM-DRIP 205 (527-774)	Expression plasmid of the fusion protein GAL4-DBD/DRIP205-RID (a.a.527-774)	Arnold *et al.*,2004
pVP16-CARSV2 (105-353)	Expression plasmid of the fusion protein VP16-AD/CARSV2-LBD (a.a.105-353)	Arnold *et al.*,2004
pM-SRC1 (583-783)	Expression plasmid of the fusion protein GAL4-DBD/SRC1-RID (a.a.583-783)	Arnold *et al.*,2004
pVP16-PXR (108-434)	Expression plasmid of the fusion protein VP16-AD/PXR-LBD (a.a.108-434)	Burk *et al.,* 2005
pGL3-CYP3A4	CYP3A4 luciferase promoter reporter construct	Hustert *et al.,* 2001
pcDhuPXR	Expression plasmid of human PXR	Geick *et al* ,2001

2.1.7. OLIGONUCLEOTIDES

The oligonucleotides used during this project are described in tables 5 and 6. For the re-sequencing of PXR specific primers had to be designed and amplification conditions had to be optimized.

Table 5: Oligonucleotides used for quantitative real time RT (reverse transcription)-PCR. The primers were obtained from Biomers (Ulm, Germany), and dissolved to a final concentration of 100µM according to the indications from the supplier. Probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA, USA).

Gene	*Oligonucleotide*	*Sequence (5'-3')*	*Label*	*Assay conditions (nM)*	*Reference*
18S	**Fw Primer**	ACCGCAGCTAGGAATAATGGA		400	
	Rv Primer	GCCTCAGTTCCGAAAACCA		400	
	Probe	ACCGCGGTTCTATTT	5'VIC/3' MGB/NFQ	200	
CYP3A4	**Fw Primer**	TGTCCTACCATAAGGGCTTTTGTAT		400	Wolbold *et al.*, 2003
	Rv Primer	TTCACTAGCACTGTTTTGATCATGTC		400	
	Probe	CTTTTATGATGGTCAACAGCCTGTGCTG	5'FAM/3'TAMRA	200	
CYP2B6	**Fw Primer**	GCTGAACTTGTTCTACCAGACTTTTTC		400	Burk *et al.*, 2005
	Rv Primer	GAAAGTATTTCAAGAAGCCAGAGAAGAG		400	
	Probe	TGTATTCGGCCAGCTGT	5'FAM/3'MGB/NFQ	400	
MDR1	**Fw Primer**	CTGGTGTTTGGAGAAATGACAGATA		900	Burk *et al.*, 2005
	Rv Primer	TGGTCATGTCTTCCTCCAGATTC		900	
	Probe	TCAAACATCACTAATAGAAG	5'FAM/3'MGB/NFQ	240	

Table 6: Primers, annealing temperatures and fragment sizes used for the re-sequencing of PXR. Primers where dissolved to a concentration of 100µM with water according with the information of the supplier (Thermo Fischer Scientific, Ulm, Germany). The primers used for sequencing are highlighted in bold.

Name	*Sequence (5'-3')*	*T (°C)*	*Fragment size (bp)*	**Reference**
Promoter1Fw	**CCCAGCAGTGAGCTGTGTAA**	56	644	Zhang et al. 2001
Promoter1 Rv	AGCTGAGGGCTCTTTCCTCT			Zhang et al. 2001
Promoter2Fw	**GCACCTGCTGCTAGGGAATA**	56	568	Zhang et al. 2001
Promoter2 Rv	CCCCTCCTAAGTCCCAAGTC			Newly designed
Exon1a Fw	**TCAAGTGCTGGACTTGGGAC**	69	460	Hustert et al. 2001
Exon1a Rv	CCCACTATGATGCTGACCTC			Hustert et al. 2001
Exon1b Fw	**TGCTCTCTGGTCCTGCACTA**	56	411	Newly designed
Exon1b Rv	CCACATGCAGGCAAGACTC			Hustert et al. 2001
Exon2 Fw	**CTGAGGCCTCTACACATC**	64	487	Hustert et al. 2001
Exon2 Rv	GAAGGAGACCTTTCCTGGGT			Newly designed
Exon3 Fw	CTGGGACGCAAAGGCTAGTG	64	417	Hustert et al. 2001
Exon3 Rv	**CCTGTTGCACACGGACAC**			Hustert et al. 2001
Exon4 Fw	**TAACGGCTTCTGCTGCCTTG**	64	367	Newly designed
Exon4 Rv	ATCCTTGGGGAACCTCAGTT			Newly designed
Exon5 Fw	**GCTGTGTGTGTATATGTGTGAGGA**	60	566	Ferreira et al. 2008
Exon5 Rv	TTGGTGTCAGAAGACCCTCC			Ferreira et al. 2008
Exon6/7 Fw	**GAGATGAGAGGCAGCCAGAC**	56	695	Newly designed
Exon6/7 Rv	GACTGGGACCTTCCCTGG			Hustert et al. 2001
Exon8 Fw	**TATGGCCTTGCTCCTCATTC**	56	505	Newly designed
Exon8 Rv	CCCCTGTTTGCTTGTGTTTT			Newly designed
Exon9(1) Fw	**AATCTTTTCTCTGGCTGGCA**	56	770	Newly designed
Exon9(1) Rv	TGTCAGAAGCTTGGCATGAC			Newly designed
Exon9 (2)Fw	**CAAATGTCAGAAGCTTGGCA**	56	754	Newly designed
Exon9(2) Rv	TATTTCCACACCCCCACATT			Newly designed

2.1.8. SOFTWARE

Statistical analysis was performed using GraphPad Prism v.4 (GraphPad Software, San Diego, USA). Primer design was performed using Primer Blast in NCBI Homepage. Sequence alignment and new SNP search was performed using Geneious Pro V.4.8.

2.1.9. HUMAN SAMPLES

The human hepatocytes used in subproject 1 were kindly provided by Dr. Andreas Nüssler from Charité, Humbold University, Berlin. These hepatocytes were obtained from liver resections of patients with primary or secondary liver tumors. The harvesting and following procedures were done according to the institutional guidelines of the Charité, and with the written informed consent of each patient. The hepatocytes were isolated from the liver tissue and cultivated as reported elsewhere (Yuan *et al.*, 2004).

For subproject 2 a group of 75 healthy Vietnamese volunteers, described previously by Asimus et al 2007, were used in the study. The group comprises 72 subjects from the predominant Kinh ethnic group and 3 from Thai ethnicity.The participants were all judged to be healthy on the basis of medical history, physical examination and routine clinical laboratory determinations. Subjects who had taken any antimalarial drug within 1 month or any other drug within 2 weeks before the start of the study and those with a history of alcohol abuse were excluded from the study. Oral contraceptive use was not recorded. Thirty-six of the subjects were smokers of no more than 10 cigarettes per day.

All participants provided written informed consent prior to enrolment. Study protocols were approved by the Ministry of Health, Hanoi, Vietnam, the Swedish Medical Products Agency, Uppsala, Sweden and the Ethics Committee of University of Gothenburg, Göteborg, Sweden. The studies were performed according to the principles of the Declaration of Helsinki.

2.2. METHODS

2.2.1. PLASMID PREPARATION

2.2.1.1. CHEMICALLY COMPETENT BACTERIAL CELLS

TOP 10 F' E.coli bacteria were transferred into 6ml of Ψ-broth medium and grown at 37°C up to an optical density at 550nm (OD550) of 0.3. One hundred ml of pre-warmed Ψ-broth medium was then inoculated with 5 ml of this pre-culture, and incubated up to an OD550 of 0.45-0,5. This culture was then incubated on ice for 10 min and centrifuged at 3000 g, 5 min at 4°C. The pellet was ressuspended in 20ml ice-cold TfB I buffer, filled up to 60 ml and incubated on ice for 2h. This suspension was then centrifuged at 3000 g, 5min, 4°C. The pellet was ressuspended in 4ml of ice-old TfB II buffer and further incubated on ice for 15 min. The bacteria were aliquoted and stored at -80°C until usage.

Ψ-Broth-Medium: 2% Bacto-Trypton; 10 mM KCl; 0.5% yeast extract; autoclave; at the end add 0.2% $MgSO_4$·7 H2O; adjust pH with KOH to 7.6

TfB I buffer: 100 mM $RbCl_2$; 50 mM $MnCl_2$; 30 mM K-acetate; 10 mM $CaCl_2$; 15% (v/v) glycerin; adjust pH with 0.2 M acetic acid to 5.8

TfB II buffer: 10 mM MOPS, pH 7.0; 10 mM $RbCl_2$; 75 mM $CaCl_2$; 15% (v/v) glycerine

2.2.1.2. BACTERIAL CELL TRANSFORMATION WITH PLASMID DNA

One µL of plasmid DNA (100-500ng) was incubated with 50µL of pre-aliquoted TOP 10F' competent cells on ice for 30 min, followed by a incubation at 42°C for 35 sec and a final incubation on ice for 2 min. 250 µl SOC media were added and the cells were incubated at 37°C with shaking for 1 h. The bacteria were then spread on a LB agar plate containing ampicillin (0.2mg/ml), or directly inoculated in 50 ml of LB medium containing ampicillin and incubated over-night at 37°C. The purification of the plasmid was performed in the following day.

SOC medium: 2% Bacto-Trypton; 0.5% yeast extract; 0.06% NaCl; 2.5 mM KCl; autoclave; + 2 mM $MgCl_2$

LB- ("Luria-Bertani") medium: 2% Bacto-Trypton; 1% NaCl; 0.5% yeast extract;

LB agar plates: LB-medium with 1.5% Bacto-Agar; autoclave, and divide by the plates.

2.2.1.3. ISOLATION OF PLASMID DNA

The cells from an overnight culture were harvested by centrifugation and plasmid isolation was performed using the PureYield^{TM} Plasmid Midiprep System. This system uses silica-membrane columns for a rapid and efficient extraction of high quality DNA for use in eukaryotic transfections and in vitro expression experiments.

The extractions were performed according to the manufacturer's instructions.

2.2.1.4. QUANTIFICATION AND CONFIRMATION OF THE IDENTITY OF THE PLASMID DNA

The plasmid DNA was quantified using a microvolume spectrophotometer. After assessing the DNA amount, this was diluted to a final concentration of 0.2µg/µL.

0.5µg of plasmid DNA was digested with appropriate restriction endonucleases to confirm its identity by analysis of its restriction pattern. DNA digestions were carried out according to the manufacturer's instructions. The digested DNA was analyzed by agarose gel electrophoresis.

2.2.2. BASIC CELL CULTURE PROTOCOL

For gene reporter and mammalian two hybrid assays, immortalized cell lines were used, specifically COS1 and HeLa.

These cells lines were cultured in DMEM medium, supplemented with 10% FBS, 2mM L-glutamine and 1% of penicillin/streptomycin. Twice a week they were subcultured in a ratio of 1:10.

2.2.3. TRANSIENT TRANSFECTION USING EFFECTENE®

Effectene® is a non-liposomal lipid formulation transfection reagent optimized to achieve high transfection efficiencies. This is achieved by an initial step of DNA condensation by interaction with the Enhancer® followed by the addition of the Effectene and formation of condensed Effectene-DNA complexes. The Effectene forms micelles with uniform size, significantly independent of batch variation, ensuring high reproducibility of DNA transfection.

Twenty hours before transfection COS1 and HeLa cells were seeded in 24-well plates at a density of $3x10^4$ cells / well for both cell lines. On the day of transfection, 200 ng of DNA/well was diluted in Buffer EC up to a final volume of 60µL. 1.6 µL of enhancer was added and the final mixture incubated at room temperature for 5 min. Following this incubation, 2µL of Effectene transfection reagent was added, followed by incubation at room temperature for 10 minutes, meanwhile the cells where re-fed with 500µl of new medium. Finally the DNA mixture was diluted with 200µl of fresh medium and the transfection complexes where transferred dropwise to the cells re-fed with new medium. Cells were incubated with the transfection mixture for 8 hours, after this period the cells were washed with PBS and fed with the induction medium.

2.2.4. MAMMALIAN TWO HYBRID ASSAY

Mammalian two hybrid is a method used to analyse protein-protein interactions. In this project we intended to study the ligand dependent interaction between the LBD of a specific nuclear receptor and the RID of a co-activator. For this purpose the LBD of the nuclear receptor was fused to the VP16-activation domain and the RID of the co-activator fused with the GAL4-DNA binding domain. In the presence of a specific ligand the interaction between the NR-LBD and the co-activator-RID is induced, bringing into close proximity the VP16-AD and the GAL4-DBD, forming an active transcription activation complex and inducing the expression of the reporter target gene, the firefly luciferase (Fig.9). Concomitant, the cells were also co-transfected with a plasmid containing a constitutively active β-galactosidase, used as a transfection efficiency control.

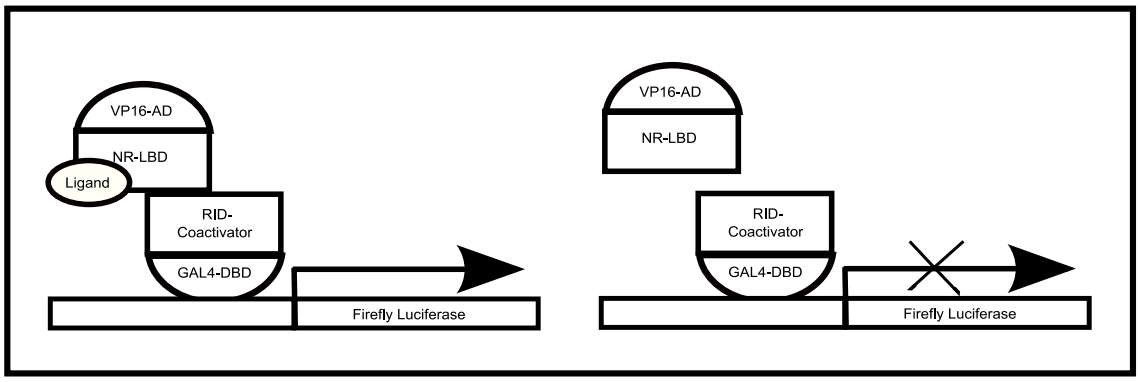


Figure 9: Brief description of the mammalian two hybrid assay.

Based on previously obtained data from our laboratory, two systems were chosen to study the ligand binding of the antimalarials to the xenosensors, PXR and CAR. (Burk *et al.*, 2005). The selection of the co-activator to be tested was based on the previoulsy data from our lab, concerning interaction intensity. For these studies it was chosen the co-activator with the highest interaction values with the PXR/CAR, upon exposure to a prototypical ligand, namely rifampicin for PXR and CITCO for CAR. So, based on these criteria, it was chosen as targets of study the interactions between PXR and SRC1 and of CAR SV2 and PPARBP. These systems were used to perform an initial screening of the ligand capacity of the antimalarials and further to determine their specific EC50's. For an initial screening two concentrations were used, 100µM and 10µM. Compounds that revealed an induction of the interaction between the nuclear receptor and the co-activator at this two initial concentrations, were further tested using five additional concentrations (1, 3, 30, 50 and 300µM) to determine the EC50's by graphical analysis of the dose-response curves, using the GraphPad software. All compounds were tested using this system, except for the compounds from the artemisinin family in the PXR case that were already described as not inducing the interaction of co-activators with PXR (Burk *et al.*, 2005); for these compounds a gene reporter assay approach was used.

Briefly, the plasmids described in table 7 were transfected into COS1 (CARSV2-PPARBP) or HeLa Cells (PXR-SRC1) according to the description in section 2.2.3. All transfection were performed in triplicates and repeated at least twice in independent experiments.

Table 7: Plasmid amounts, used for the mammalian two hybrid assays.

Plasmid name	CARSV2-PPARBP	PXR-SRC1
pGL3-G5	0.10µg	0.10µg
pCMVß	0.02µg	0.02µg
pUC18	-	0.02µg
pM-PPARBP	0.01µg	-
pVP16-CARSV2	0.08µg	-
pM-SRC1	-	0.05µg
pVP16-PXR	-	0.01µg
Total amount	0.21µg	0.20µg

The induction procedure was performed as described in section 2.2.6.

2.2.5. GENE REPORTER ASSAY

Reporter gene assays are powerful tools to access the regulation of a specific gene. In this technique the DNA regulatory region of interest is fused to a reporter gene, in our specific case, the firefly luciferase gene. The enzyme coded by this gene is able to catalyze a light emitting reaction. Upon induction by the promoter region under test, the light emitted is quantified as a direct measurement of the induction capacity of the molecule on study.
If the cell line used for the assay did not express the transcription factor mediating the activation of the promoter region, it may be necessary to co-transfect an episomal expression vector carrying the gene coding for this protein. This was the situation in our system - the study of the induction of the promoter region of *CYP3A4* by PXR. As COS1 cells do not express PXR, co-transfection of the pcDhuPXR expression plasmid carrying the gene for this nuclear receptor had to be performed.

Briefly, COS1 cells were transfected as described in section 2.2.3, with the plasmids described in table 8. All transfection were performed in triplicates and repeated at least twice in independent experiments.

Table 8: Plasmid amounts, used for the gene reporter assay.

Plasmid name	Amount
pGL3-CYP3A4	0.15µg
pCMVß	0.02µg
pUC18	0.02µg
pcDhuPXR	0.01µg
Total amount	0.20µg

The induction procedure was performed as described in section 2.2.6.

2.2.6. INDUCTION BY ANTIMALARIALS OF THE MAMMALIAN TWO HYBRID AND GENE REPORTER SYSTEMS

After 8 hours of incubation with the specific transfection complexes, the cells were treated with induction mediums. For the initial screening two concentrations of the antimalarials were tested, namely, 100µM and 10 µM. The compounds and their solvent (Me_2SO) were diluted to their final concentration, in phenol red-free DMEM medium, supplemented with 10% FCS-DCC (fetal calf serum that was previously treated with dextran-coated charcoal to eliminate steroids), 2mM L-glutamine and 1% of penicillin/streptomycin. The same was performed to assess dose-curve response curves for all compounds that revealed induction in the first screening. For the dose-curve response curves concentrations of 1, 3, 30, 50, and 300 µM were tested. After ~40h of induction the cells were harvested (see section 2.2.7.) and firefly luciferase and β-galactosidase activities were measure according to sections 2.2.7. and 2.2.8.

2.2.7. CELL HARVESTING

After ~40h of induction the cells were harvested by aspirating the medium and washing the cells twice with 500 µl 1x PBS. Cell lyses was performed using 150 µl 1x Passive Lyses Buffer (Promega) added to each well and incubated at room temperature for 20 min with shaking. The lysates were homogenized and centrifuged at 12000 x g. The samples were maintained on ice until the measurement of ß-galactosidase/luciferase activity was performed (alternatively the extracts can be stored at – 80°C in 24-well plates).

2.2.8. DETERMINATION OF THE FIREFLY LUCIFERASE ACTIVITY

The firefly luciferase is an oxidative enzyme, which is frequently used for measuring promoter activity. This protein catalyzes luciferin oxidation, with concomitant emission of light which is measured using a luminometer. The quantitative level of light emission is proportional to the promoter activity of the reporter gene.

For firefly luciferase activity determination, 20µl of the sample obtained from the mammalian two hybrid or gene reporter assay were transferred to a 4 ml tube. Meanwhile the reaction injection mix + was prepared and introduced in a luminometer.

Reaction Injection Mix+ (RIM+): 0,005mM luciferin ; 2mM ATP ; 10mM $MgCl_2$; 0,027mM coenzyme A ; DTT 10mM ; 25mM glycylglycine pH7.8

2.2.9. DETERMINATION OF THE β-GALACTOSIDASE ACTIVITY

This assay is based on fact that β-galactosidase is an enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides, in our particular case the hydrolysis of galacton, followed by light production at a pH>9.5. This was used to assay the efficiency of transfection. The assay was performed according to what was described by Jain and Magrath (1991) with modifications. Briefly, 10µl of cell extract was incubated with 100µl β-Gal assay buffer for 30 min followed by the injection of 300µl β-Gal assay stop buffer mix. Luminescence was measured for 5 sec in a luminometer Autolumat Plus (Berthold, Bad Wildbad, Germany)

β-Gal assay buffer: 100mM Na-phosphate pH 8.0 ; 1mM $MgCl_2$; 1,25µg/ml galacton

β-Gal assay stop buffer: 0.2 M NaOH; 2.5% Emerald™ Enhancer

2.2.10. COACTIVATOR-DEPENDENT RECEPTOR LIGAND ASSAY (C.A.R.L.A.)

CARLA is an *in vitro* assay based on the principle that the binding of a ligand to a nuclear receptor will induce its interaction with a co-activator. The assay relies on the pull-down of a

GST-coactivator fusion protein interacting in a ligand-dependent way with an *in vitro* transcribed (35S-methionine-labelled) NR. The GST-complex is then immobilized to glutathione-sepharose beads.

2.2.10.1. BACTERIAL EXPRESSION OF GST-FUSION PROTEINS

The E.coki bacterial strain BL21 (DE3) pLysS was transformed with the plasmid enconding a fusion protein of GST and the human PPARBP-RID according to the protocol described in section 2.2.1.2. This strain allows a high-efficiency protein expression of any gene that is under the control of a T7 promoter and has a ribosome binding site. The bacteria were inoculated in 5ml of LB-Amp medium and incubated overnight at 37°C. On the following day, 50ml of LB-Amp medium were inoculated with 1.5ml of the pre-culture and further incubated at 37°C until the culture reaches an OD600 of 0.7-0.8. Following this period the culture was induced with 0.5mM IPTG (a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the *lac* operon), and the bacteria were incubated for ~20h at 16°C.

The culture was then centrifuged at 4°C, 2000xg for 15 min. The bacterial pellet was diluted in 2.5ml of lysis buffer supplemented with protease inhibitors. The lysis was performed by freezing/thawing cycles in liquid N_2. The bacteria were further sonicated on ice for 40 sec. To remove the bacterial debris a final centrifugation was performed at 4°C, 40000xg for 30 min. The supernatant, containing the recombinant protein, was stored at -80°C.

Lysis Buffer: 20mM Tris-HCl pH8.0; 100mM NaCl; 1mM EDTA; 1mM DTT; 0.5% NP-40 (supplemented with protease inhibitors)

2.2.10.2. BINDING OF GST-FUSION PROTEINS TO GLUTATHIONE-SEPHAROSE BEADS

Before usage, the glutathione-sepharose beads were washed 3 times with NETN buffer by centrifugation. After the final washing the beads were ressuspended in 1ml of NETN buffer.

500µl of the beads were centrifuged and the supernatant removed. 2ml of the corresponding bacterial protein lysate (PPARBP) were added to the beads and incubated 1h at 4°C in an

over-head shaker. Finally the beads were washed 3 times with NETN and ressuspended in 5ml NETN + 0.5%MM.

Glutathione-Sepharose 4B (GE Healthcare # 17-0756-01): 75% slurry in 20% ethanol

NETN buffer: 20 mM Tris-Cl pH 8.0 / 100 mM NaCl / 1 mM EDTA / 1 mM DTT / 0.5% NP-40

NETN buffer + 0.5% MM: NETN / 0.5% (w / v) skimmed milk powder

2.2.10.3. 35S-METHIONINE LABELING

The labeling of CAR was performed according to the recommendations for *in vitro* transcription / translation of the TNT Quick Coupled Transcription / Translation Kit.

2.2.10.4. BINDING REACTION OF GST-FUSION PROTEIN TO 35S-LABELED PROTEIN

To 0.5ml of the GST-protein-bead slurry in NETN + 0.5%MM was added 0.5ml NETN + 0.5% MM, 2µl of CAR 35S-Met labelled and 10µl of the specific ligand (from a stock solution of 100x) or DMSO up to a final concentration of 1%. This mixture was further incubated in an over-head shaker for 4 hours at 4°C. The samples were then centrifuged and washed 3 times with NETN plus the specific ligand or DMSO. After the last washing the supernatant was completely removed and 35µl 1x Lämmli buffer + β- mercaptoethanol were added to the pellet. The samples were stored at -80 °C until protein gel electrophoresis.

2.2.10.5. PROTEIN GEL ELECTROPHORESIS AND ANALYSIS

The final step of a C.A.R.L.A. assay is the analysis of the proteins in an polyacrylamide gel. For this purpose a sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. After preparing the specific gel, 30µl of pre-boiled sample was loaded. As an input control it was also loaded 10µl of the corresponding 35S-Met labelling-reaction (1:50 diluted in 1x Lämmli buffer +β- mercaptoethanol).

After the electrophoresis the gel was stained for 15 min in Coomassie, de-stained for 45 min, and finally it was washed with water. In a final step the gel was incubated for 30 min in 0.5M Na-slicylate. Following this steps the gel was dried for 1h, and exposed to an image-plate over-night.

Coomassie-staining solution: 0.125% Coomassie Brilliant Blue / 45% ethanol / 10% acetic acid

fixing / destaining: 9% ethanol / 6% acetic acid

2.2.11. INDUCTION EXPERIMENTS WITH PRIMARY HUMAN HEPATOCYTES

2.2.11.1. HUMAN PRIMARY HEPATOCYTES CULTURE AND INDUCTION

The hepatocytes used in this study were isolated from liver tissue and cultivated as reported elsewhere (Yuan *et al.*, 2004). The isolation procedure was performed by the provider.
After arrival the hepatocytes were supplied with pre-warmed WilliamsE/ITS/Dex culture medium. 24 hours after this initial medium change the induction was started by adding fresh medium containing the respective inducers and solvent controls. For amodiaquine and desethylchloroquine a treatment of only 24 hours was performed due to toxicity of the compounds. For all other inducers tested cells were re-fed after ~24h and cells were induced for ~48hours. After induction the cells were processed for RNA extraction.

WilliamsE/ITS/Dex culture medium: 10nM dexamethasone; 2mM L-glutamine; 1% ITS-G; 100U/ml penicillin; 100mg/ml streptomycin

2.2.11.2. RNA EXTRACTION

At the end of the induction phase, cells were washed once with PBS. Cells were lysed in 600µl of lysis/binding buffer (mirVana® Kit). The extraction was performed according to the instructions of the manufacturer.

2.2.11.3. ASSESSMENT OF RNA QUALITY AND QUANTITY

RNA quality was assessed by the integrity and intensity of the 28S and 18S bands observed by electrophoresis in a denaturating agarose gel. For this purpose a 1% agarose gel was prepared, by dissolving the agarose in 1xMOPS running buffer and DEPC water containing 0.365M formaldehyde. The samples were prepared by adding 0.15-0.5µg of RNA to RNA sample buffer dye to a volume of 16µl, 0.8µl of ethidium bromide (0.5µg/µl) was also added. This sample was heat denaturated at 60°C for 10 minutes, and chilled on ice. Prior to loading the gel, 4µl of RNA loading dye containing bromophenol blue was added to the samples. The electrophoresis was performed in 1x MOPS running buffer at 4V/cm. The RNA was visualized on a UV transilluminator. The integrity of the RNA was assessed by observation of sharp 28S and 18S bands and by observing a ratio of intensity of 2:1 (28S:18S).
RNA was further quantified using a Nanodrop.

1x MOPS Buffer: 20mM MOPS pH 7.0; 1mM EDTA; 5mM Na-acetat pH 7.0 adjusted with NaOH

RNA sample buffer: 50% deionised formamid; 1xMOPS buffer; 2.2M formaldehyde.

RNA loading dye: 10% ficoll; 0.1% bromophenolblue

2.2.12. QPCR (TAQMAN)

2.2.12.1. CDNA SYNTHESIS

For the synthesis of cDNA from human hepatocytes the TaqMan reverse transcription reagents were used according to the manufacturer's instructions. Briefly, 1µg of total RNA was mixed with the following reagents: 5µl 10x TaqMan RT Buffer, 11µl $MgCl_2$ (25mM), 10µl dNTP Mix (2.5mM each), 2.5 µl random hexamers (50µM), 1µl RNase inhibitor, 1.25 µl MultiScribe reverse transcriptase (50U/µl) and RNase-free water up to a total volume of 50µl. This mixture was incubated for 10 minutes at 22°C for the annealing of the random hexamers. This initial incubation was followed by an additional incubation at 48°C for 1 hour and an enzyme inactivation at 95°C for 5 min. The cDNA was diluted to a final concentration of 5ng/µl and stored at -20°C.

2.2.12.2. QUANTITATIVE REAL-TIME PCR (TAQMAN®)

TaqMan® quantitative real-time PCR was developed to increase specificity in real-time PCR assays. Shortly, this method relies on the 5'-3' nuclease activity of Taq polymerase to cleave a dual labeled probe. This probe is labeled with a fluorophore at the 5'-end and a quencher at the 3'-end. Upon probe cleavage by *Taq* Polymerase, physically displacing the quencher from the fluorophene, the latter is allowed to emit fluorescence after excitation by the cycler's light source. The resulting signal allows the quantitative measurement of the accumulation of the amplification product during the exponential stages of the PCR.

TaqMan® -based quantitative real-time PCR was used to quantify the expression of the genes under study in induced primary human hepatocytes. The specific sequences and labels of the oligonucleotides are described in table 5. A calibration curve was used in parallel to the samples, allowing absolute quantification. This calibration curve was obtained by serial dilution of linearized plasmids containing the cDNA of the genes of interest.

The reactions were performed according to the standard protocol for the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, USA). The assay conditions are listed in table 5. All samples were measured in triplicate and induction values were obtained using the absolute quantification method, after normalization with the normalizing gene (18S rRNA coding gene).

2.2.13. DETERMINATION OF GENETIC VARIABILITY IN PXR.

2.2.13.1. DESCRIPTION OF THE SAMPLES

Samples used in sub-project 2 were obtained from a clinical study previously described by Asimus and colleagues. Briefly, 75 healthy volunteers (51 men and 24 women) were repeatedly treated with one of the following artemisinin drugs; artemisinin (500 mg), DHA (60 mg), artemeether (100 mg), arteether (100 mg) or artesunate (100 mg) for five days (day 1 -5). A cocktail of six probe drugs, caffeine (100 mg), coumarin (5 mg), mephenytoin (100 mg), metoprolol (100 mg), chlorzoxazone (250 mg) and midazolam (7.5 mg), were given orally six days before (day -6) administration of the artemisinin drugs. On day 1 and day 5 of artemisinin drug intake, the cocktail drugs were given again, 1 hour after the artemisinin drugs. The cocktail drugs were then administered once more after a wash-out period of five

days (day 10). Probe compounds were measured in blood samples taken immediately before, and at 4 hours after administration of the cocktail drugs on days -6, 1, 5 and 10 (Figure 10).

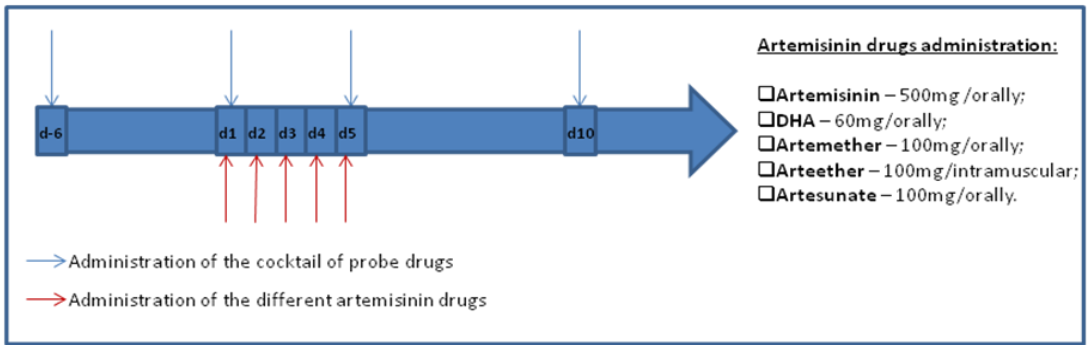


Figure 10: Description of the clinical study.

DNA was extracted from these samples, and further analysed.

2.2.13.2. WHOLE GENOME AMPLIFICATION

In recent years a new PCR based technique has arisen that allows the replication of the entire DNA of a specific sample, from very limited starting material.

Due to the lack of sufficient amounts of DNA, whole genome amplification was performed with the samples. The amplification was performed as described in the protocol provided with the illustra GenomiPhi® V2 DNA Amplification Kit. Briefly, 3-5µl of each sample was dried overnight in an open 96 well plate. After this step, the samples were re-hydrated with 1 µl of Sigma Delta Select water and 9 µL of sample buffer, and the DNA was denaturated for 3 min at 95°C, and cooled to 4°C. To each cooled sample 9µl of reaction buffer and 1 µl of enzyme mixtures were added. Samples were then incubated at 30°C for 1.5 hours and subjected to an enzyme heat inactivation for 10 min at 65°C. In parallel to our samples, a "no template control" was used, to ensure that all solutions in the protocol were DNA free.

After amplification, samples were diluted to 10 fold with water. The efficiency of the whole genome amplification was determined by PCR.

2.2.13.3 AMPLIFICATION OF DNA FRAGMENTS AND RE-SEQUENCING

Our approach to study the genetic variability of *PXR* was to re-sequence all exons, the 3'UTR region and 1kb of the 5' promoter region (Figure 11). PCR assays were developed to analyze these regions. For that purpose specific oligonucleotides were either newly developed or used from already published data. The newly developed primers were designed according to the sequence AF364606 (table 4).

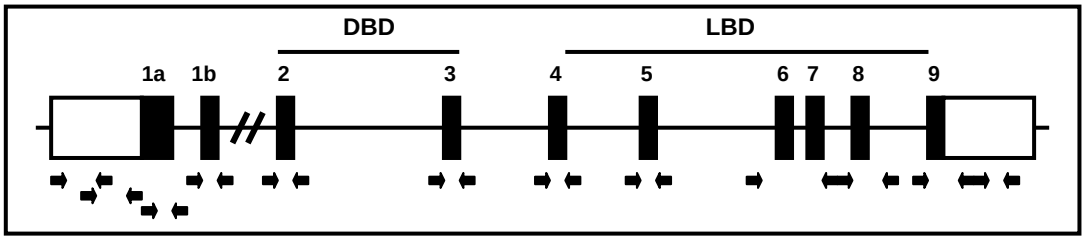


Figure 11: Linear graphic representation of the PXR gene, with all the amplified fragments annotated. The arrows symbolize the position of the specific oligonucleotides. It is also highlighted the position of the coding regions for the DBD (DNA binding domain) and the LBD (ligand binding domain) of PXR. The white boxes represent the 1kb of the promoter region and the 3'UTR region.

The amplifications were performed in a GenAmp® PCR System 2700 (Applied Biosystems, Fresno, CA, USA). Each amplification reaction started with an denaturation step at 95°C for 2 min, followed by a cycle, repeated 40x, of 30sec at 94°C, 30 sec at the specific annealing temperature (see table 6), and 60 sec at 72°C, the PCR reaction ending with a final extension of 5' min at 72°C. The amplifications were performed in a master mix containing, 1x GoTaq® reaction buffer, 0.2µM of each dNTP, 0.4µM of each specific primer and 1.25U of GoTaq® polymerase. After amplification the presence of the specific fragment was confirmed in a 2% agarose gel. All amplicons were sequenced by Macrogen Inc. (www.macrogen.com, Seoul University, Seoul, South Korea). All PCR fragments were single strand sequenced; the specific primers used for sequencing are annotated in table 4.

3. RESULTS

3.1. STUDY OF THE CAPACITY OF CURRENTLY USED ANTIMALARIAL DRUGS TO ACTIVATE THE PXR/CAR XENOBIOTIC SIGNALLING PATHWAY.

3.1.1. ACTIVATION OF PXR BY ANTIMALARIALS

3.1.1.1. SCREENING

A mammalian two hybrid assay was used to study the induction of the interaction of PXR with the co-activator protein SRC1, upon treatment with antimalarials. All available antimalarials were tested, together with the induction control, rifampicin. The values obtained for each compound were normalized to a control performed with the respective concentration of the vehicle (Me_2SO). Due to their toxicity at screening concentrations (10 and 100µM) in HeLa cells, mefloquine, dihydro-artemisinin and pyronaridine were removed from the study. Data concerning all other compounds screened is described in Figure 12.

A cut-off value of 2 fold induction was defined to be the minimum value to consider a drug as inducing the interaction of PXR with SRC1. In this assay the compounds above this cut-off value were lumefantrine (mean F.I. = 3.59±1.71), carboximefloquine (mean F.I. = 3.38±1.21), chloroquine (mean F.I. = 2.13±0.48), at the maximum concentration tested (100µM), DEAQ (mean F.I. = 2.57±0.70) and amodiaquine (mean F.I. = 2.08±0.56) at 10 µM. For these two compounds data is not available for the 100 µM concentration due to their observed toxicity. Taken these fold inductions in consideration, these 5 compounds were further tested in a dose-response study. Results are described in chapter 3.1.1.2.

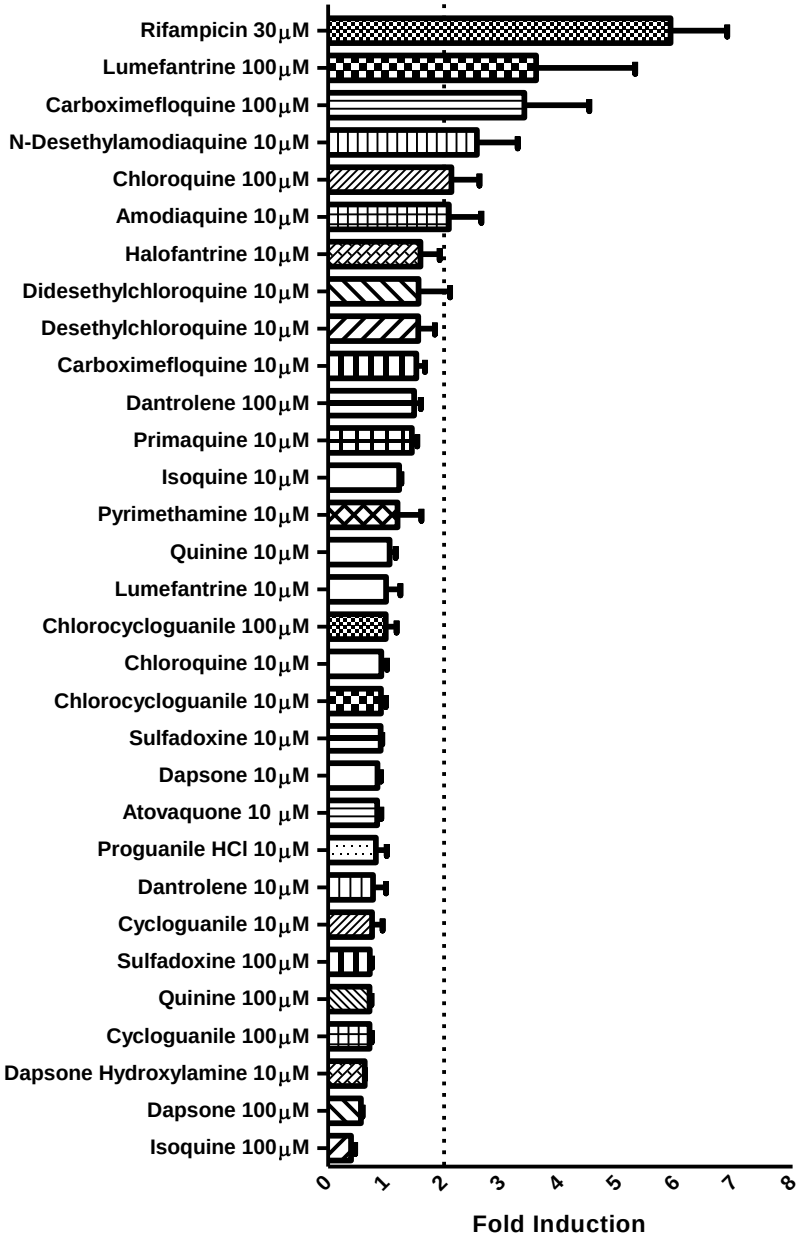


Figure 12: Modulation of the interaction of PXR with the co-activator SRC1 by antimalarials, in a mammalian two hybrid assay. Two concentrations were analyzed (10 and 100 μM). The horizontal bars represent the mean induction factors (± S.D.). The baseline activity of the nuclear receptor in the presence of the co-activator, treated only with Me_2SO (negative control) was defined as 1.

The compounds of the artemisinin family were previously described as not inducing significantly the interaction of PXR with SRC1 in a mammalian two hybrid assay (Burk *et al.*, 2005), hence were not analyzed by this approach. For this class of compounds a reporter gene

assay was further performed, by co-transfecting the expression plasmids encoding for human *PXR* and the *CYP3A4* promoter gene reporter plasmid into COS1 cells. In this assay artemisinin, arteether, artemether, deoxy-arteether and deoxy-artemisinin showed induction of the nuclear receptor PXR. Artemisinin had the highest induction at 100µM (mean F.I. = 4.12±1.08), but did not show significant induction at the lower concentration of 10µM (mean F.I. = 0.90±0.10). Both arteether and artemether show induction at both 100 µM (ARE mean F.I. =3.04±0.64; ARM mean F.I. =2.61±0.44) and 10 µM (ARE mean F.I. =2.25±0.32; ARM mean F.I. =1.63±0.07). No induction was observed at both concentrations tested for artesunate, and at the lowest concentrations of deoxy-arteether and deoxy-artemisinin (Figure 13).

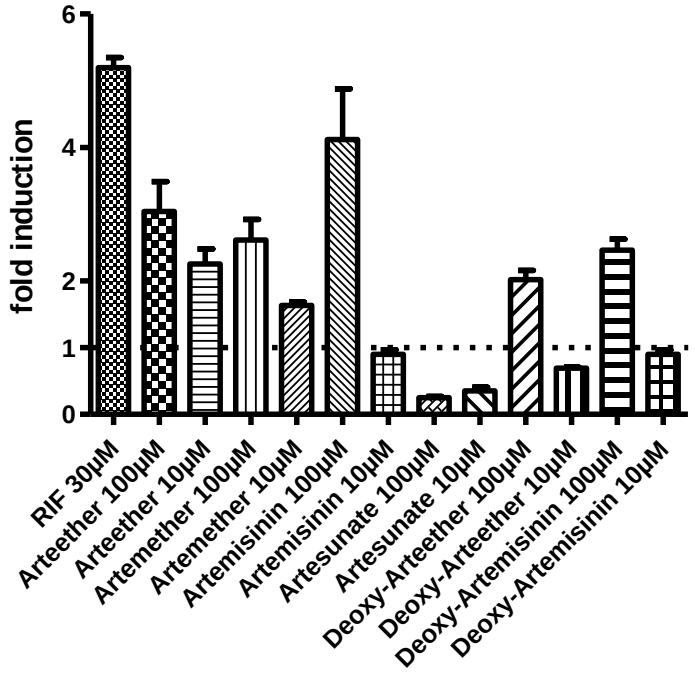


Figure 13: Screening by gene reporter assay of artemisinin and its derivatives ligand capacity for PXR. Columns show the mean induction factors (± S.D.). The activity of the nuclear receptor in the presence of Me_2SO (negative control) was given the value 1.

3.1.1.2. EC_{50} DETERMINATION

From the initial screening, all the compounds with fold induction above 2 were chosen to be tested in a dose-response assay in order to further confirm induction of the nuclear receptor

PXR at different drug concentrations, and to determine their specific half maximal effective concentration (EC_{50}). This value represents the concentration of agonist necessary to reach 50% of the maximum induction.

Briefly, this value was determined by performing a dose-response curve, using 7 different concentrations (1, 3, 10, 30, 50, 100, 300µM) of the compounds, using the same assays as applied for their screening. The EC_{50}'s were extrapolated using the software GRAPHPAD PRISM5, plotting the log concentration antimalarial *vs*. response (fold induction), in cases where the plateau phase of induction was not reached, the presented EC_{50} value is only a graphically approximation. The specific values obtained for these compounds are shown in table 9.

Table 9: Dose-response analysis of PXR activation by antimalarials. Data obtained by gene reporter assays for the artemisinins; mammalian two-hybrid assay for the other antimalarials. Several concentrations of compounds were tested (1, 3, 10, 30, 50, 100, 300µM). Results were obtained using the software GRAPHPAD PRISM5, and are presented as mean ± 95% CI.

Compound	*EC_{50}(95% CI)μM*	*Maximum fold induction*	*Maximum concentration(μM)*
Artemisinin	53.54 (11.46-250.20)	4.12	100
Arteether	8.92 (3.62-21.98)	3.18	30
Artemether	14.15 (10.32-19.42)	3.41	50
Deoxy-Arteether	32.03 (24.46-41.94)	2.02	100
Deoxy-Artemisinin	25.13 (18.74-33.71)	2.48	300
Lumefantrine	> 64	3.56	100
Carboximefloquine	27.44 (10.70-70.34)	3.37	300
DEAQ	> 20	41.18	30
Chloroquine	> 54	2.13	100
Amodiaquine	17.20 (0.53 – 556.50)	34.50	50

3.1.2. ACTIVATION CAPACITY OF CAR BY ANTIMALARIALS

3.1.2.1. SCREENING

For this nuclear receptor a mammalian two hybrid assay was used, to screen the influence of the antimalarials on the interaction of the ligand-dependent CAR splice variant CAR-SV2 with the co-activator DRIP 205. In this assay CITCO was used as the positive induction control. The values obtained for each compound were normalized to a control performed with the respective concentration of the vehicle (Me_2SO).

In this assay atovaquone, debutyl-benflumetol, pyrimethamine and pyronaridine were removed from the study due to their high toxicity in COS1 cells at both concentrations tested (10 and 100µM).

A cut-off value of 2 fold induction was defined to be the minimum induction to consider a drug as inducing the interaction of CARSV2 with DRIP205. This group of drugs includes artemether with fold induction of 106.90±28.50 and 29.60±1.27, respectively at 100 and 10µM, arteether, also inducing the interaction at both concentrations (mean F.I. =102.00±15.56 (100µM); mean F.I. =40.45±2.48 (10µM)). The metabolites deoxy-arteether (mean F.I. = 68.65±0.07 (100µM); mean F.I. =15.73±18.20 (10µM)) and deoxy-artemisinin (mean F.I. =62.10±36.44 (100µM); mean F.I. =7.36±6.05 (10µM)) also induced CAR. The parent compound, artemisinin induced the interaction at 100 µM (mean F.I. =33.43±21.35). Together with these compounds, amodiaquine at a concentration of 100 µM (mean F.I. =34.65±17.76) was also able to induce the interaction of CARSV2 with DRIP205 (Fig.14).

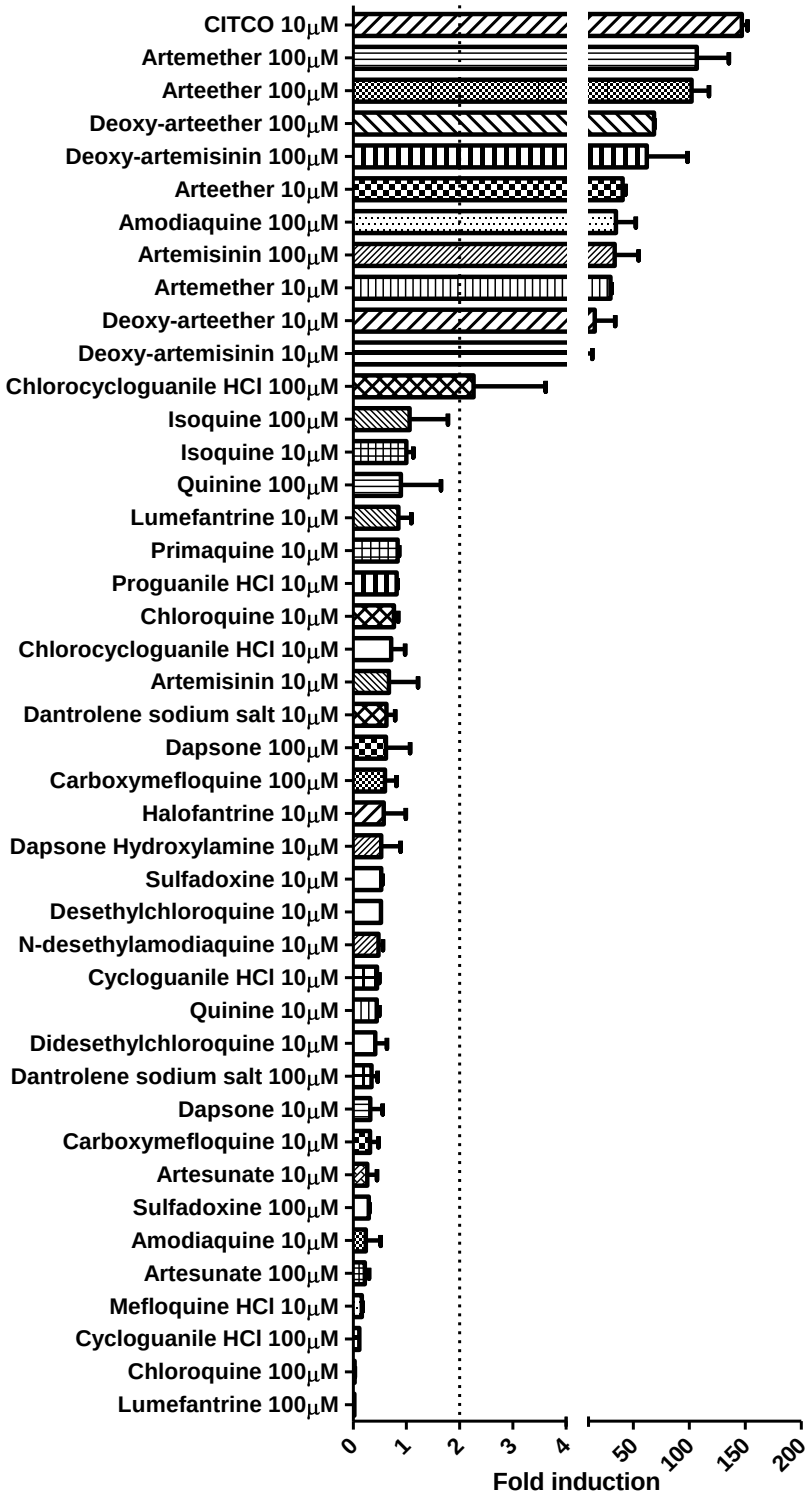


Figure 14: Antimalarial modulation of the interaction of CAR-SV2 with the co-activator DRIP205 by mammalian two hybrid. Two concentrations were analyzed 10 and 100 μM. Columns show the mean induction factors (± S.D.). The activity of the nuclear receptor in the presence of the co-activator treated only with Me_2SO (negative control) was given the baseline value of 1.

3.1.2.2. EC_{50} DETERMINATIONS

All the compounds with fold induction above 2 were further tested in a dose-response assay in order to confirm induction of the nuclear receptor CAR at different drug concentrations, and to determine their specific half maximal effective concentration (EC_{50}).

Briefly, this value was determined by performing a dose-response curve, using 7 different concentrations (1, 3, 10, 30, 50, 100, 300µM) of the compounds and EC_{50} were extrapolated using the software GRAPHPAD PRISM5, plotting the log concentration antimalarial vs. response (fold induction). If the plateau phase of induction was not reached, the presented EC_{50} value is only a graphically approximation. The specific values obtained for these compounds are showed in table 10.

Table 10: Dose-response analysis of the induction of CAR-SV2 interaction with DRIP205 by antimalarials. Data obtained by mammalian two hybrid assay. Results are presented as mean ± 95% CI. In cases where the plateau phase of induction was no reachable, EC50 value is only a graphically approximation, and CI was not determined.

Compound	*EC_{50} (μM)*	*Maximum fold induction*	*Maximum concentration(μM)*
Amodiaquine	> 85	49.04	100
Arteether	20.19 (16.23 – 25.12)	249.93	50
Artemeether	12,44 (0.95 – 162.60)	105.85	100
Artemisinin	71,26 (57.56 – 88.21)	62.52	300
Deoxy-Artemisinin	> 141	270.30	300
Deoxy-Arteether	48.48 (21.19 – 110.90)	82.26	300

3.1.2.3. *IN VITRO* INDUCTION STUDIES

For further confirmation of assess the capacity of CAR by the artemisinins and amodiaquine, an *in vitro* approach was used. For this purpose a C.A.R.L.A. assay was performed using the same concentrations of ligands as for the previous assays. No induction was observed for these compounds, except for the induction control CITCO and arteether (Figure 15). Due to this fact, this approach was not further continued and only one experiment was performed. Further increases in the concentrations of ligands would probably be necessary to obtain induction by these compounds in this system.

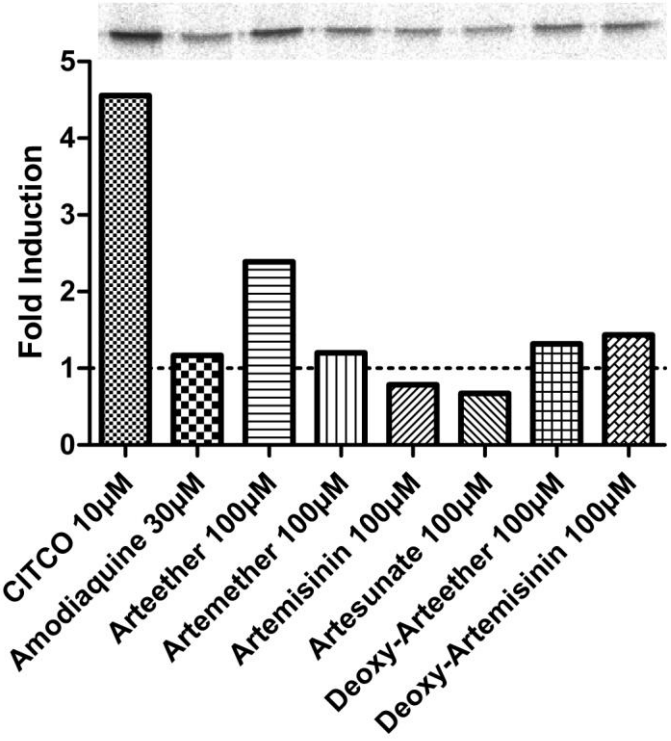


Figure 15: *In vitro* induction of selected antimalarials. Data was obtained performing a CARLA assay. In the image is also shown the original IP.

3.1.3. INDUCTION OF KEY DME'S IN HUMAN HEPATOCYTES BY SELECTED ANTIMALARIALS.

Further induction studies were performed to access the capacity of these drugs to interact with *PXR* and CAR *in vivo*, in this particularly case in primary human hepatocytes. For this study the expression of 3 known PXR/CAR target genes, *CYP3A4*, *CYP2B6 and MDR1*, were measured by real time RT-PCR, upon treatment with the antimalarials.

The concentrations to be tested were chosen to be the highest not showing toxicity, asseessed by microscopic inspection, in the dose-response analysis. In this study dihydro-artemisinin was also included, due to the fact that is was described as non-toxic in primary human hepatocytes (Burk *et al.,* 2005). Induction controls were performed with the prototypical PXR and CAR ligands, rifampicin and CITCO, repectively. The induction values were also normalized with the values for the dilution vehicle alone Me_2SO (negative control).

Taking in consideration that the hepatocyte cultures were derived from different donors and thus may show interindividual variability in response, and that only a very small number of cultures was used, statistical analysis was not performed. Thus the compounds were regarded as inducing/repressing, if the majority of the cultures were modulated accordingly. The cultures were considered as induced if an increase of 1.50 fold in induction and repressed if a decrease of 0.67 in expression upon treatment with the respective antimalarial, as compared to expression in the presence of Me_2SO only, was observed (table11).

For *CYP3A4* we could see a clear induction by the prototypical inducers, rifampicin and CITCO, at all tested conditions. A clear repression of this P450 was observed for amodiaquine (24h), with all 3 cultures being repressed (mean F.I. = 0.48±0.11). The same was observed for DEAQ, with 2 of the cultures being clearly repressed (mean F.I. = 0.55±0.28). No clear tedency of induction was observed for carboximefloquine, chloroquine and lumefantrine. All artemisinin compounds, except artesunate and DHA, were inducing in all cultures. The highest mean fold induction was observed for artemisinin (11.73±7.17), folowed by artemether (10.25±6.07), deoxy-artemisinin (4.11±1.52), arteether (4.05±1.81) and deoxy-arteether (3.04±0.74). Both artesunate and DHA were repressing in this set of hepatocytes: artesunate was repressing in all cultures (mean F.I. = 0.59±0.35) and DHA was repressing in 3 out of 5 cultures with a mean repression of 0.50±0.33 (Figure 16).

Also for *CYP2B6* a clear induction by the prototypical inducers, rifampicin and CITCO, was observed. Regarding amodiaquine, no clear induction tendency was observed, being the results very divergent between cultures. Its metabolite DEAQ clearly repressed the expression of *CYP2B6* in 2 of the 3 cultures studied (mean F.I.= 0.59±0.17). A clear induction was observed for carboxymefloquine with a mean fold indution of 7.52±8.30. No clear conclusion regarding the regulation of *CYP2B6* expression by chloroquine, lumefantrine and artesunate could be obtained. The highest induction of CYP2B6 was observed by the treatment with artemisinin (mean F.I. 9.85±7.77), the other compounds from this family had lower inductions, e.g., deoxy-arteether (mean F.I. 7.10±3.11), deoxy-artemisinin (mean F.I. 6.05±1.60), artemether (mean F.I. 5.68±2.04) and arteether (mean F.I. 2.89±1.60). A clear repression of CYP2B6 expression was observed by the treatment with DHA in 3 of the 5 cultures (mean F.I. 0.65±0.43) (Figure 16).

Induction of the expression of *MDR1* was not as efficient as for the other two genes. Induction was not observed in all cultures for the ligands rifampicin and CITCO (Table11). No clear induction or repression was observed for amodiaquine, DEAQ, carboximefloquine, chloroquine, lumefantrine, deoxy-artemisinin, deoxy-arteether and artesunate. Induction of the expression of this gene was clearly observed for artemisinin (mean F.I. 2.50±1.50), and arteether (mean F.I. 2.63±1.59). For artemether and DHA, induction was only observed in 2 out of 5 cultures, with mean fold inductions of respectively, 2.22±1.46 and 1.60±0.72 (Figure 16).

Table11: Summary of the induction of gene expression by antimalarials. Cultures were considered to be induced above the thresholf of 1.5-fold inductions, and repressed bellow the threshold 0.67.

	CYP3A4		*CYP2B6*		*MDR1*	
Xenobiotic	Induction responsive cases	Repression responsive cases	Induction responsive cases	Repression responsive cases	Induction responsive cases	Repression responsive cases
Rifampicin	12/12	0/12	12/12	0/12	11/12	0/12
Rifampicin (24h)	3/3	0/3	3/3	0/3	2/3	0/3
Rifampicin (0.8%Me_2SO)	3/3	0/3	3/3	0/3	1/3	0/3
CITCO	3/3	0/3	3/3	0/3	0/3	0/3
Amodiaquine	1/4	2/4	1/4	1/4	2/4	1/4
Amodiaquine (24h)	0/3	3/3	0/3	1/3	0/3	0/3
DEAQ	0/3	2/3	0/3	2/3	0/3	1/3
Carboxime floquine	1/3	1/3	3/3	0/3	1/3	0/3
Chloroquine	1/3	1/3	0/3	1/3	1/3	0/3
Lumefantrine	0/3	1/3	1/3	1/3	0/3	1/3
Artemisinin	8/8	0/8	8/8	0/8	6/8	0/8
Deoxy-arteminin	3/3	0/3	3/3	0/3	1/3	0/3
Arteether	5/5	0/5	4/5	0/5	3/5	0/5
Deoxy-arteether	3/3	0/3	3/3	0/3	0/3	0/3
Artemether	5/5	0/5	5/5	0/5	2/5	0/5
Artesunate	0/3	3/3	0/3	0/3	0/3	0/3
DHA	0/5	3/5	0/5	3/5	2/5	0/5

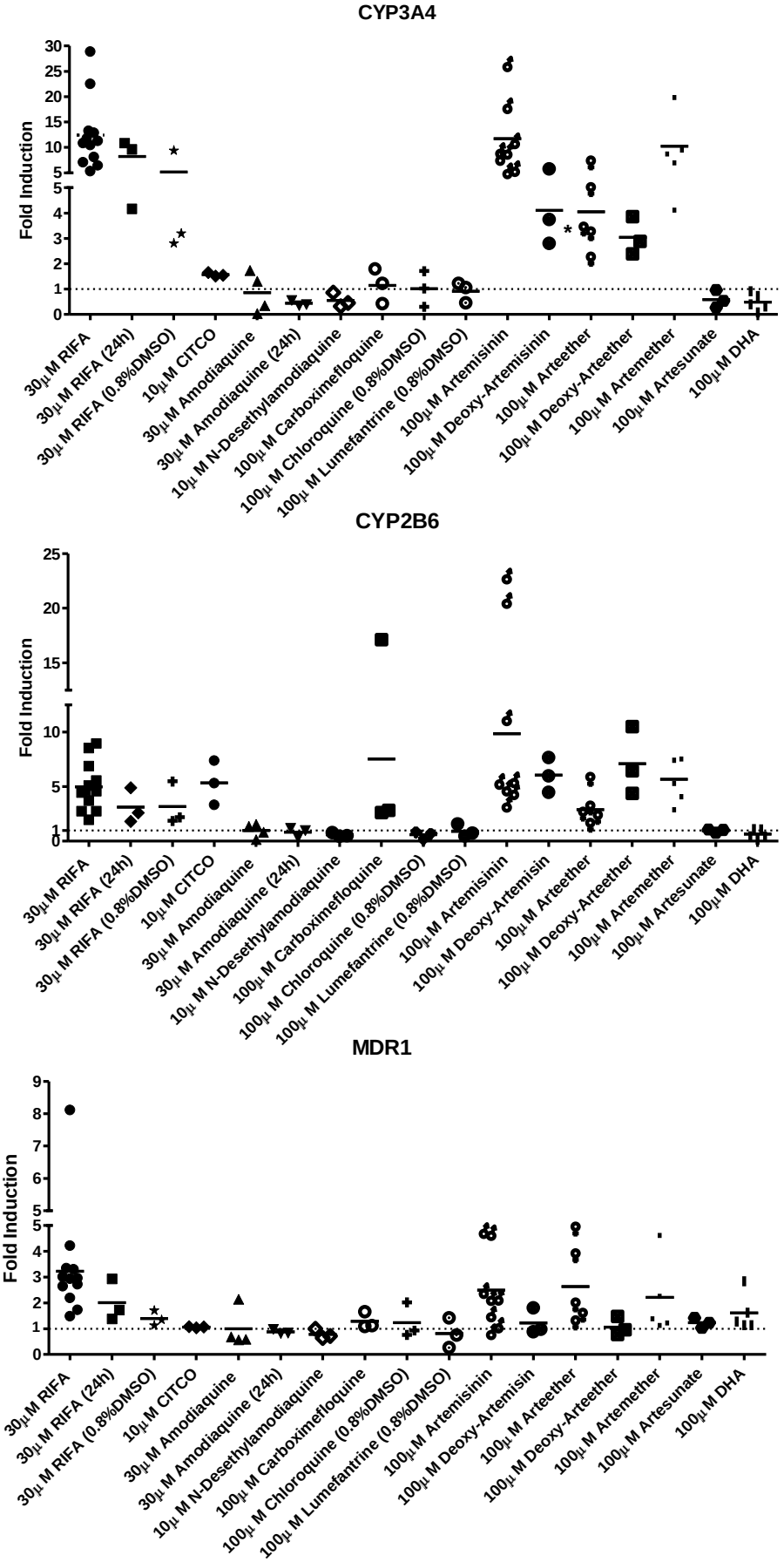

CYP3A4
Fold Induction
30
25
20
15
10
5
5
4
3
2
1
0
*
30µM RIFA
30µM RIFA (24h)
30µM RIFA (0.8%DMSO)
10µM CITCO
30µM Amodiaquine
30µM Amodiaquine (24h)
10µM N-Desethylamodiaquine
100µM Carboximefloquine
100µM Chloroquine (0.8%DMSO)
100µM Lumefantrine (0.8%DMSO)
100µM Artemisinin
100µM Deoxy-Artemisinin
100µM Arteether
100µM Deoxy-Arteether
100µM Artemether
100µM Artesunate
100µM DHA
CYP2B6
Fold Induction
25
20
15
10
5
1
0
30µM RIFA
30µM RIFA (24h)
30µM RIFA (0.8%DMSO)
10µM CITCO
30µM Amodiaquine
30µM Amodiaquine (24h)
10µM N-Desethylamodiaquine
100µM Carboximefloquine
100µM Chloroquine (0.8%DMSO)
100µM Lumefantrine (0.8%DMSO)
100µM Artemisinin
100µM Deoxy-Artemisin
100µM Arteether
100µM Deoxy-Arteether
100µM Artemether
100µM Artesunate
100µM DHA
MDR1
Fold Induction
9
8
7
6
5
5
4
3
2
1
0
30µM RIFA
30µM RIFA (24h)
30µM RIFA (0.8%DMSO)
10µM CITCO
30µM Amodiaquine
30µM Amodiaquine (24h)
10µM N-Desethylamodiaquine
100µM Carboximefloquine
100µM Chloroquine (0.8%DMSO)
100µM Lumefantrine (0.8%DMSO)
100µM Artemisinin
100µM Deoxy-Artemisin
100µM Arteether
100µM Deoxy-Arteether
100µM Artemether
100µM Artesunate
100µM DHA

Figure 16: Induction of *CYP3A4, CYP2B6* and *MDR1* mRNA expression by treatment of different antimalarials. Primary hepatocytes were derived from 3-12 donors. Except if otherwise stated, the cultures were treated for 48 hours. The expression was analyzed by real time RT-PCR and normalized to the 18S sRNA expression. Data represents fold induction relative to the vehicle, 0.1% Me_2SO alone (if different, it is stated in the graphic).

3.2. STUDY OF THE GENETIC VARIABILITY IN PXR IN A VIETNAMESE POPULATION

Re-sequencing of all *PXR* exons, flanking intronic regions and 1kb of the promoter region was performed in all 75 healthy Vietnamese individuals included in the Asimus *et al.*, 2007 clinical study. Drop-outs were observed for some SNPs due to technical problems during sequencing and limited amount of DNA sample. Data was obtained by MALDI-TOF-MS by Dr. Elke Scheffler, Dr.Margarete Fischer Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany, for further selected SNPs located in the promoter and intron1.

This method allowed the study of 79 SNPs, but only 32 of them are present in this population in a polymorphic form, with minor allelic frequency >0.01. Six of these polymorphism were not described in previously published data, being to our knowledge newly identified SNPs, namely; - 24910 G>A and -23925 C>T, in the promoter regions of PXR-1 and PAR2 respectively; 8582T>G in the intronic region; 9915T>A (F420Y) in the open reading frame; finally 10098 C>T and 10976 G>A in the 3' UTR region. Data concerning the genotypic and allelic frequencies of these SNPs is provided in table 12, a complete list of all SNPs studied in these samples, including the ones observed as monomorphic in this population are found in appendix 2. All SNPs with MAF >0.01% are described in figure 17.

Among the SNPs identified -4356 T>C, 7635 A>G, 10331 A>G and 10483 T>C were the highest prevalent with minor allelic frequencies above 0.50, being the most common polymorphic alleles in this population. The newly identified SNPs, 8582 T>G and 10976 G>A showed minor allelic frequencies above 0.20. All other newly identified SNPs were only found in a heterozygous form in one individual, hence with allele frequencies below 1%.

All SNPs, except PXR 8055 C>T and 10976 G>A were in Hardy –Weinberg equilibrium. The assessment of the Hardy-Weinberg equilibrium was performed using the chi-squared test and fisher exact test in the GraphPad Quickcalcs software (data not shown).

Table 12: Genotyping data of the Vietnamese Population for SNPS with MAF>0.01. Genotype and allele frequencies are described, as well as their position and number of individuals analyzed; c Value in parenthesis is the 95% confidence interval determine with GraphPad Quickcalcs.In red are described the SNPs newly identified, and in blue SNPs genotyped by MALDI-TOF-MS.

					Allele frequency [c]		Genotype frequencies [c]		
Position [a]	**Putative effect**	**Identifier**	**Sequence**	**N [b]**	**wt**	**mut**	**wt/wt**	**wt/mut**	**mut/mut**
-25913 C>T	STAT1, 3, 6 and NAT binding sites			71	0.84 (0.77-0.89)	0.16 (0.11-0.23)	0.70 (0.59-0.80)	0.27 (0.18-0.38)	0.03 (0.00-0.10)
-25385 C>T	NFKB, ISGf-3 binding sites	rs3814055	CAGGTT[C/T]TCTTTT	75	0.83 (0.76-0.88)	0.17 (0.12-0.24)	0.69 (0.58-0.79)	0.28 (0.19-0.39)	0.03 (0.00-0.10)
- 24910 G>A	Lost of Oct-1 binding site anda CCAAT box		TGATTG[G/A]CACCGT	75	0.99 (0.96-0.99)	0.01 (0.00-0.04)	0.99 (0.92-0.99)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
-24381 A>C		rs1523127	CCTGAA[A/C]AAGGCA	75	0.83 (0.76-0.88)	0.17 (0.12-0.24)	0.69 (0.58-0.79)	0.28 (0.19-0.39)	0.03 (0.00-0.10)
-23925 C>T			TACCAC[C/T]TCCAAG	56	0.99 (0.94-1.00)	0.01 (0.00-0.05)	0.98 (0.89-1.00)	0.02 (0.00-0.10)	0.00 (0.00-0.08)
-23913 T>G		rs3814056	GGACTG[T/G]GGGAGC	56	0.71 (0.61-0.78)	0.29 (0.22-0.38)	0.48 (0.36-0.61)	0.45 (0.32-0.58)	0.07 (0.02-0.17)
-14042 C>A	DR3 bindingsite			71	0.65 (0.57-0.73)	0.35 (0.27-0.43)	0.41 (0.30-0.53)	0.49 (0.38-0.61)	0.10 (0.05-0.19)
-4356 T>C				69	0.33 (0.25-0.41)	0.67 (0.59-0.75)	0.09 (0.04-0.18)	0.48 (0.36-0.59)	0.43 (0.32-0.55)
-601A>G	HNF4 binding site			70	0.56 (0.47-0.64)	0.44 (0.36-0.53)	0.31 (0.22-0.43)	0.49 (0.37-0.60)	0.20 (0.12-0.31)

252 A>G		rs1464603	GGTAAC[A/G]TCTCAG	75	0.55 (0.47-0.62)	0.45 (0.38-0.53)	0.29 (0.20-0.41)	0.51 (0.40-0.62)	0.20 (0.12-0.30)
275 A>G		rs144602	TGACCT[A/G]TCCCCC	75	0.57 (0.49-0.65)	0.43 (0.35-0.51)	0.32 (0.23-0.43)	0.51 (0.40-0.62)	0.17 (0.10-0.28)
308 A>T			TGGCCC[A/T]CCCAAA	75	0.99 (0.96-1.00)	0.01 (0.00-0.04)	0.99 (0.92-1.00)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
3015 T>G			GGTGTG[T/G]GCATGC	61	0.84 (0.76-0.89)	0.16 (0.11-0.24)	0.74 (0.62-0.83)	0.19 (0.12-0.32)	0.07 (0.02-0.16)
5761 A>G			GTGGCC[A/G]GGAGGT	67	0.99 (0.94-1.00)	0.01 (0.00-0.06)	0.97 (0.89-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)
7635 A>G		rs6785049	CCTCTC[A/G]CCCCCA	66	0.41 (0.33-0.49)	0.59 (0.51-0.67)	0.18 (0.11-0.29)	0.45 (0.34-0.57)	0.36 (0.26-0.48)
7675C>T		rs6797879	TGCCGG[T/C]CTGTGG	67	0.81 (0.74-0.87)	0.19 (0.13-0.26)	0.67 (0.55-0.77)	0.28 (0.19-0.40)	0.04 (0.01-013)
8055C>T		rs2276707	CTCCAT[C/T]CTGTTA	73	0.60 (0.52-0.67)	0.40 (0.33-0.49)	0.44 (0.33-0.55)	0.31 (0.22-0.43)	0.25 (0.16-0.36)
8118 C>T	Y328Y	rs2229856	CCACTA[C/T]ATGCTG	68	0.74 (0.66-0.80)	0.26 (0.20-0.35)	0.47 (0.36-0.59)	0.53 (0.41-0.64)	0.00 (0.00-0.06)
8582T>G			CATAGG[T/G]GAGCAC	70	0.79 (0.72-0.85)	0.21 (0.15-0.28)	0.59 (0.47-0.70)	0.41 (0.31-0.53)	0.00 (0.00-0.06)
9863A>G	I403V		CGCAGC[A/G]TCAATG	60	0.99 (0.95-1.00)	0.01 (0.00-0.05)	0.98 (0.90-1.00)	0.02 (0.00-0.10)	0.00 (0.00-0.07)
9915T>A	**F420Y**		ACCCCT[T/A]TGCTAC	65	0.99 (0.95-1.00)	0.01 (0.00-0.05)	0.98 (0.91-1.00)	0.02 (0.00-0.09)	0.00 (0.00-0.07)
9932C>G	Q426 E	rs56162473	CTCATG[C/G]AGGAGT	65	0.98 (0.94-1.00)	0.02 (0.00-0.06)	0.97 (0.88-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)

9976G>A	rs3732358	CCTTGG[G/A]TGACAC	68	0.98 (0.95-1.00)	0.01 (0.00-0.06)	0.97 (0.89-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.06)
9987G>A		ACCTCC[G/A]AGAGGC	68	0.99 (0.96-1.00)	0.01 (0.00-0.05)	0.98 (0.91-1.00)	0.02 (0.00-0.09)	0.00 (0.00-0.06)
10058C>G		AAGAGC[C/G]GACAAT	65	0.98 (0.94-1.00)	0.02 (0.00-0.06)	0.97 (0.88-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)
10098 C>T		TTCCTG[C/T]TATGAC	71	0.99 (0.96-1.00)	0.01 (0.00-0.04)	0.99 (0.92-1.00)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
10331A>G	rs3732359	AAGGAT[A/G]GGCCAT	68	0.36 (0.28-0.44)	0.64 (0.56-0.72)	0.22 (0.14-0.33)	0.28 (0.19-0.40)	0.50 (0.38-0.62)
10483 T>C	rs3732360	GGCAGG[C/C]GCATGA	66	0.36 (0.29-0.45)	0.64 (0.55-0.71)	0.23 (0.14-0.34)	0.27 (0.18-0.39)	0.50 (0.38-0.62)
10719 A>G	rs6438550	ACAAAC[A/G]ATTTGG	64	0.82 (0.74-0.88)	0.18 (0.12-0.26)	0.75 (0.63-0.84)	0.14 (0.07-0.25)	0.11 (0.05-0.21)
10976 G>A		GGCTAC[G/A]CTGACA	75	0.65 (0.57-0.72)	0.35 (0.38-0.43)	0.29 (0.20-0.41)	0.71 (0.60-0.80)	0.00 (0.00-0.06)
11156 A>C	rs3814057	CACCTA[A/C]GAACTA	75	0.56 (0.48-0.64)	0.44 (0.36-0.52)	0.40 (0.30-0.51)	0.32 (022-0.43)	0.28 (0.19-0.39)
11193 T>C	rs3814058	TTAATG[T/C]CAAATC	75	0.56 (0.48-0.64)	0.44 (0.36-0.52)	0.40 (0.30-0.51)	0.32 (022-0.43)	0.28 (0.19-0.39)

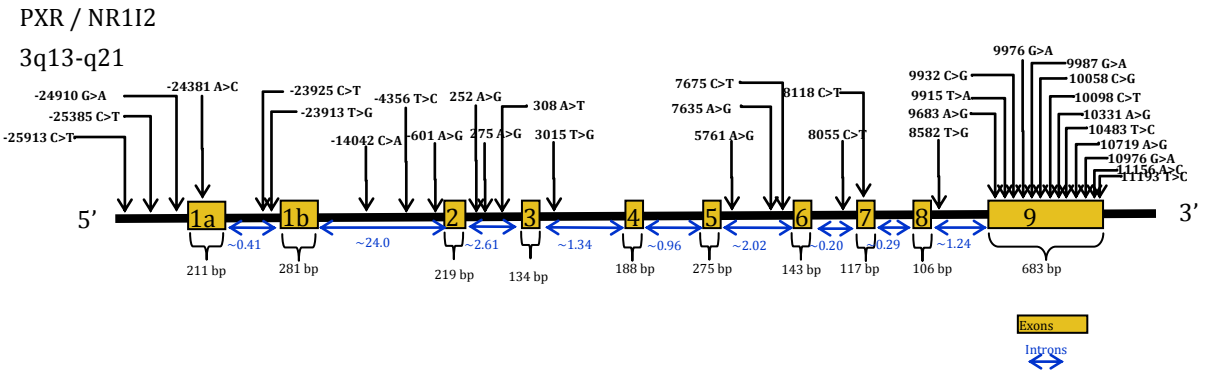


Figure 17: Schematic representation of the SNPS with MAF>0.01 in the PXR gene.

The population prevalence of most of the identified SNPs is not documented. Concerning the data obtained for SNP -4356 T>C, our population was very similar with the only description of this SNP in a Caucasian population (p=0.8814) (Lamba *et al.*, 2008). The prevalence of the 7635 A>G SNPs in Vietnam is different from all previously described population (appendix1) (*p*=<0.0001-0.01), except for the Asian-American population described by King *et al.*, 2007 (*p*=0.1561), the Indian population described by Sandaranaj *et al.*, 2008 (*p*=0.5684) and the Scandinavian population described by Karlsen *et al.*, 2006 (*p*=0.5612). The frequency of the 10331 A>G SNP was significantly or near significantly different from all Caucasian populations previously described (appendix1) (p=<0.0001-0.0623). Similarities were observed with the African-American (*p*=0.5492) and Asian-American (*p*=0.5612) population described by King *et al.*, 2007. The comparison between our population and the one described by Sandaranaj et al. 2008, showed statistically significant differences with the Chinese (*p*=0.0045) and Malay populations (*p*=0.001), the distribution of this SNPS was similar to the one observed in the Indian population (*p*=0.6622). The same was observed for the 10483 T>C SNPs. Similarities for this SNPs were only observed between the Vietnamese population and a previously described Asian-American (*p*=1.000) population, all other previously described population were statistically significantly different (*p*=<0.001-0.0258) (King *et al.*, 2007) (appendix 1).

The linkage between the SNPs identified was also studied. Using the software Haploview, five main linkage disequilibrium blocks were identified in this population:1) -25913 C>T, -

25385 C>T, -24910 G>A and -24381 A>C; 2) -14042 C>A, -4356 T>C, -601 A>G; 3) 252 A>G, 275 A>G; 4) 10331A>G, 10483 T>C; 5) 11156 A>C, 11193 T>C (Figure 18).

Complete LD between the SNPs in block 2 and 3, and the SNP 7675 was also observed. These SNP was also found to be in linkage with the SNPs in Block 4 and 5. The SNPs in these blocks were in linkage with the SNP 7635. It was also observed a linkage between the SNPs in block 1 and in block 3. In figure 19 it is described the haplotype frequencies based on these linkage blocks.

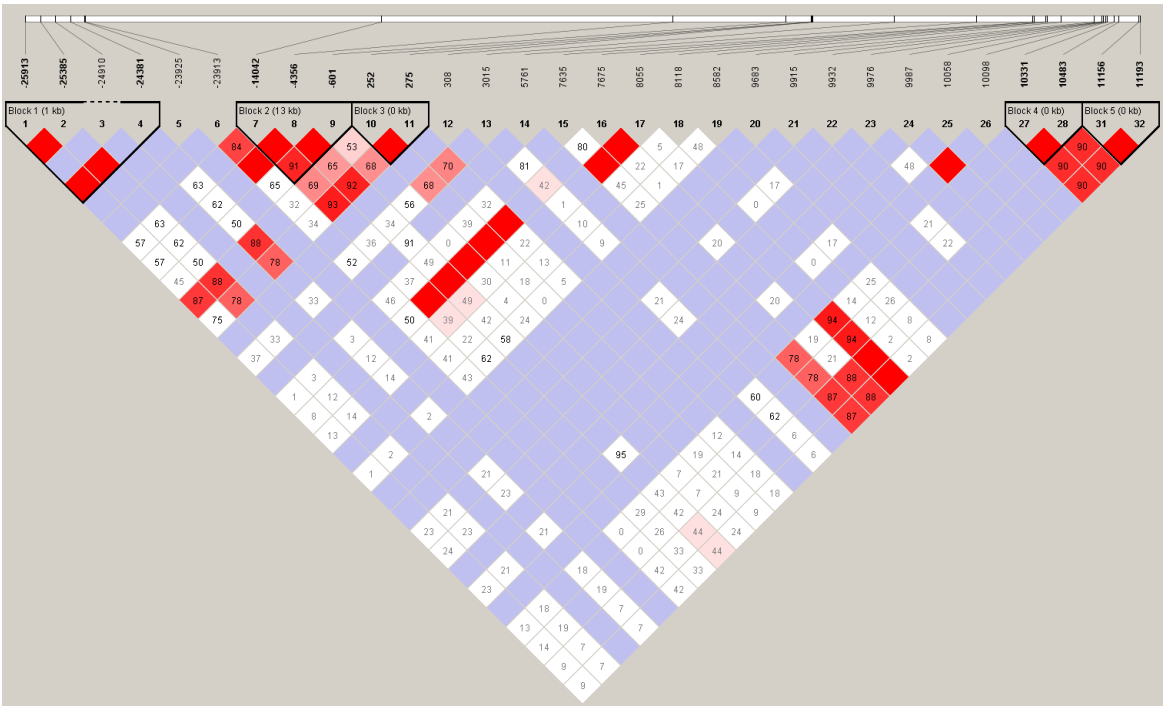


Figure 18: Linkage disequilibrium analysis of the PXR SNPS with variant allelic frequency > 0.01. The analysis was performed using haploview. Red squares indicate statistically significant (LOD>2) allelic association (linkage disequilibrium, LD) between the pair of SNPs, as measured by the D' statistic; darker colors of red indicate higher values of D', up to a maximum of 1. White squares indicate pairwise D' values of <1 with no statistically significant evidence of LD. Blue squares indicate pairwise D' values of 1 but without statistical significance.

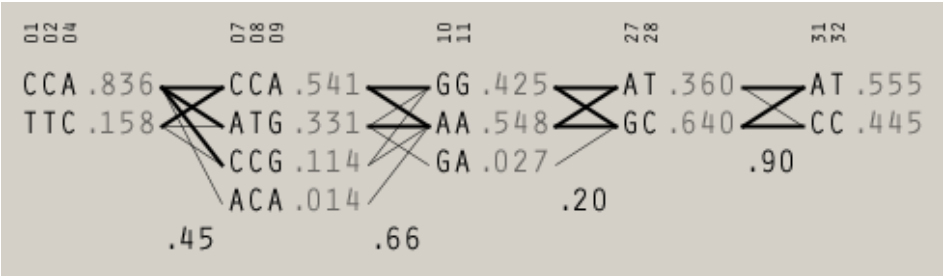


Figure 19: Frequencies of the main haplotypes observed in the Vietnamese population. The analysis was performed using haploview.

3.3. ROLE OF PXR SINGLE NUCLEOTIDE POLYMORPHISMS IN THE INTER-INDIVIDUAL VARIABILITY OF CYP3A INDUCTION UPON TREATMENT WITH BY ARTEMISININ AND ITS DERIVATIVES.

3.3.1. INTER-INDIVIDUAL VARIABILITY IN THE INDUCTION OF CYP3A ACTIVITY UPON EXPOSURE TO ARTEMISININ AND ITS DERIVATIVES IN A CLINICAL STUDY.

A high inter-individual variability for CYP3A activity induction was observed in the study described by Asimus *et al.*, 2007, for the individuals treated with 500 mg/orally artemisinin during a 5 day period as described in section 2.2.12.1. The groups of individuals treated with other compounds from the artemisinin family, showed lower inter-individual variability and CYP3A activity induction (Figure 20). The activity of this family of proteins was previously determined by the measurement of the 1-OH-midazolam/midazolam 4 hr plasma concentration ratio. This data was kindly provided by Prof. Dr. Michael Aston and Dr. Sarah Asimus, Gothenburg University, Sweden.

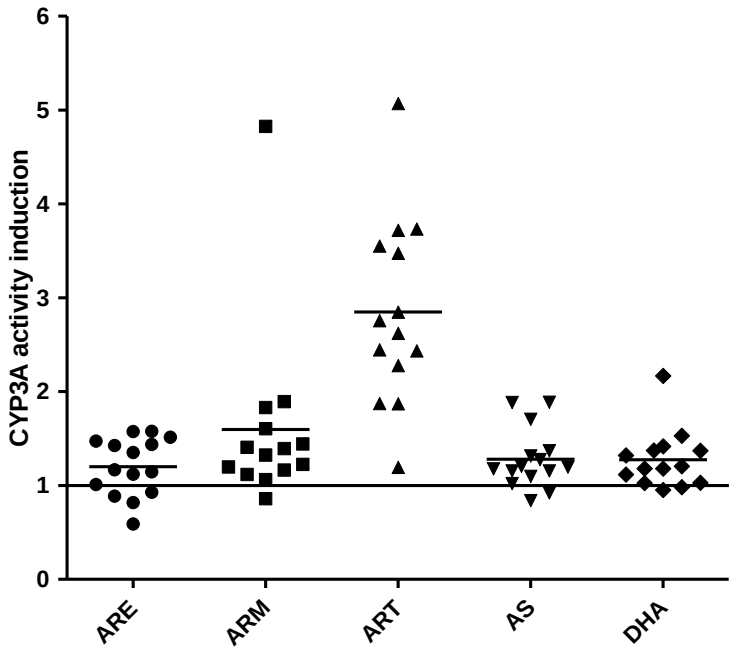


Figure 20: Inter-individual variability in CYP3A activity of Vietnamese individuals treated with different artemisinin related compounds. Induction of CYP3A activity was measured as the 3A 1-OH-midazolam/midazolam 4 hr plasma concentration ratio at day5 normalized to day-6. These pharmacokinetic parameters were determined as described by Asimus *et al.*, 2007.

A closer look at the individuals treated with artemisinin showed that the degree of CYP3A induction was not dependent on its basal levels, having individuals with the same basal level and with the highest (5.07) and lowest (1.19) inductions of CYP3A activity. No correlation was observed using a spearman correlation test (Figure 21).

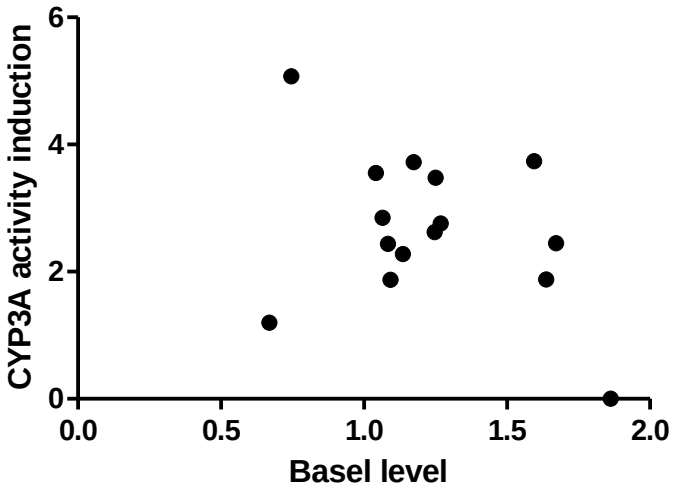


Figure 21: Relation between CYP3A activity basal levels and its induction in Vietnamese individuals treated with artemisinin. Basal level data corresponds to 3A 1-OH-midazolam/midazolam 4 hr plasma concentrationr atio at day-6. Induction data was obtained by the 3A 1-OH-midazolam/midazolam 4 hr plasma concentration ratio at day5 normalized to day-6.These pharmacokinetic parameters were determined as described by Asimus *et al.*, 2007.

This led us to study if genetic variability in CYP3A known activator, and main xenosensor PXR, was involved in this inter-individual variability. The results of these studies are described in the following chapters.

3.3.2. ASSOCIATION BETWEEN PXR GENETIC VARIABILITY AND CYP3A ACTIVITY INDUCTION BY ARTEMISINS IN A VIETNAMESE POPULATION

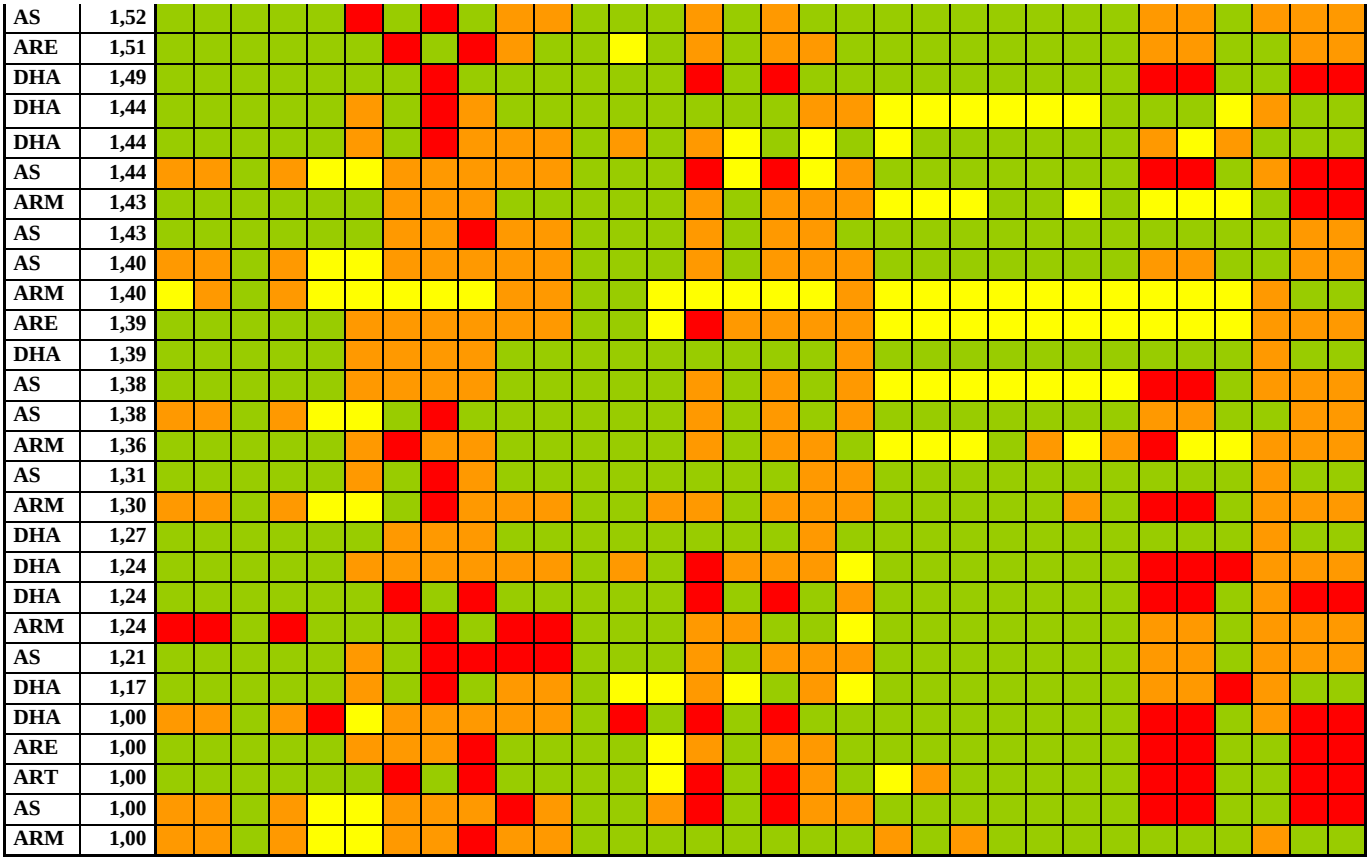
A comparison between the group of individuals with higher (50%) and lower CYP3A (50%) activity induction upon exposure to one of the antimalarials aforementioned did not show any statistically significant difference between the two groups concerning the genetic variability in *PXR* (data not showed).

To eliminate the effect of the different treatments, the induction of CYP3A activity for all compounds was normalized to the individualas showing the lowest induction in each group. A

trend could be observed for the SNPs 252 and 275, the prevalence of individuals homozygous for reference allele being higher among the low induction group of individuals (Table 13).

Table 13: Association between the PXR genotypes and the CYP3A activity induction. For each drug the induction were normalizedto the individual showing the lowest induction in each group (green: homozygous for the reference allele; orange: heterozygous; red: homozygous for the variant allele; yellow: unknown). The table is organized in terms of progressive decrease of induction. ART: artemisinin; ARE: arteether; ARM: artemeether; AS: artesunate; DHA: dihydro-artemisinin.

Drug	Normalized induction	-25913 C/T	-25385 C>T	-24910 G>A	-24381 A>C	-23935 C>T	-23913 T>G	-14042 C>A	-4356 T>C	-601 A>G	252 A>G	275 A>G	308 A>T	3015 T>G	5761 A>G	7635 A>G	7675 C>T	8055 C>T	8118 C>T	8582 T>G	9863 A>G	96915 T>A	9932 C>G	9976 G>A	9987 G>A	10058 C>G	10098 C>T	10331 A>G	10483 T>C	10719 A>G	10976 G>A	11156 A>C	11193 T>C
ARM	5,62																															
ART	4,24																															
ART	3,12																															
ART	3,11																															
ART	2,97																															
ART	2,91																															
ARE	2,68																															
ARE	2,67																															
ARE	2,57																															
ARE	2,50																															
ARE	2,44																															
ARE	2,42																															
ART	2,38																															
ART	2,31																															
ARE	2,30																															
DHA	2,28																															
AS	2,25																															
AS	2,24																															
ARM	2,21																															
ART	2,19																															
ART	2,05																															
ARM	2,13																															
ART	2,04																															
AS	2,03																															
ARE	1,98																															
ARE	1,95																															
ART	1,91																															
ARE	1,90																															
ARM	1,87																															
ARE	1,72																															
ARM	1,68																															
ARM	1,64																															
AS	1,63																															
ARM	1,62																															
DHA	1,61																															
ARE	1,57																															
ART	1,57																															
AS	1,57																															
ART	1,56																															
ARM	1,54																															



A step-wise comparison was performed between subsets of individuals showing high and low induction using fisher exact test and are shown in figure 22. A higher frequency of the G allele was observed in this subset of individuals, statistical significant differences were observed for the SNPs 252 and 275 A>G when comparing the 40% higher inducers with the 60% lower inducers (p=0.0138). The same was observed until the fractioning of 60% higher inducers and 40% lower inducers (p=0.0216).

Analysis of the 10331 A>G and 10483 T>C also revealed a high prevalence of the polymorphic allele the in the fraction of 30 and 40% of high inducers compared with the rest of the population (p=0.0490 and 0.0434, respectively).

Statistically significant differences were also obtained for the stratification of the population according to the -601 A>G SNPs, when the 60% higher inducers were compared with the rest of the population (p=0.0463). The same was observed for the subset 90%/10% (p=0.0451).

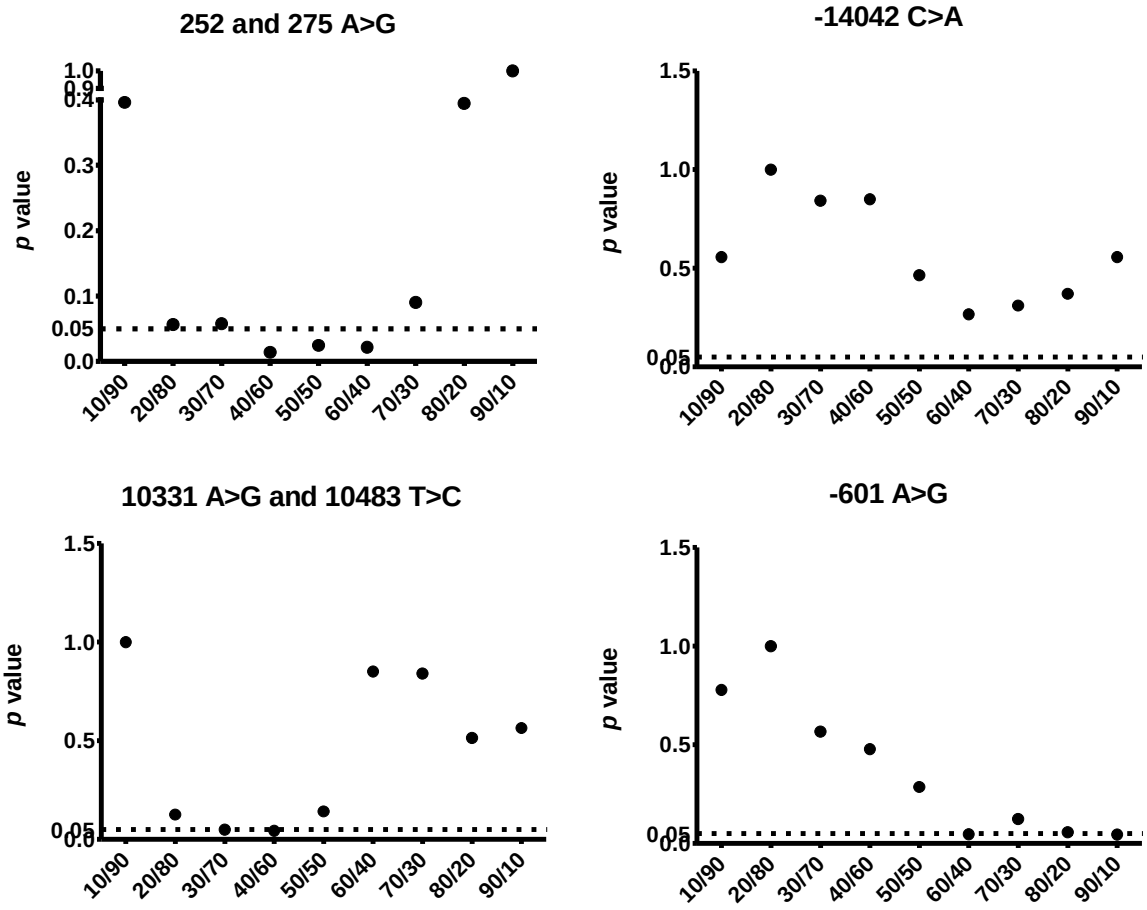


Figure 22: Comparison of the genetic diversity of selected SNPs in subgroups of individuals treated with artemisinins. The p values represent the comparison between the percentages of high and low inducers. The values were obtained using the fisher test.

A special attention was given to the individuals treated with artemisinin in order to assess the correlation between *PXR* SNPs and their high inter-individual variability in induction of CYP3A activity (table 14). No association between any of the SNPs and CYP3A induction was observed, a fact that might be due to the small number of individuals treated with this drug, not allowing significant statistic associations.

Table 14: Genotyping data of PXR in Vietnamese individuals treated with 500 mg/orally artemisinin and mean CYP3A4 activity induction; [a] Position of SNP in GenBank sequence AF364606.1 with +1 being the first nucleotide of the start codon (CTG) in exon2 (nucleotide 70390); [b] Number of individuals analyzed; [c] Value in parenthesis is the 95% confidence interval determine by the modified Wald method using GraphPad Quickcalcs software; [d] Value in parenthesis is the Std. deviation determined using the software GraphPad Prism.

Position[a]	*Aminoacid*	*Identifier*	*n*[b]	*Genotypic frequencies*[c]		*Mean CYP3A4*[d]
-25913 C>T		rs1523130	14	C/C	0.57 (0.32- 0.79)	2.76 (±0.83)
				C/T	0.36 (0.16- 0.61)	2.82 (±1.32)
				T/T	0.07 (0.00- 0.33)	3.74
-25385 C>T		rs3814055	14	C/C	0.57 (0.32- 0.79)	2.76 (±0.83)
				C/T	0.36 (0.16- 0.61)	2.82 (±1.32)
				T/T	0.07 (0.00- 0.33)	3.74
-24381 A>C		rs1523127	14	A/A	0.57 (0.32- 0.79)	2.76 (±0.83)
				A/C	0.36 (0.16- 0.61)	2.82 (±1.32)
				C/C	0.07 (0.00- 0.33)	3.74
-23913 T>G			10	T/T	0.50 (0.24- 0.76)	2.56 (±1.13)
				T/G	0.40 (0.17- 0.69)	3.06 (±0.54)
				G/G	0.10 (0.00- 0.43)	2.62
-14042 C>A			14	C/C	0.29 (0.11- 0.55)	2.87 (±0.62)
				C/A	0.57 (0.32- 0.79)	2.77 (±0.74)
				A/A	0.14 (0.03- 0.41)	3.13 (±2.74)
-4356 T>C		rs13059232	14	T/T	0.14 (0.03- 0.41)	3.13 (±2.74)
				T/C	0.50 (0.27- 0.73)	2.90 (±0.70)
				C/C	0.36 (0.16- 0.61)	2.67 (±0.70)
-601 A>G		rs7643645	14	A/A	0.29 (0.11- 0.55)	2.77 (±0.77)
				A/G	0.57 (0.32- 0.79)	2.82 (±0.68)
				G/G	0.14 (0.03- 0.41)	3.13 (±2.74)
252 A>G		rs1464603	14	A/A	0.21 (0.07- 0.48)	2.34 (±1.18)
				A/G	0.58 (0.32- 0.79)	3.01 (±1.07)
				G/G	0.21 (0.07- 0.48)	2.93 (±0.70)
275 A>G		rs144602	14	A/A	0.29 (0.11- 0.55)	3.03 (±1.67)
				A/G	0.50 (0.27- 0.73)	2.71 (±0.72)
				G/G	0.21 (0.07- 0.48)	2.93 (±0.70)
3015 T>G			13	T/T	0.54 (0.29- 0.77)	2.88 (±1.31)
				T/G	0.31 (0.12- 0.58)	2.94 (±0.55)
				G/G	0.15 (0.03- 0.43)	3.05 (±0.60)
7635 A>G		rs6785049	14	A/A	0.07 (0.00- 0.33)	2.28
				A/G	0.64 (0.39- 0.84)	2.98 (±0.67)
				G/G	0.29 (0.11- 0.55)	2.69 (±1.69)
7675 C>T		rs6797879	13	C/C	0.46 (0.23- 0.71)	2.91 (±1.40)
				C/T	0.54 (0.29- 0.77)	2.80 (±0.69)
				T/T	0 (0.00- 0.27)	-
8055 C>T		rs2276707	14	C/C	0.58 (0.32- 0.79)	2.76 (±0.67)
				C/T	0.21 (0.07- 0.48)	3.22 (±0.52)
				T/T	0.21 (0.07- 0.48)	2.71 (±2.07)
8118 C>T			13	C/C	0.54 (0.29- 0.77)	3.29 (±0.99)
				C/T	0.46 (0.23- 0.71)	2.34 (±0.88)
				T/T	0 (0.00- 0.27)	-
8582 T>G			13	T/T	0.54 (0.29- 0.77)	2.85 (±1.26)
				T/G	0.46 (0.23- 0.71)	2.95 (±0.74)
				G/G	0 (0.00- 0.27)	-
9915 T>A	F420Y		14	T/T	0.93 (0.67-1.00)	2.98 (±0.90)
				T/A	0.07 (0.00-0.34)	1.19 (±0.00)
				A/A	0.00 (0.00-0.25)	-
9932 C>G	Q426E		14	C/C	0.93 (0.67-1.00)	2.78 (±1.00)
				C/G	0.07 (0.00-0.34)	3.72
				G/G	0.00 (0.00-0.25)	-

9976 G>A	rs3732358	14	G/G	0.86 (0.59-0.96)	2.96 (±1.02)
			G/A	0.14 (0.03-0.41)	2.16 (±0.40)
			A/A	0.00 (0.00-0.25)	-
10058 C>G		14	C/C	0.93 (0.67-1.00)	2.88 (±1.02)
			C/G	0.07 (0.00-0.34)	2.45
			G/G	0.00 (0.00-0.25)	-
10331 A>G	rs3732359	14	A/A	0.14 (0.03-0.41)	2.08 (±0.29)
			A/G	0.29 (0.11-0.55)	3.06 (±0.54)
			G/G	0.57 (0.33-0.79)	2.94 (±1.21)
10483 T>C	rs3732360	14	T/T	0.14 (0.03-0.41)	2.08 (±0.29)
			T/C	0.29 (0.11-0.55)	3.06 (±0.54)
			C/C	0.57 (0.33-0.79)	2.94 (±1.21)
10719 A>G	rs6438550	13	A/A	0.62 (0.35-0.82)	2.74 (±1.26)
			A/G	0.23 (0.07-0.51)	2.61 (±0.16)
			G/G	0.15 (0.03-0.44)	3.28 (±0.62)
10976 G>A		14	G/G	0.29 (0.11- 0.55)	2.31 (±1.01)
			G/A	0.71 (0.45-0.89)	3.07 (±0.94)
			A/A	0 (0.00- 0.25)	-
11156 A>C	rs3814057	14	A/A	0.50 (0.27- 0.73)	2.62 (±0.58)
			A/C	0.29 (0.11- 0.55)	3.35 (±0.50)
			C/C	0.21 (0.07- 0.48)	2.71 (±2.07)
11193 T>C	rs3814058	14	T/T	0.50 (0.27- 0.73)	2.62 (±0.58)
			T/C	0.29 (0.11- 0.55)	3.35 (±0.50)
			C/C	0.21 (0.07- 0.48)	2.71 (±2.07)

3.3.3. ASSOCIATION BETWEEN *PXR* GENETIC VARIABILITY AND *CYP3A4* INDUCTION BY ARTEMISININ IN HUMAN PRIMARY HEPATOCYTES

Taken in consideration the group of individuals treated with artemisinin comprised only 14 subjects, a further test was performed to assess the association between SNPs in *PXR* and CYP3A4 induction by artemisinin in a larger group of individuals. For this purpose cultures of primary human hepatocytes were treated with 100 µM artemisinin, and induction of CYP3A4 mRNA expression was measured and associated with *PXR* genotype (Table 15). SNPs with MAF > 0.05 in the first group of patients were analyzed by MALDI-TOF-MS (data kindly provided by Dr. Kathrin Klein and Dr. Elke Schaeffler, Dr.Margarete Fischer Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany) or by sequencing.

Although a trend can be observed for -14042 C>A, 252 A>G, 275 A>G, 7635A>G, 10331 A>G where the induction of CYP3A4 expression is suggested to be decreased in homozygotic carriers of the variant allele, particularly when compared with homozygous for the wt allele, no statistically significant association was observed between the analyzed SNPs and *CYP3A4*

mRNA expression induction most likely due to the high interindividual variability of induction.

Table 15: Genotyping data of human primary hepatocytes treated with 100µM artemisinin and their mean CYP3A4 induction; [a] Position of SNP in GenBank sequence AF364606.1 with +1 being the first nucleotide of the start codon (CTG) in exon2 (nucleotide 70390); [b] Number of individuals analyzed; [c] Value in parenthesis is the 95% confidence interval determine by the modified Wald method using GraphPad Quickcalcs software; [d] Value in parenthesis is the Std. deviation determined using the software GraphPad Prism.

Position[a]	***Identifier***	***n***[b]	***Genotypic frequencies***[c]		***Mean CYP3A4***[d]
-25913 T>C	rs1523130	37	T/T	0.22 (0.11-0.37)	13.73 (±12.73)
			T/C	0.51 (0.36-0.67)	18.58 (±26.59)
			C/C	0.27 (0.15-0.43)	12.58 (±10.93)
-25385 C>T	rs3814055	37	C/C	0.32 (0.19-0.49)	12.23 (±9.93)
			C/T	0.49 (0.33-0.64)	19.92 (±27.25)
			T/T	0.19 (0.09-0.34)	11.90 (±12.57)
-24381 A>C	rs1523127	37	A/A	0.32 (0.19-0.49)	12.23 (±9.93)
			A/C	0.49 (0.33-0.64)	19.92 (±27.25)
			C/C	0.19 (0.09-0.34)	11.90 (±12.57)
-14042 C>A		37	C/C	0.54 (0.38-0.69)	20.81 (±25.98)
			C/A	0.38 (0.24-0.54)	10.45 (±9.88)
			A/A	0.08 (0.02-0.22)	8.71 (±5.99)
-4356 T>C	rs13059232	37	T/T	0.11 (0.04-0.25)	17.39 (±14.46)
			T/C	0.46 (0.31-0.62)	9.44 (±11.40)
			C/C	0.43 (0.29-0.59)	22.42 (±27.22)
-601 A>G	rs7643645	37	A/A	0.41 (0.26-0.56)	16.79 (±19.12)
			A/G	0.35 (0.22-0.51)	16.76 (±27.23)
			G/G	0.24 (0.13-0.40)	13.22 (±11.82)
252 A>G	rs1464603	35	A/A	0.37 (0.23-0.54)	15.37 (±18.29)
			A/G	0.46 (0.31-0.62)	11.40 (±10.09)
			G/G	0.17 (0.08-0.33)	8.48 (±4.22)
275 A>G	rs144602	35	A/A	0.37 (0.23-0.54)	15.37 (±18.29)
			A/G	0.46 (0.31-0.62)	11.40 (±10.09)
			G/G	0.17 (0.08-0.33)	8.48 (±4.22)
7635 A>G	rs6785049	37	A/A	0.24 (0.13-0.40)	16.63 (±21.33)
			A/G	0.60 (0.43-0.74)	17.64 (±23.02)
			G/G	0.16 (0.07-0.31)	8.47 (±3.039)
8055 C>T	rs2276707	37	C/C	0.54 (0.38-0.69)	16.18 (±18.27)
			C/T	0.41 (0.26-0.56)	16.25 (±25.10)
			T/T	0.05 (0.01-0.19)	10.62 (±0.14)
10331 A>G	rs3732359	37	A/A	0.43 (0.29-0.59)	18.45 (±19.76)
			A/G	0.46 (0.31- 0.62)	15.26 (±23.73)
			G/G	0.11 (0.04- 0.25)	8.57 (±3.56)
11156 A>C	rs3814057	37	A/A	0.54 (0.38- 0.69)	16.18 (±18.27)
			A/C	0.41 (0.26- 0.56)	16.25 (±25.10)
			C/C	0.05 (0.01- 0.19)	10.62 (±0.14)

4. DISCUSSION

4.1. STUDY OF THE CAPACITY OF CURRENTLY USED ANTIMALARIAL DRUGS TO ACTIVATE THE PXR/CAR XENOBIOTIC SIGNAL TRANSDUCTION SIGNAL.

This study confirms the previously described data concerning the activation of PXR by artemether and arteether, and the activation of PXR and CAR by artemisinin (Burk *et al.*, 2005; Simmonson *et al.*, 2006). However the EC_{50} value obtained in this study for the induction of PXR by artemisinin is 20µM higher (53.64 µM vs. 34µM) compared to the value obtained by Burk and colleagues. This may be due to the different cell lines used, the previously described EC_{50} was obtained performing the gene reporter assay in LS174T cells, and in this study the assay was performed in COS1 cells. This difference in values may be an indication of a different permeability of the cells for this drug. Comparatively the values obtained for the half maximal effective concentration for artemisinin and derivatives were higher for the activation of CAR compared with the values obtained for the activation of PXR, except for artemether (EC_{50}(CAR)=12.44µM; EC_{50} (PXR)= 14.15µM). This may indicate preferential activation of PXR as compared to CAR by most artemisinins.

Due to its short half live and absorption through the liver, it is to expect a higher concentration of artemisinin in the liver, comparatively with its systemic plasma concentration. Together with artemisinin, also its metabolite deoxy-artemisinin was also identified as a ligand for PXR and CAR, in cellular assays, and further induced *CYP3A4* and *CYP2B6* in the hepatocytes. Taken in consideration this fact, the induction of CYP3A activity observed *in vivo* by artemisinin in the Asimus study, may result from the combined action of artemisinin and its metabolite in the cells.

Together with these compounds also DHA was studied in human hepatocytes. No data was obtained from the cellular assays due to the high toxicity of this compound, however this characteristic was not observed in human primary hepatocytes. This may be due to the presence of drug metabolizing enzymes and transporters in the hepatocytes, helping to eliminate DHA, thereby reducing its intra-cellular availability and consequent toxicity. This

conversion is mediated by UGT1A6 and UGT2B7 (Kerb *et al.,* 2009). A repression of the expression of *CYP3A4* and *CYP2B6* was observed in the human hepatocytes, however this was not corroborated with the data from the clinical study performed in Vietnam, where a slight induction of CYP3A activity was observed (Asimus *et al.*, 2007). This may be the result of the action of other compounds on PXR *in vivo*.

Another member of this family of compounds, artesunate, also repressed the induction of CYP3A4 and CYP2B6 in human hepatocytes. But contrary to the previously described compound, for artesunate, data from in vitro assays is available, and corroborates the repression observed in human hepatocytes.

Taken together the *in vitro* data obtained from this study with the *in vivo* data obtained from the only clinical study involving artemisinin and cytochrome P450 induction (Asimus *et al.,* 2007), the high induction observed by artemisinin, as compared to artemether and arteether, may result from the combined action of the parent drug and its metabolite deoxy-artemisinin in the hepatocytes. Not happening with the other compounds, as these are metabolized to DHA, a compound that in human hepatocytes showed a repression of CYP3A mRNA expression and thus may counteract the induction of the parent compounds.

Together with these compounds, also, amodiaquine induced simultaneously PXR and CAR. However, no induction was observed in human hepatocytes, and even a repression of CYP3A4 was observed. This may be partially explained by its toxicity and disruption of the normal cell machinery, but is also an indication that although the drug may have the capacity to induce the interaction of the nuclear receptor with its co-activator; it may not be able to induce the release of co-repressor. This was previously observed for docetaxel, which was showed to promote the interaction of PXR with its coactivators, however did not activate this nuclear receptor. This was associated with a failure to disrupt to interaction of PXR with its co repressors (Synould *et al.,* 2001). Further tests, e.g., co-repressor release assay, are necessary to confirm this hypothesis.

This work also provides the first evidences for activation/ligand binding of PXR by the quinoline antimalarials lumefantrine, carboximefloquine, DEAQ and chloroquine, in a mammalian two hybrid assay. From these compounds only carboximefloquine was observed to induce *CYP2B6* in the hepatocytes culture, confirming the data obtained in the cellular

assay (table 16). This suggests a gene specific effect of this drug, since no induction was observed for *CYP3A4*, and *MDR1*. This compound is formed, from the parent antimalarial mefloquine, by the action of CYP3A4 (Fontaine *et al.*, 2000). No data is available regarding the plasma concentration of carboxymefloquine in patients, but considering the plasma concentrations observed for mefloquine ranging from 2.500ng/ml-2.830ng/ml depending on the drug supplier (Gutman *et al.*, 2009), and its long half time (14-15 days), we may expect that high concentrations of this metabolite may be reachable in the patients liver, increasing their probability of drug interactions due to the induction of the PXR system

Lumefantrine and chloroquine did not show any induction in human primary hepatocytes. This observation may result from the influence of the high amount of Me_2SO, 0.8% compared with the normal 0.1% regularly used for the other compounds. This was also observed in the induction control rifampicin; when usend with 0.1% Me_2SO its mean induction of *CYP3A4* was 2.5x higher compared with the treatment containing 0.8% Me_2SO (Figure 14). This was due to a higher basal level of the measured genes in the presence of a high amount of Me_2SO, and consequent lower induction. Another factor that has to be taken in consideration to explain this data is the uptake transport of these drugs, no data is available at the moment about this mechanism. A further test that should be performed with these compounds should be the measurement of its concentrations in the hepatocytes, to confirm that the uptake of the drugs is occurring, and exclude the possibility that the non-induction is resulting from the low concentration of compounds inside the cell. No quantifiable value for the maximum induction for lumefantrine was obtained, due to the fact that the plateau phase was not reached. Nevertheless, the *in vitro* induction data obtained for this drug is of particular interest due to its long high half life time (up to 6 days) and common utilization in malaria therapeutics; combined with artemeether (Coartem®) it is the most prevalent first line treatment in the world (WHO, 2010). The *in vitro* induction of PXR is an indication that lumefantrine may suffer also from auto-induction of its detoxification, since CYP3A4/5 are the main enzymes involved in this process and known targets for PXR. This is of concern if the concentration necessary to activate PXR is lower than the maximum concentration in the liver. No data is available regarding the concentration of this drug in the liver, however its plasma concentration is described as being 6.757 ng/ml (Mwesigwa *et al.*, 2010). Further tests are necessary to determine the correct concentration necessary to induce the PXR system. Together with malaria, a high incidence of HIV and TB co-exists in the same regions(WHO,

2010) making patients treated with lumefantrine more prone to develop drug-drug interactions in the case of drugs metabolized by enzymes regulated by PXR.

Amodiaquine and its active metabolite DEAQ activate PXR with EC_{50}'s of, respectively, 17.20µM (7.99 mg/ml) and ~24.74 µM (8.11mg/ml). These values are much higher than the observed maximum plasma concentration observed for amodiaquine (5.2ng/ml) and DEAQ (253ng/ml) (Mwesigwa *et al.*, 2010). However liver concentrations of these compounds may be much higher than plasma concentrations, as so, concern should be taken in the future, if a dosage increase is necessary, and preliminary tests to assess the intra-hepatic concentration of these compounds should be performed.

Table 16: Compilation of the results concerning the capacity of currently used antimalarials to activate the PXR/CAR system obtained during this project.

	Human Hepatocytes				
Drug	***Nuclear Receptor***	***In vitro induction***	***Induction***	***Repression***	***In vivo***
Artemisinin	PXR/CAR	√	*CYP3A, CYP2B6* and *MDR1*	-	CYP3A activity induction
Arteether	PXR/CAR	√	*CYP3A4, CYP2B6* and *MDR1*	-	CYP3A activity induction
Artemether	PXR/CAR	√	*CYP3A4 and CYP2B6*	-	CYP3A activity induction
Deoxy-arteether	PXR/CAR	√	*CYP3A4 and CYP2B6*	-	-
Deoxy-artemisinin	PXR/CAR	√	*CYP3A4 and CYP2B6*	-	-
DHA	-	-	-	*CYP3A4 and CYP2B6*	CYP3A activity induction
Artesunate	PXR/CAR	repression	-	*CYP3A4 and CYP2B6*	CYP3A activity induction
Lumefantrine	PXR	√	-	-	-
Carboximefloquine	PXR	√	*CYP2B6*	-	-
DEAQ	PXR	√		*CYP3A4 and CYP2B6*	-

Chloroquine	PXR	√	-	-	-
Amodiaquine	PXR/CAR	√	-	*CYP3A4*	-

A further methodological approach was used to confirm the interaction of the aforementioned compounds with CAR. A C.A.R.L.A. assay was chosen for this purpose, but due to low sensitivity no induction was observed for these compounds at the same concentrations used in the hepatocytes, except for arteether (Figure 17). Due to this observation, only one experiment was performed.

In mammalian two hybrid experiments, only the activation of CAR by ligand binding of the compounds was studied. Further studies are necessary to identify the compounds that act as CAR activators by inducing its translocation to the nucleus. One known compound that acts by this particular mechanism is phenobarbital (Kodama and Negishi, 2006). Taken this in consideration we cannot exclude completely the hypothesis that one of the other antimalarials may act in the same way as phenobarbital, as so, we may only state that the compounds identified in this study induce the CAR system, but we cannot conclude anything about the compounds not positive in our experiments. The translocation of CAR could be studied using, e.g., yellow fluorescent protein-tagged-human constitutive androstane receptor, by the direct observation of the translocation of CAR to the nucleus (Li *et al.*, 2009).

4.2. STUDY OF THE GENETIC VARIABILITY IN PXR IN A VIETNAMESE POPULATION

To the best of our our knowledge, this is the first extensive description of *PXR* genetic variability in a Southeast Asian population, being actually the first study re-sequencing of this gene after the first publication describing its genetic variability almost one decade ago (Hustert *et al.*, 2001; Zhang *et al.*, 2001). The re-sequencing technique chosen in this project, revealed to be an appropriate approach, which, complemented with the use of MALDI-TOF-MS, allowed the analysis of 79 positions of genetic variability, six of them not described previously. All SNPs were in Hardy-Weinberg equilibrium, except the PXR 8055C>T and

10976 G>A SNP. This anomaly may be either due to not sufficiently isolated populations, or just a reflection of the small group of samples analyzed, since the hypothesis of methodological error during the determination of this genotype was discarded by the use of two independent genotyping methods, re-sequencing and MALDI-TOF-MS (data kindly provided by Dr. Elke Schaeffler, Dr.Margarete Fischer-Bosch- Intitute of ClinicalPharmacology, Stuttgart, Germany).

The gene was highly conserved in this population (from the 79 SNP positions studied, only 32 could be identified). Only 4 SNPs in the ORF of the gene were observed. Being the synonymous SNP 8118C>T the most prevalent (MAF=0.26), the other 3 non-synonymous SNPs were only found in the heterozygous form in 1-2 individuals. The newly identified SNP 9915 T>A (F240Y) is localized in exon9, in the region encoding the LBD of the protein; as so, it may be hypothesized that this SNP may influence ligand binding. This was confirmed by molecular docking studies that revealed a disruption of ligand binding in the presence of the tyrosine (Y) (data kindly provided by Pedro Ferreira, Department of Medicine, Karolinska Institutet, Stockholm, Sweden).

All other both new as well as previously described SNPs observed in this study, are localized in intronic and promoter regions, as well as in the 3'UTR region. The lack of genetic diversity in the protein coding sequences suggests that the stability of the protein sequence is essential for its function, being kept free of genetic variability during evolution. This is corroborated by previously published data (see appendix 1).

Four hundred and fourteen SNPs are described for this gene (http://www.ncbi.nlm.nih.gov/sites/entrez, accessed on 17 May 2010), but only for a few of them there is data available regarding their prevalence in other populations. From the data available, it is possible to observe that the Vietnamese population shows more similarities to an Indian and Asian-American populations then to Caucasians populations (see section 3.2), as expected. Surprisingly statistically significant differences were observed for the 10331 and 10483 SNP, between our population and a Chinese and Malay population. It should be expected a higher similarity between the Vietnamese population and the Chinese population, due to the fact that Vietnamese are mainly derived from individuals from southern Chinese and Thai-Indonesian populations (Ivanova *et al.*, 1999). One possible explanation is the unknown origin of the Chinese population that could be originated in more northern parts of

China, and being more diverse to the southern population, and not influencing the Vietnamese population.

4.3. ROLE OF PXR SINGLE NUCLEOTIDE POLYMORPHISMS IN THE INTER-INDIVIDUAL VARIABILITY OF CYP3A INDUCTION UPON TREATMENT WITH BY ARTEMISININ AND ITS DERIVATIVES

This study was based on a previously described clinical study, involving 75 Vietnamese individuals treated with 5 different compounds of the artemisinin family (arteether, artemeether, artemisinin, artesunate and dihydro-artemisinin). From this study we could see a high variability in induction of CYP3A activity, particularly for artemisinin. This compound demonstrated the highest induction of CYP3A activity, thereby corroborating the results we obtained *in vitro* (section 3.1.1.). Artemisinin shows the highest induction capacity of all compounds of this family of drugs in the PXR system. This variability was not correlated with the basal CYP3A activity, being only visible upon induction with artemisinin (figure 21). Altogether, this led us to hypothesize that the observed variability in CYP3A induction was a result of genetic variability in its main genetical regulator, PXR.

In order to normalize induction values for each drug, the data from the induction of each individual was normalized to the individual showing the lowest induction in the respective group. An increased prevalence of the 252G and 275G alleles was observed in the group of high inducers. No data is available concerning the *in vivo* effect of the aforementioned SNPs. To confirm if the higher CYP3A activity was correlated with the induction of expression of its mRNA, further induction studies were performed in human hepatocytes. Contrary to expected we observed a higher CYP3A4 mRNA content for the homozygous for the wt allele (A/A) comparatively with the homozygous for the variant allele (G/G). This is an indication that the influence of this SNPs does not came directly from the induction of mRNA expression. It has to be taken in consideration also, that these are intronic SNPs.

The newly identified SNP 9915 T>A (F420Y) is located in the ligand binding domain of the nuclear receptor. Docking studies revealed a disruption of ligand binding in the presence of the amino acid tyrosine (data kindly provided by Pedro Ferreira, Department of medicine,

Karolinska Institutet, Stockholm, Sweden). The data was obtained for the ligands rifampicin and colupulone. Our experimental data supports this result - the individual carrying this SNP showed no induction (1.02) in the group of individuals treated with artemisinin. Further studies are necessary to confirm the phenotypic outcome of this SNP, namely directed mutagenesis of this aminoacid in the PXR protein coupled with gene reporter assays.

Also for the SNPs 10331 A>G and 10483 T>C, it was observed a high prevalence of variant allele carriers in the high inducers group. A similar association was also previously reported by Oleson *et al.,* 2009. Their work showed that individuals that carried these two linked polymorphic alleles had 80% higher oral midazolam clearence, an indication of higher CYP3A activity. However further analysis in human primary hepatocytes treated with artemisinin, showed an opposite trend for SNP 10331, the association between the presence of this SNP and *CYP3A4* mRNA induction was quite the opposite in the hepatocytes, an increase of the induction of *CYP3A4* mRNA was observed in the carriers of the wt allele. However the statistical analysis of the human hepatocyte data is hampered by the high interindividual variability in induction, probably due to diverse origins and quality of the cells. Also the underlying pathologies of the patients may also be influencing *CYP4A4* induction response to these compounds. This may also be an indication that the phenotypic effect from this SNP may come from the association with other genetical factors, not only the aforementioned SNPs together.

Our hepatocyte treatment data regarding the polymorphism -14042C>A, could confirm the trend described previously by Lamba *et al.*, 2008, were the presence of the polymorphism decreases *CYP3A4* induction. This was also observed in our hepatocyte data, however no association was observed *in vivo* upon treatment with artemisinin.

The SNP -601 A>G, was previously reported as being associated with a lower CYP3A4 activity and lower CYP3A4 mRNA levels. We could observe a decrease in the induction of the mRNA levels in the hepatocytes, but could not confirm the decrease in CYP3A4 activity in the clinical study. We actually observe a slight increase in its activity upon treatment with artemisinin. This may also be explained by the other non-genetical factors influencing genetical expression, e.g. food, environmental pollutants.

Our data could not confirm the previously described report on the 7635A/G allele (Zhang *et al.*, 2001). We could not observe an increase of CYP3A4 induction in the carries of the G/G allele, as reported previously, neither in the clinical study nor in the hepatocytes treatment experiment.

Also to highlight is the presence of the SNPs 9863 A>G (I403V) and 9932 C>G (Q426E) in the individual with the lowest induction for the treatment with artesunate. This non-induction may come directly from the I403V polymorphism, since this was previously described to reduce ligand dependent transactivation of *CYP3A4* (Koyano *et al.,* 2002 and 2004). It seems that our data confirms the previously described phenotypic association. For the other polymorphism no phenotypic association is known, since it has not been studied in this context.

Although some of these results are suggestive of an influence of these specific alleles in the in vivo induction capacity of their carriers, one should be cautious when interpreting the data. The main overall observation of this analysis is that actually the final phenotype might be the result of many small SNP influences. The determination of the precise influence of each one awaits further specifically designed studies.

Taken together we could confirm some of the data published concerning phenotypic changes induced by PXR SNPs. However no clear results were obtained for the newly identified SNPs due to a limited number of samples, except for the SNP F420Y.

REFERENCES

Alin MH, Kihamia CM, Bjorkman A, Bwijo BA, Premji Z, Mtey GJ, Asthon M. Efficacy of oral and intravenous artesunate on male Tanzanian adults with Plasmodium falciparum malaria and in vitro susceptibility to artemisinin, chloroquine and mefloquine. *Am J Trop Med Hyg.* 1995; 53(6):639-45.

Alkadi HO. Antimalarial drug toxicity: a review. *Chemotherapy.* 2007; 53(6):385-91.

Aly ASI, Vaughan AM, Kappe SHI. Malaria parasite development in the mosquito and infection of the mammalian host. *Annu Rev Microbiol.* 2009; 63:195-221.

Amre DK, Mack DR, Israel D, Morgan K, Krupoves A, Costea I, Lambrette P, Grimard G, Deslandres C, Levy E, Seidman EG. Investigation of associations between the pregnane-X receptor gene (NR1I2) and Crohn's disease in Canadian children using a gene-wide haplotype-based approach. *Inflamm Bowel Dis.* 2008; 14(9):1214-8.

Arnold KA, Eichelbaum M, Burk O. Alternative splicing affects the function and tissue-specific expression of the human constitutive androstane receptor. *Nucl Recept.* 2004; 2:1.

Asimus S, Elsherbiny D, Hai TN, Jansson B, Huong NV, Petzold MG, Simonsson US, Ashton M. Artemisinin antimalarials moderately affect cytochrome P450 enzyme activity in healthy subjects. *Fundam Clin Pharmacol.* 2007; 21(3):307-16.

Axelrod J. The enzymatic demethylation of ephidrine. *J. Pharmacol*. 1955; 114:430-8.

Baes M, Gulick T, Choi HS, Martinoli MG, Simha D, Moore DD. A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol Cell Biol.* 1994; 14(3):1544-52.

Baune B, Flinois JP, Furlan V, Gimenez F, Taburet AM, Becquemont L, Farinotti R. Halofantrine metabolism in microsomes in man: major role of CYP 3A4 and CYP 3A5. *J Pharm Pharmacol.* 1999; 51(4):419-26.

Benkali K, Prémaud A, Picard N, Rérolle JP, Toupance O, Hoizey G, Turcant A, Villemain F, Le Meur Y, Marquet P, Rousseau A. Tacrolimus population pharmacokinetic-

pharmacogenetic analysis and Bayesian estimation in renal transplant recipients. *Clin Pharmacokinet.* 2009;48(12):805-16.

Bertz RJ, Granneman, GR. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet.* 1997; 32 (3):210-58.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A.* 1998; 95:12208-13.

Blumberg B, Sabbagh W Jr, Juguilon H, Bolado J Jr, van Meter CM, Ong ES, Evans RM. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* 1998; 12:3195-205.

Bosch TM, Deenen M, Pruntel R, Smits PH, Schellens JH, Beijnen JH, Meijerman I. Screening for polymorphisms in the PXR gene in a Dutch population. *Eur J Clin Pharmacol.* 2006; 62(5):395-9.

Brodie B, Axelrod J, Cooper JR, Gaudette L, LaDu BN, Mitoma C, Udenfriend S. Detoxication of drugs and other foreign compounds by liver microsomes. *Science*. 1955; 121:603-4.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF, Eichelbaum M. Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol*. 2005; 67(6):1954-65.

Burckhalter JH, Tendick FH, Jones FH, Jones PA, Holcombe WF, Rawlins AL. Aminoalkylphenols as antimalarials (heterocyclicamino)-alpha-amino-o-cresols; the synthesis of camoquin. *J Am Chem Soc.* 1948; 70(4):1363-73.

Carnahan VE, Redinbo MR. Structure and function of the human nuclear xenobiotic receptor PXR. *Curr Drug Metab.* 2005; 6:357-67.

Cavaco I. Molecular determinants of the Response to malaria therapeutics. *University of the Algarve*. 2008.

Choi HS, Chung M, Tzameli I, Simha D, Lee YK, Seol W, Moore DD. Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. **J Biol Chem.** 1997; 272(38):23565-71.

Cox FE. History of the discovery of the malaria parasites and their vectors. *Parasit Vectors.* 2010; 3(1):5.

Cui L, Su XZ. Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Rev Anti Infect Ther*. 2009; 7(8):999-1013.

di Masi A, Marinis ED, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Aspects Med.* 2009; 30(5):297-343.

Dring MM, Goulding CA, Trimble VI, Keegan D, Ryan AW, Brophy KM, Smyth CM, Keeling PW, O'Donoghue D, O'Sullivan M, O'Morain C, Mahmud N, Wikström AC, Kelleher D, McManus R. The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. *Gastroenterology*. 2006; 130(2):341-8.

Eastman RT, Fidock DA. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat. Rev. Microbiol.* 2009; Nov 2. [Epub ahead of print]

Falco EA, Goodwin LG, Hitchings GH, Rollo IM, Russell PB. 2:4-diaminopyrimidines- a new series of antimalarials. *Br J Pharmacol Chemother.* 1951; 6(2):185-200.

Famin O, Ginsburg H. Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in Plasmodium falciparum-infected erythrocytes. *Biochem Pharmacol.* 2002; 63(3):393-8.

Ferreira PE, Veiga MI, Cavaco I, Martins JP, Andersson B, Mushin S, Ali AS, Bhattarai A, Ribeiro V, Björkman A, Gil JP. Polymorphism of antimalaria drug metabolizing, nuclear receptor, and drug transport genes among malaria patients in Zanzibar, East Africa. *Ther Drug Monit.* 2008; 30(1):10-5.

Fontaine F, de Sousa G, Burcham PC, Duchêne P, Rahmani R. Role of cytochrome P450 3A in the metabolism of mefloquine in human and animal hepatocytes. *Life Sci.* 2000; 66(22):2193-212.

Fromm E, Wittmann J. Derivate des *p*-Nitrothiophenols *Berichte der deutschen chemischen Gesellschaft* 1906**;** 41(2): 2264 – 2273.

Garfinkel D. Studies on pig liver microsomes. I. enzymic and pigment composition of different microsomal fractions. *Arch Biochem Biophys*. 1958; 77:493-509.

Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 2001; 276: 14581-14587.

Germain P, Staels B, Dacquet C, Spedding M, Laudet V. Overview of nomenclature of nuclear receptors. *Pharmacol Rev.* 2006; 58(4):685-704.

Giguère V. Orphan Nuclear Receptors: From gene to function. *Endocr Rev.* 1999; 20:689-725.

Gil JP, Gil Berglund E. CYP2C8 and antimalaria drug efficacy. *Pharmacogenomics.* 2007; 8(2):187-98.

Gil JP. Amodiaquine pharmacogenetics. *Pharmacogenomics.* 2008; 9(10):1385-90.

Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 1999; 56:1329-1339.

Goodwin B, Moore LB, Stoltz CM, McKee DD, Kliewer SA. Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* 2001; 60:427-431.

Gronemeyer H, Gustafsson JA, Laudet V. Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov*. 2004; 3(11):950-64.

Gutman J, Green M, Durand S, Rojas OV, Ganguly B, Quezada WM, Utz GC, Slutsker L, Ruebush TK 2nd, Bacon DJ. Mefloquine pharmacokinetics and mefloquine-artesunate effectiveness in Peruvian patients with uncomplicated Plasmodium falciparum malaria. *Malar J.* 2009; 8:58.

Hempelmann E. Hemozoin biocrystallization in Plasmodium falciparum and the antimalarial activity of crystallization inhibitors. *Parasitol Res.* 2007; 100(4):671-6.

Ho GT, Soranzo N, Tate SK, Drummond H, Nimmo ER, Tenesa A, Arnott ID, Satsangi J. Lack of association of the pregnane X receptor (PXR/NR1I2) gene with inflammatory bowel disease: parallel allelic association study and gene wide haplotype analysis. *Gut.* 2006; 55(11):1676-7.

Hor SY, Lee SC, Wong CI, Lim YW, Lim RC, Wang LZ, Fan L, Guo JY, Lee HS, Goh BC, Tan T. PXR, CAR and HNF4alpha genotypes and their association with pharmacokinetics and pharmacodynamics of docetaxel and doxorubicin in Asian patients. *Pharmacogenomics J.* 2008; 8(2):139-46.

Hustert E, Zibat A, Presecan-Siedel E, Eiselt R, Mueller R, Fuss C, Brehm I, Brinkmann U, Eichelbaum M, Wojnowski L, Burk O. Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. Drug Metab Dispos. 2001; 29(11):1454-9.

Hyde JE. Drug-resistant malaria - an insight. *FEBS J.* 2007; 274(18):4688-98.

Ivanova R, Astrinidis A, Lepage V, Djoulah S, Wijnen E, Vu-Trieu AN, Hors J, Charron D. Mitochondrial DNA polymorphism in the Vietnamese population. *Eur J Immunogenet.* 1999; 26(6):417-22.

Jain VK, Magrath IT. A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range: application to quantitation of beta-galactosidase in lacZ-transfected cells. *Anal Biochem.* 1991; 199:119-124.

Jensen EV. On the mechanism of estrogen action. *Perspect Biol Med* 1962; 6:47–59.

Jensen M, Mehlhorn H. Seventy-five years of Resochin in the fight against malaria. *Parasitol Res.* 2009; 105(3):609-27.

Josephy, PD. Molecular Toxicology. *Oxford University Press.* 1997;1st edition

Karlsen TH, Lie BA, Frey Frøslie K, Thorsby E, Broomé U, Schrumpf E, Boberg KM. Polymorphisms in the steroid and xenobiotic receptor gene influence survival in primary sclerosing cholangitis. *Gastroenterology.* 2006; 131(3):781-7.

Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA. Regulation of multidrug resistance-associated protein 2 (ABCC2)

by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem* 2002; 277: 2908-2915.

Kenakin, TP. A Pharmacology Primer: Theory, Applications and Methods. 2009; Elsevier:3rd edition.

Kerb R, Fux R, Mörike K, Kremsner PG, Gil JP, Gleiter CH, Schwab M. Pharmacogenetics of antimalarial drugs: effect on metabolism and transport. *Lancet Infect Dis.* 2009; 9(12):760-74.

King CR, Xiao M, Yu J, Minton MR, Addleman NJ, Van Booven DJ, Kwok PY, McLeod HL, Marsh S. Identification of NR1I2 genetic variation using resequencing. *Eur J Clin Pharmacol.* 2007; 63(6):547-54.

Klingenberg M. Pigments of rat liver microsomes. Arch Biochem Biophys. 1858; 75:376-86.

Kitchen LW, Vaughn DW, Skillman DR. Role of US military research programs in the development of US Food and Drug Administration--approved antimalarial drugs. *Clin Infect Dis.* 2006; 43(1):67-71.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T, Lehmann JM. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell.* 1998; 92:73-82.

Kodama S, Negishi M. Phenobarbital confers its diverse effects by activating the orphan nuclear receptor CAR. *Drug Metab Rev.* 2006; 38(1-2):75-87.

Koyano S, Kurose K, Ozawa S, Saeki M, Nakajima Y, Hasegawa R, Komamura K, Ueno K, Kamakura S, Nakajima T, Saito H, Kimura H, Goto Y, Saitoh O, Katoh M, Ohnuma T, Kawai M, Sugai K, Ohtsuki T, Suzuki C, Minami N, Saito Y, Sawada J. Eleven novel single nucleotide polymorphisms in the NR1I2 (PXR) gene, four of which induce non-synonymous amino acid alterations. *Drug Metab Pharmacokinet.* 2002; 17(6):561-5.

Koyano S, Kurose K, Saito Y, Ozawa S, Hasegawa R, Komamura K, Ueno K, Kamakura S, Kitakaze M, Nakajima T, Matsumoto K, Akasawa A, Saito H, Sawada J. Functional characterization of four naturally occurring variants of human pregnane X receptor (PXR):

one variant causes dramatic loss of both DNA binding activity and the transactivation of the CYP3A4 promoter/ enhancer region. *Drug Metab Dispos* 2004; 32:149–54.

Kurose K, Koyano S, Ikeda S, Tohkin M, Hasegawa R, Sawada J. 5' diversity of human hepatic PXR (NR1I2) transcripts and identification of the major transcription initiation site. *Mol Cell Biochem.* 2005; 273:79-85.

Lacher M, Kappler R, Schroepf S, Berkholz S, Ballauff A, Bufler P, Baurecht H, von Schweinitz D, Koletzko S. Nuclear pregnane X receptor single nucleotide polymorphism (-25385C/T) is not associated with inflammatory bowel disease in pediatric patients. *J Pediatr Gastroenterol Nutr*. 2009; 49(1):147-50.

Lamba J, Lamba V, Schuetz E. Genetic variants of PXR (NR1I2) and CAR (NR1I3) and their implications in drug metabolism and pharmacogenetics. *Curr Drug Metab.* 2005; 6:369–83.

Lamba J, Lamba V, Strom S, Venkataramanan R, Schuetz E. Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos.* 2008; 36(1):169-81.

Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S, Schuetz EG. PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators. *Toxicol Appl Pharmacol.* 2004; 199:251-65.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 1998; 102**:**1016-1023.

Li H, Chen T, Cottrell J, Wang H. Nuclear Translocation of Adenoviral-Enhanced Yellow Fluorescent Protein-Tagged-Human Constitutive Androstane Receptor (hCAR): A Novel Tool for Screening hCAR Activators in Human Primary Hepatocytes. *Drug metab dispos.* 2009; 37:1098-1106.

Li H, Wang H. Activation of xenobiotic receptors: driving into the nucleus. *Expert Opin Drug Metab Toxicol.* 2010 Jan 29. [Epub ahead of print]

Li Y, Wu YL. An over four millennium story behind qinghaosu (artemisinin)--a fantastic antimalarial drug from a traditional chinese herb. *Curr Med Chem.* 2003; 10(21):2197-230.

Lim YP, Liu CH, Shyu LJ, Huang JD. Functional characterization of a novel polymorphism of pregnane X receptor, Q158K, in Chinese subjects. *Pharmacogenet Genomics.* 2005; 15(5):337-41.

Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol.* 2002; 62:638-46.

Makanga M, Premji Z, Falade C, Karbwang J, Mueller EA, Andriano K, Hunt P, De Palacios PI. Efficacy and safety of the six-dose regimen of artemether-lumefantrine in pediatrics with uncomplicated Plasmodium falciparum malaria: a pooled analysis of individual patient data. *Am J Trop Med Hyg.* 2006; 74(6):991-8.

Mariga ST, Gil JP, Wernsdorfer WH, Björkman A. Pharmacodynamic interactions of amodiaquine and its major metabolite desethylamodiaquine with artemisinin, quinine and atovaquone in Plasmodium falciparum in vitro. *Acta Trop.* 2005; 93(3):221-31.

Martínez A, Márquez A, Mendoza J, Taxonera C, Fernández-Arquero M, Díaz-Rubio M, de la Concha EG, Urcelay E. Role of the PXR gene locus in inflammatory bowel diseases. *Inflamm Bowel Dis.* 2007; 13(12):1484-7.

Mårtensson A, Strömberg J, Sisowath C, Msellem MI, Gil JP, Montgomery SM, Olliaro P, Ali AS, Björkman A. Efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood Plasmodium falciparum malaria in Zanzibar, Tanzania. *Clin Infect Dis*. 2005; 41(8):1079-86.

Meshnick, SR. Artemisinin: mechanisms of action, resistance and toxicity. *Int J Parasitol.* 2002; 32:1655-60.

Meshnick SR, Alker AP. Amodiaquine and combination chemotherapy for malaria. *Am J Trop Med Hyg.* 2005; 73:821-3.

Meyer zu Schwabedissen HE, Kim RB. Hepatic OATP1B transporters and nuclear receptors PXR and CAR: interplay, regulation of drug disposition genes, and single nucleotide polymorphisms. *Mol Pharm.* 2009; 6(6):1644-61.

Mitra AK, Thummel KE, Kalhorn TF, Kharasch ED, Unadkat JD, Slattery JT. Metabolism of dapsone to its hydroxylamine by CYP2E1 in vitro and in vivo. *Clin Pharmacol Ther.* 1995; 58(5):556-66.

Modell W. Malaria and victory in Vietnam. The first battle against drug-resistant malignant malaria is described. *Science.* 1968; 162(860):1346-52.

Moore JT, Collins JL, Pearce KH. The nuclear receptor superfamily and drug discovery. *ChemMedChem.* 2006; 1(5):504-23.

Mwesigwa J, Parikh S, McGee B, German P, Drysdale T, Kalyango JN, Clark TD, Dorsey G, Lindegardh N, Annerberg A, Rosenthal PJ, Kamya MR, Aweeka F.Pharmacokinetics of artemether-lumefantrine and artesunate-amodiaquine in children in Kampala, Uganda. *Antimicrob Agents Chemother.* 2010; 54(1):52-9.

Na-Bangchang K, Karbwang J. Current status of malaria chemotherapy and the role of pharmacology in antimalarial drug research and development. *Fundam Clin Pharmacol.* 2009; 23(4):387-409.

Neiva A. Ueber die bildung einer chininresistenten rasse des malaria parsiten. *Mems. Inst. Oswaldo Cruz.* 1910; 2: 131-140.

Nelson, DR. Cytochrome P450 and the individuality of species. *Arch Biochem Biophys.* 1999; 369:1-10.

Nelson AL, Purfield A, McDaniel P, Uthaimongkol N, Buathong N, Sriwichai S, Miller RS, Wongsrichanalai C, Meshnick SR. pfmdr1 genotyping and in vivo mefloquine resistance on the Thai-Myanmar border. *Am J Trop Med Hyg.* 2005; 72(5):586-92.

Nicosia V, Colombo G, Consentino M, Di Matteo S, Mika F, De Sanctis S, Ratti S, Vinci M. Assessment of acceptability and ease of use of atovaquone/proguanil medication in subjects undergoing malaria prophylaxis. *Ther Clin Risk Manag.* 2008; 4(5):1105-10.

Nzila A. The past, present and future of antifolates in the treatment of Plasmodium falciparum infection. *J Antimicrob Chemother*. 2006; 57(6):1043-54.

Olefsky JM. Nuclear Receptor Minireview Series. *J Biol Chem*. 2001; 276:36863-36864.

Oleson L, von Moltke LL, Greenblatt DJ, Court MH. Identification of polymorphisms in the 3'-untranslated region of the human pregnane X receptor (PXR) gene associated with variability in cytochrome P450 3A (CYP3A) metabolism. *Xenobiotica.* 2010; 40(2):146-62.

Oliver P, Lubomirov R, Carcas A. Genetic polymorphisms of CYP1A2, CYP3A4, CYP3A5, pregnane/steroid X receptor and constitutive androstane receptor in 207 healthy Spanish volunteers. *Clin Chem Lab Med*. 2010; 48(5):635-9.

Ollomo B, Durand P, Prugnolle F, Douzery E, Arnathau C, Nkoghe D, Leroy E, Renaud F. A new malaria agent in African hominids. *PLoS Pathog.* 2009; 5(5):e1000446.

Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem.* 1964; 239:2370-8.

Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. II. solubilization, purification and properties. *J Biol Chem.* 1964; 239:2378-85.

Op den Buijsch RA, de Vries JE, Loots WJ, Landt O, Wijnen PA, van Dieijen-Visser MP, Bekers O.Genotyping of the PXR A11156C polymorphism with locked nucleic acid containing fluorogenic probes. *Pharmacogenomics J.* 2005;5(2):72-4.

Orans J, Teotico DG, Redinbo MR. The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges. *Mol Endocrinol.* 2005; 19:2891-900.

Owen BM, Van Mil SW, Boudjelal M, McLay I, Cairns W, Elias E, White R, Williamson C, Dixon PH. Sequencing and functional assessment of hPXR (NR1I2) variants in intrahepatic cholestasis of pregnancy. *Xenobiotica.* 2008; 38(10):1289-97.

Powell RD. Development of new antimalarial drugs. *Am J Trop Med Hyg.* 1972; 21(5):744-8.

Premji ZG. Coartem: the journey to the clinic. *Malar J.* 2009; 8:Suppl 1:S3.

Sachs JD. Achieving the Millennium Development Goals-the case of malaria. *N Engl J Med.* 2005; 352:115-7.

Sandanaraj E, Lal S, Selvarajan V, Ooi LL, Wong ZW, Wong NS, Ang PC, Lee EJ, Chowbay B. PXR pharmacogenetics: association of haplotypes with hepatic CYP3A4 and ABCB1 messenger RNA expression and doxorubicin clearance in Asian breast cancer patients. *Clin Cancer Res.* 2008; 14(21):7116-26.

Schlitzer M. Malaria chemotherapeutics part I: History of antimalarial drug development, currently used therapeutics, and drugs in clinical development. *ChemMedChem*. 2007; 2(7):944-86.

Siccardi M, D'Avolio A, Baietto L, Gibbons S, Sciandra M, Colucci D, Bonora S, Khoo S, Back DJ, Di Perri G, Owen A. Association of a single-nucleotide polymorphism in the pregnane X receptor (PXR 63396C-->T) with reduced concentrations of unboosted atazanavir. *Clin Infect Dis.* 2008; 47(9):1222-5.

Simonsson US, Lindell M, Raffalli-Mathieu F, Lannerbro A, Honkakoski P, Lang MA. In vivo and mechanistic evidence of nuclear receptor CAR induction by artemisinin. *Eur J Clin Invest.* 2006; 36(9):647-53.

Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. *Lancet.* 2004; 363(9414):1017-24.

Skinner TS, Manning LS, Johnston WA, Davis TM. In vitro stage-specific sensitivity of Plasmodium falciparum to quinine and artemisinin drugs. *Int J Parasitol.* 1996; 26:519-25.

Sookoian S, Castaño GO, Burgueño AL, Gianotti TF, Rosselli MS, Pirola CJ. The nuclear receptor PXR gene variants are associated with liver injury in nonalcoholic fatty liver disease. *Pharmacogenet Genomics.* 2010; 20(1):1-8.

Sonoda J, Xie W, Rosenfeld JM, Barwick JL, Guzelian PS, Evans RM. Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR). *Proc Natl Acad Sci U S A. 2002*; 99(21):13801-6.

Sonoda J, Pei L, Evans RM. Nuclear receptors: decoding metabolin disease. *FEBS Lett.* 2008; 582(1):2-9.

Synold TW, Dussault I, and Forman BM. The orphan nuclear receptor SXR co-ordinately regulates drug metabolism and efflux. *Nat Med* 2001; **7:**584–590.

Ter Kuile F, White NJ, Holloway P, Pasvol G, Krishna S. Plasmodium falciparum: in vitro studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. *Exp Parasitol.* 1993; 76:85-95.

Tirona RG, Kim RB. Nuclear receptors and drug disposition gene regulation. *J Pharm Sci.* 2005; 94:1169-86.

Tham LS, Holford NH, Hor SY, Tan T, Wang L, Lim RC, Lee HS, Lee SC, Goh BC. Lack of association of single-nucleotide polymorphisms in pregnane X receptor, hepatic nuclear factor 4alpha, and constitutive androstane receptor with docetaxel pharmacokinetics. *Clin Cancer Res.* 2007; 13(23):7126-32.

Uno Y, Sakamoto Y, Yoshida K, Hasegawa T, Hasegawa Y, Koshino T, Inoue I. Characterization of six base pair deletion in the putative HNF1-binding site of human PXR promoter. *J Hum Genet*. 2003; 48(11):594-7.

Ursing J, Schmidt BA, Lebbad M, Kofoed PE, Dias F, Gil JP, Rombo L. Chloroquine resistant P. falciparum prevalence is low and unchanged between 1990 and 2005 in Guinea-Bissau: an effect of high chloroquine dosage? *Infect Genet Evol.* 2007; 7(5):555-61.

Vale N, Moreira R, Gomes P. Primaquine revisited six decades after its discovery. Eur J Med Chem. 2009; 44(3):937-53.

Wang D, Sadée W. Searching for polymorphisms that affect gene expression and mRNA processing: example ABCB1 (MDR1). *AAPS J.* 2006; 8(3):E515-20.

Wang P, Wang Q, Aspinall TV, Sims PF, Hyde JE. Transfection studies to explore essential folate metabolism and antifolate drug synergy in the human malaria parasite Plasmodium falciparum. *Mol Microbiol.* 2004; 51:1425-38.

Wang XD, Deng XY, Chen J, Li JL, Chen X, Zhao LZ, Lu Y, Chowbay B, Su QB, Huang M, Zhou SF. Single nucleotide polymorphisms of the pregnane x receptor gene in Han Chinese and a comparison with other ethnic populations. *Pharmacology*. 2008; 81(4):350-4.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science.* 2001; 292:2329-33.

Wei P, Zhang J, Dowhan DH, Han Y, Moore DD. Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. Pharmacogenomics J 2002; 2:117-126.

White NJ. Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. *Antimicrob Agents Chemother*. 1997; 41:1413-22.

White NJ. Delaying antimalarial drug resistance with combination chemotherapy. *Parasitologia.* 1999; 41(1-3):301-8.

White NJ. Plasmodium knowlesi: the fifth human malaria parasite. *Clin Infect Dis.* 2008; 46:172-173.

WHO. World Malaria Report. World Health Organization. 2006.

WHO. World Malaria Report. World Health Organization. 2009.

WHO. Guidelines for the treatment of malaria, second edition. Geneva. World Health Organization. 2010

Wolbold R, Klein K, Burk O, Nussler AK, Neuhaus P, Eichelbaum M, Schwab M, Zanger UM. Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* 2003; 38:978-988.

Yuan J, Liu L, Shimada M, Wang A, Ruhnke M, Heeckt P, Muller AR, Nussler NC, Neuhaus P, Nussler A. Induction, expression and maintenance of cytochrome P450 isoforms in long-term cultures of primary human hepatocytes. *ALTEX* 2004; 21 Suppl 3:3-11.

Young MD. Amodiaquine and hydroxychloroquine resistance in Plasmodium falciparum. *Am J Trop Med Hyg.* 1961; 10:689-93.

Young MD. Contacos PG, Stitcher JE, Millar JW. Drug resistance in plasmodium falciparum from Thailand. *Am J Trop Med Hyg.* 1963; 12:305-14.

Zhang J, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Wrighton SA, Hancock M, Kim RB, Strom S, Thummel K, Russell CG, Hudson JR Jr, Schuetz EG, Boguski MS. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics.* 2001; 11:555-72.

Zanger UM, Turpeinen M, Klein K, Schwab M. Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem.* 2008; 392(6):1093-108

APPENDIX1: DESCRIPTION OF THE POLYMORPHISM DESCRIBED IN PUBLICATIONS IN *PXR*, UNTIL THE PRESENT MOMENT

Allele	SNP Position in the Sequence AF364606 (GeneBank) (Position +1, translatiom starting site)	Rs numbering	Position in the gene	effect	Functional consequence	Population studied and allelic frequency	Reference
	-33045 G>A		upstream			C=0.01	Lamba *et al.* 2008
	-32911 T>C		upstream			PDR=0.10	Lamba *et al.* 2008
	-31539 G>A		upstream			PDR=0.02	Lamba *et al.* 2008
	-31408 G>A	r4688036	upstream			PDR=0.08	Lamba *et al.* 2008
	-31333 G>A		upstream			PDR=0.04	Lamba *et al.* 2008
	-31273 A>G	r9832958	upsteram			PDR=0.22	Lamba *et al.* 2008
	-26966 C>T		upstream			AA=0.16, C=0.00, AS=0.00	King *et al.* 2007
	-26612 T>C	rs4688038	upstream			AA=0.43, C=0.00, AS=0.00	King *et al.* 2007
	-26479 T>C	rs9813490	upstream			AA=0.03, C=0.00, AS=0.00	King *et al.* 2007
	-26405 C>T	rs4687883	upstream			AA=0.06, C=0.00, AS=0.00	King *et al.* 2007
	-25913 T>C	rs1523130	upstream	STAT1, 3, 6, NFAT sites lost in Tallele	Increase Basal CYP3A4, and decrease in its induction in hepatocytes	AA=0.28, C=0.70, AS=0.67; PDR=0.45, C=0.27; C=0.55	King *et al.* 2007; Lamba *et al.* 2008; Siccardi *et al.* 2008
	-25812 G>A	rs1523129	upstream			AA=0.16, C=0.00, AS=0.01; PDR=0.06	King *et al.* 2007; Lamba *et al.* 2008
	-25726 C>A					PDR=0.02	Lamba *et al.* 2008
	-25564 G>A		upstream	HSTF COMP1		C=0.01, AA=0.09 ; IE=0.018(IBD), IE=0.030 (Controls); C*=0.03	Zhang *et al.* 2001; Dring *et al.* 2006; Bosch *et al.* 2006
	-25385 C>T	rs3814055	upstream	NFKB, ISGF-3	Rif induced ERMBT and fold induction after rif: TT>CC; Frequency of the minor allele is decreased in IBD; Decrease of PXR mRNA in males	C=0.39, AA=0.32; IE=0.321 (IBD), IE=0.433 (Controls); C*=0.36; AA=0.34; C=0.50; AS=0.21; ES=0.38 PDR=0.29, C=0.31; CN=0.17; MY=0.21; IN=0.33; FR*=0.40 C=0.405, C(IBD)=0.356; ES=0.41 AR=0.39	Zhang *et al.* 2001; Dring *et al.* 2006; Bosch *et al.* 2006; King *et al.* 2007; Martinez *et al.* 2007; Lamba *et al.* 2008; Sandanaraj *et al.* 2008; Khaled *et al.* 2009; Lacher *et al.* 2009; Oliver *et al.* 2010; Sookian *et al.* 2010
	-25048 C>G	rs12721614	upstream			C=0.01, AA=0.00; C*=0.00	Zhang *et al.* 2001; Bosch *et al.* 2006
	-25036 G>C		5'UTR			PDR=0.02	Lamba *et al.* 2008

	-25003 C>T		Upstream (5'UTR)			PDR=0.02	Lamba *et al*. 2008
	-24755 C>T					AA=0.02, C=0.00, AS=0.00	King *et al*. 2007
	-24756 G>A	rs1523128	upstream	C/EBP, HNF1		C=0.01, AA=0.14; IE= 0.003(IBD), IE=0.004 (Controls); C*=0.01 AA=0.13, C=0.00, AS=0.00: PDR=0.08	Zhang *et al.* 2001; Dring *et al*. 2006; Bosch *et al*. 2006; King *et al*. 2007; Lamba *et al*. 2008
	-24737 A>G		upstream	GBF		C=0.01, AA=0; C*=0.005	Zhang *et al.* 2001; Bosch *et al*. 2006
	-24719 C>G		upstream	PR		C=0.01, AA=0; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	-24586 T>C		upstream			C=0.01, AA=0; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	-24462G>A		5'UTR			C=0.01, AA=0; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	-24381 A>C	rs1523127	5'UTR		Frequency of the minor allele is decreased in IBD; No association with UC, CD, or IBD; Associated with ABCB1 and CYP3A4 mRNA expression in colon tumor.	C=0.41, AA=0.73; IE=0.357(IBD), IE=0.452(Controls); C*=0.54; SF=0 AA=0.50, C=0.69, AS=0.33; SG*=0.26; CA= CN=0.26, MY=0.13, IN=0.57; CN=0.17, MY=0.26, IN=0.35	Zhang *et al.* 2001; Dring *et al.* 2006; Bosch *et al*. 2006; Ho *et al*. 2006; King *et al*. 2007; Tham *et al.* 2007; Amre *et al*. 2008; Hor *et al.* 2008* Sandaranaj *et al.* 2008
	-24113 G>A	rs2276706	Intron1		Rif induced ERMBT and fold induction after rif: AA>GG	C=0.39, AA=0.32 AA=0.32, C=0.41, AS=0.28; CN=0.18, MY=0.23, IN=0.32	Zhang *et al.* 2001; King *et al*. 2007; Sandaranaj *et al.* 2008
PAR.2*2	-24020 6bp del	rs3842689	5'UTR	HNF1 site lost with deletion	Complete loss of PAR2 promoter activity in HepG2 cells	J= 0.27; SK=0.40; C=0.29 (D)	Uno *et al*. 2003; Karlsen *et al.* 2006; Lamba *et al*. 2008;
	-23839 Cins	rs11421631	5'UTR	C insertion present in SRE site		C=0.26(6C)	Lamba *et al*. 2008
	-23700 C>T					J=0.024	Koyano *et al.* 2002
	-23350C>	rs12488820				AR=0.42	Sookian *et al.* 2010
	-22373 C>T	rs2472671				AR=0.14	Sookian *et al.* 2010
	-20349 A>T	rs4234666	Intron1			PDR=0.09	Lamba *et al*. 2008
	-20102 G>C		Intron1			PDR=0.23	Lamba *et al.* 2008
	-19744 C>T	r4472074	Intron1			PDR=0.08	Lamba *et al*. 2008
	-19724 A>G		Intron1			PDR=0.23	Lamba *et al.* 2008
	-19715 insC	r11409387	Intron1			PDR=0.23 (n)	Lamba *et al.* 2008
	-19118 A>G		Intron1			PDR=0.02	Lamba *et al*. 2008
	-19043 C>A		Intron1			PDR=0.02	Lamba *et al*. 2008
	-18835 del	r3030845	Intron1			PDR=0.26 (n)	Lamba *et al.* 2008
	-18500 G>T		Intron1			PDR=0.05	Lamba *et al.* 2008
	-18445 (TTA ins)		Intron1			PDR=0.10 (TTA/5)	Lamba *et al*. 2008
	-18433 C>A		Intron1	Repeat is present in FOXJ2 site		PDR=0.02	Lamba *et al*. 2008
	-18432 G>A		Intron1			PDR=0.02	Lamba *et al*. 2008

	-18334 C>A		Intron1			PDR=0.02	Lamba *et al.* 2008
	-17910 C>T		Intron1			PDR=0.02	Lamba *et al.* 2008
	-17889 C>T	r4566573	Intron1	SNP present in DR4 site		PDR=0.02	Lamba *et al.* 2008
	-17785 T>C		Intron1			C=0.03	Lamba *et al.* 2008
	-14974 A>G		Intron1			PDR=0.10, C=0.99	Lamba *et al.* 2008
	-14882 C>T		Intron1			C=0.01	Lamba *et al.* 2008
	-14555 T>C		Intron1	PPARγ site gained in C allele		C=0.01	Lamba *et al.* 2008
	-14479 T>A		Intron1			C=0.02	Lamba *et al.* 2008
	-14438 G>C		Intron1	SNP present in DR4 site		PDR=0.04, C=0.02	Lamba *et al.* 2008
	-14260 C>G		Intron1			C=0.01	Lamba *et al.* 2008
	-14165 G>C		Intron1			PDR=0.02, C=0.00	Lamba *et al.* 2008
	-14080 T>G		Intron1			C=0.01	Lamba *et al.* 2008
	-14042 C>A		Intron1	SNP present in DR3 site	Increase basal CYP3A4 and decrease in its fold induction	PDR=0.44, C=0.29	Lamba *et al.* 2008
	-13451 A>G		Intron1			PDR=0.02	Lamba *et al.* 2008
	-13389 C>T		Intron1			PDR=0.02	Lamba *et al.* 2008
	-13354 C>G		Intron1			PDR=0.08	Lamba *et al.* 2008
	-13043 C>T		Intron1			PDR=0.04	Lamba *et al.* 2008
	-12838 G>A		Intron1			PDR=0.08, C=0.02	Lamba *et al.* 2008
	-12780 C>G		Intron1			PDR=0.02	Lamba *et al.* 2008
	-12665 T>C		Intron1			PDR=0.02	Lamba *et al.* 2008
	-12585 A>G	rs6773295	Intron1			PDR=0.10, C=0.02	Lamba *et al.* 2008
	-12565 G>T	rs6438545	Intron1			PDR=0.10, C=0.02	Lamba *et al.* 2008
	-12554 A>T		Intron1			PDR=0.02	Lamba *et al.* 2008
	-12285 T>G		Intron1			C=0.01	Lamba *et al.* 2008
	-12202 T>C	rs13085558	Intron1		Increase in basal CYP3A4 activity and decrease in its inducibility	PDR=0.10, C=0.09	Lamba *et al.* 2008
	-12156 G>A		Intron1			PDR=0.04	Lamba *et al.* 2008
	-11752 G>A		Intron1			PDR=0.08, C=0.03	Lamba *et al.* 2008
	-11540 A>T		Intron1	HNF4 site lost in T allele		PDR=0.06	Lamba *et al.* 2008
	-11443 G>A		Intron1			PDR=0.02, C=0.01	Lamba *et al.* 2008
	-11413 C>T		Intron1			PDR=0.28	Lamba *et al.* 2008
	-11381 G>T		Intron1			PDR=0.36	Lamba *et al.* 2008
	-9513 T>C		Intron1	HEB site gained in C allele		PDR=0.21, C=0.03	Lamba *et al.* 2008
	-7945 G>A		Intron1	SNP present in HNF4α and NF1 sites		PDR=0.09, C=0.02	Lamba *et al.* 2008
	-7941 A>G		Intron1	SNP present in NF1 site		PDR=0.09, C=0.02	Lamba *et al.* 2008
	-7938 A>T		Intron1	SNP present in NF1 site		PDR=0.09, C=0.02	Lamba *et al.* 2008
	-7930 C>A		Intron1			PDR=0.09, C=0.02	Lamba *et al.* 2008
	-7924 A>G		Intron1			PDR=0.17, C=0.04	Lamba *et al.* 2008

	-7923 A>G		Intron1			PDR=0.09, C=0.02	Lamba *et al*. 2008
	-6994 C>T	rs2472677	Intron1	SNP present in HNF3β site	Increases CYP3A4 activity and decrease in its fold induction; Increase in PAR2 and PXR mRNA; Correlated with reduced concentrations of atazanavir	PDR=0.35, C=0.62; C=0.66	Lamba *et al*. 2008; Siccardi *et al.* 2008
	-6942 C>A		Intron1			C=0.01	Lamba *et al*. 2008
	-6827 T>G		Intron1			PDR=0.06	Lamba *et al*. 2008
	-6686 A>G	rs12492296	Intron1			PDR=0.35, C=0.62	Lamba *et al*. 2008
	-6577(CAAA)5/6. (CA) 12/14	rs4267673, rs7372335	Intron1	HNF3β site gained by (CAAA)CA insertion		PDR= 0.42 (6/13), C=0.38 (6/12)	Lamba *et al*. 2008
	-6513 T>C	rs6438546	Intron1			PDR=0.36, C=0.37	Lamba *et al*. 2008
	-5286 T>C	rs2461823				SF=0.62; AR=0.44	Ho *et al*. 2006 Sookian *et al*. 2010
	-4356 T>C	rs13059232	Intron1			PDR=0.44, C=0.65	Lamba *et al*. 2008
	-4129 G>A		Intron1			PDR=0.041	Lamba *et al*. 2008
	-2228 G>T	rs2416818	Intron1			PDR=0.12	Lamba *et al*. 2008
	-2009 G>T	rs4688040	Intron1	SNP present in HNF3α site		PDR=0.46, C=0.21	Lamba *et al*. 2008
	-1934 G>T		Intron1			C=0.20	Lamba *et al*. 2008
	-1855 G>A		Intron1			PDR=0.40	Lamba *et al*. 2008
	-1797 T>G		Intron1			PDR=0.02	Lamba *et al*. 2008
	-1772 T>C		Intron1	DR4 site lost in C allele		PDR=0.02	Lamba *et al*. 2008
	-1683G>A	rs4688041	Intron1			PDR=0.08	Lamba *et al*. 2008
	-1650 T>A	rs2472679	Intron1		Decrease in basal CYP3A4 and increase in its inducibility	PDR=0.08, C=0.04	Lamba *et al*. 2008
	-1586 C>T		Intron1			PDR=0.02	Lamba *et al*. 2008
	-1495 C>A		Intron1			PDR=0.02	Lamba *et al*. 2008
	-1447 C>A	rs2461817	Intron1	SNP present in DR3 and C/EBPγ sites		PDR=0.35, C=0.25	Lamba *et al*. 2008
	-1145 C>T		Intron1	CREB site lost in T allele		C=0.05	Lamba *et al*. 2008
	-977 G>A		Intron1			PDR=0.02	Lamba *et al*. 2008
	-902 A>G		Intron1			PDR=0.04, C=0.05	Lamba *et al*. 2008
	-626 G>T		Intron1			PDR=0.13, C=0.36	Lamba *et al*. 2008
	-601 A>G	rs7643645	Intron1	HNF4 site lost in G allele	Decreased CYP3A4 actiity and increase in its fold induction. Decrease in MDR, 3A4 and PXR mRNA	SF=0.37; PDR=0.55, C=0.36; C=0.37; AR=0.47	Ho *et al*. 2006; Lamba *et al*. 2008; Siccardi *et al*. 2008; Sookian *et al.* 2010;
	-114G>A		Intron1			J=0.0024	Koyano et al. 2002
	-40T>C		Intron1			C=0.00, AA=0.05; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	-42T>C	rs12721597	Intron1			C=0.00, AA=0.23; AA=0.10, C=0.00, AS=0.063	Zhang *et al.* 2001; King *et al*. 2007
	23 G>C			S8T		AA=0.03, C=0.00, AS=0.00	King *et al*. 2007
PXR*9	52 G>A		Exon2	E18K	same as WT in transactivation	C=0.0, AA=0.014;	Husert *et al.* 2001;

					assays, basal and rif and corticosterone activation	C*=0.00; CN=0.00; ZZ=0.00	Bosch *et al*. 2006; Wang *et al*. 2008; Ferreira *et al.* 2008
PXR*2	79 C>T	rs12727613	Exon2	P27S, DBD	Hepatic CYP3A4 not different.	C=0.0, AA=0.20 ; C=0.0, AA=0.15; C*=0.00; AA=0.03, C=0.00, AS=0.00; C(ICP)=0.006; CN=0.00; ZZ=0.112	Zhang *et al.* 2001; Hustert *et al*. 2001; Bosch *et al*. 2006; King *et al*. 2007; Owen *et al.* 2008; Wang *et al.* 2008; Ferreira *et al.* 2008
PXR*3	106 G>A		Exon2	G36R, DBD	same as WT in transactivation assays for basal and rif but 40 % increase with corticosterone (Hustert et al. 2001)	C=0.01, AA=0.03; C=0.03, AA=0.0; IE=0.016(IBD), IE=0.031(Controls); C*=0.01; C(ICP)=0.035; CN=0.00; ZZ=0.00	Zhang *et al.* 2001; Hustert *et al.* 2001; Dring *et al.* 2006; Bosch *et al*. 2006; Owen *et al.* 2008; Wang *et al.* 2008; Ferreira *et al.* 2008
	252 A>G	rs1464603	Intron2			C=0.28, AA=0.64; C*=0.34; SF=0.31; SK=0.36; AA=0.76, C=0.25, AS=0.40; CN=0.41, MY=0.49, IN=0.38	Zhang *et al.* 2001; Bosch *et al*. 2006; Ho *et al*. 2006; Karlsen *et al.* 2006; King *et al*. 2007; Sandaranaj *et al.* 2008
	275 A>G	rs144602	Intron2			C*=0.34; AA=0.77, C=0.28, AS=0.34; CN=0.40, MY=0.47, IN=0.38	Bosch *et al*. 2006; King *et al*. 2007; Sandaranaj *et al.* 2008
	308 A>T		Intron2			AA=0.00, C=0.00, AS=0.03	King *et al*. 2007
	2781C>T		Intron2			C=0.01, AA=0.05; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
PXR*5	2904 C>T		Exon3	R98S	Loss of DNA binding and transactivation in HepG2 cells; reduced transactivation of the CYP3A4 promoter in response to RIF, LCA, UDCA and dexamethasone	J=0.0024	Koyano *et al*. 2002, 2004; Owen *et al*. 2008
	2954 G>C		Intron 3			J=0.0024	Koyano et al. 2002
	3015 T>G		Intron3			C=0.02, AA=0.09; C*=0.06; CN=0.09, MY=0.13, IN=0.11	Zhang *et al.* 2001; Bosch *et al*. 2006; Sandaranaj *et al.* 2008
	4274 C>G		Intron3			C*=0.025	Bosch *et al*. 2006
PXR*4	4321 G>A		Exon4	R122Q, conserved	Reduced affinity in EMSA for PXR binding sequence, Reduced ligand activation in transient transfection assays	C=0.01, AA=0.0; C*=0.00; SK=0.00; CN=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006; Karlsen *et al.* 2006; Wang *et al.* 2008
PXR*10	G4374A		Exon4	V140M	50 % lower protein in LS174 T cells, Increase in basal transctivation activity in LS174 T cells, reduced induction by rif and corticosterone	C=0.002, AA=0.0; CN=0.006; C*=0.00; CN=0.00	Husert *et al.* 2001; Lim *et al.* 2005; Bosch *et al*. 2006; Wang *et al.* 2008
PXR*6	4399 G>A		Exon4	R148Q	similar transactivation as WT	J=0.0024;	Koyano *et al.* 2002, 2004;

						CN=0.00	Wang *et al.* 2008
	4418 C>A		Exon4	Q158K	The variant gave rise to much lower levels of CYP3A4 promoter activity in LS174T and HepG2 cells exposed to the PXR ligands, rifampin and paclitaxel; reduced transactivation of the CYP3A4 promoter in response to RIF, LCA, UDCA and dexamethasone	CN=0.022	Lim *et al* 2005; Owen *et al.* 2008
PXR*11	4444 A>G		Exon4	D163G	Complete loss of basal transctivation activity in LS174 T cells, reduced induction by corticosterone, but promoter dependent enhanced induction by rif	C=0.0, AA=0.014 ; CN=0.00	Husert *et al.* 2001; Wang *et al.* 2008
	4448 T>C		Exon4	Syn		C=0.01, AA=0.05; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	4760 G>A		Intron4			C*=0.685; CN=0.65, MY=0.54, IN=0.61	Bosch *et al*. 2006; Sandaranaj *et al.* 2008
	5210 C>T		Intron4			C=0.00, AA=0.05	Zhang *et al.* 2001
	5458 C>T		Exon5	Syn		C=0.01, AA=0.0; C*=0.00; ZZ=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006; Ferreira *et al.* 2008
	5611 C>T		Exon5	Syn		C*=0.01	Bosch *et al*. 2006
	5761A>G		Intron5			J=0.0073	Koyano *et al*. 2002
	6554 A>C	rs2472682	Intron5			SF=0.67	Ho *et al*. 2006
	7158 C>G	rs6784598	Intron5			ES=0.43	Martinez *et al.* 2007
	7635 A>G	rs6785049	Intron5		Rif induced intestinal CYP3A4 protein : GG>AA; Frequency of the minor allele is decreased in IBD	C=0.35, AA=0.77; IE=0.340 (IBD), IE=0.406 (Controls); C*=0.32; SK=0.64; AA=0.80, =0.33, AS=0.48; CN=0.41, MY=0.24, IN=0.54; AR=0.37	Zhang *et al.* 2001; Dring *et al.* 2006; Bosch *et al*. 2006; Karlsen *et al.* 2006; King *et al*. 2007; Sandaranaj *et al.* 2008; Sookian *et al.* 2010
	7637 C>T		Intron5			J=0.0024	Koyano *et al.* 2002
	7675 C>T	rs6797879	Intron5			C=0.04, AA=0.05; C*=0.05; AA=0.80, C=0.33, AS=0.48	Zhang *et al.* 2001; Bosch *et al*. 2006; King *et al*. 2007
	7683 C>T		Intron5			C=0.01, AA=0.00; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	7767 G>A		Exon6	Syn		C=0.01, AA=0.05; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	8055 C>T	rs2276707	Intron6		Rif induced intestinal CYP3A4 protein : TT, TC>CC	C=0.15, AA=0.18; I E=0.187 (IBD), IE=0.142 (Controls); C*=0.16; AA=0.20, C=0.39, AS=0.51; ES=0.19; CN=0.46, MY=0.44, IN=0.25	Zhang *et al.* 2001; Dring *et al.* 2006; Bosch *et al*. 2006; King *et al*. 2007; Martinez *et al.* 2007; Sandaranaj *et al.* 2008
	8224 C>T;	rs12721604	Intron7			C= 0.01, AA=0.05;	Zhang *et al.* 2001;

	8224 C/T/A					C*=0.00; AA=0.02T 0.00A, C=0.00T 0.08A, AS=0.00T 0.00A	Bosch *et al*. 2006; King *et al*. 2007
	8357 C>G		Intron7			C=0.02, AA=0.00; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
PXR*12	8528 G>A		Exon8	A370T	Increase in basal transactivation activity in LS174T cells, reduced induction by Rif and corticosterone	C=0.0, AA=0.016	Husert *et al.* 2001
	8555T>G		Exon8	C379G		C*=0.005	Bosch *et al*. 2006
PXR*7	8561 C>T		Exon8	R381W	Ligand dependent reduced transactivation ; reduced transactivation of the CYP3A4 promoter in response to RIF, LCA, UDCA and dexamethasone	J=0.0024	Koyano *et al*. 2002, 2004; Owen *et al*. 2008
	8677 G>A		Intron8			C=0.01, AA=0.00; C*=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006
	9148T>C	rs12721611	Intron8			AA=0.03, C=0.00, AS=0.00	King *et al*. 2007
	9811 A>C		Intron8			C*=0.005	Bosch *et al*. 2006
PXR*8	9863 A>G		Exon9	I403V	Ligand dependent reduced transactivation; ; reduced transactivation of the CYP3A4 promoter in response to RIF, LCA, UDCA	J=0.0024	Koyano *et al*. 2002, 2004;
	9976 G>A	rs3732358	3'UTR			C= 0.00, AA=0.36; AA=0.05, C=0.00,AS= 0.02; CN=0.005, MY=0.01, IN=0.01	Zhang *et al.* 2001; King *et al*. 2007; Sandaranaj *et al.* 2008
	9987 G>A		3'UTR			J=0.03; CN=0.02, MY=0.00, IN=0.00	Koyano *et al.* 2002; Sandaranaj *et al.* 2008
	10058 C>G		3'UTR			C*=0.005	Bosch *et al*. 2006
	10331 A>G	rs3732359	3'UTR		Heterozygous showed 80% higher Oral MDZ clearance compared with wt homozygous.	C*=0.20; AA=0.69, C=0.33, AS=0.59; CN=0.43, MY=0.34, IN=0.60; C=0.77, AA=0.18	Bosch *et al*. 2006; King *et al*. 2007; Sandaranaj et al. 2008; Oleson *et al*. 2009
	10461 C>A	rs10511395	3'UTR			C*=0.12; AA=0.06, C=0.00, AS=0.10; C=0.13, AA=0.07	Bosch *et al*. 2006; King *et al*. 2007; ; Oleson *et al.* 2009
	10477 G>T	rs61760364	3'UTR			C=0.01, AA=0.00	Oleson *et al*. 2009
	10483 T>C	rs3732360	3'UTR		Carries of the Polymorphism showed 80% higher Oral MDZ clearance compared with wt homozygous.	C*=0.24; AA=0.69, C=0.23, AS=0.65; CN=0.43, MY=0.34, IN=0.60; C=0.74, AA=0.18	Bosch *et al*. 2006; King *et al*. 2007; Sandaranaj *et al.* 2008; Oleson *et al*. 2009
	10552 T>G					AA=0.03, C=0.00, AS=0.00	King *et al*. 2007
	10620 C>T	rs1054190	3'UTR		Hepatocytes: 6-beta testosterone Hydroxylation following Rif: TT, TC<CC	C=0.11, AA=0.14; C*=0.08 SK=0.13; AA=0.03, C=0.01, AS=0.00; C=0.11, AA=0.00	Zhang *et al.* 2001; Bosch *et al*. 2006; Karlsen *et al.* 2006; King *et al*. 2007; Oleson *et al*. 2009
	10719 A>G	rs6438550	3'UTR			C=0.05, AA=0.00;	Zhang *et al.* 2001;

						C*=0.05 AA=0.18, C=0.00, AS=0.00; CN=0.09, MY=0.21, IN=0.17; C=0.05, AA=0.14	Bosch *et al*. 2006; King *et al*. 2007; Sandaranaj *et al*. 2008 Oleson *et al*. 2009
	10799 G>A	rs1054191	3'UTR		Hepatocytes: 6-beta-testosterone Hydroxylation following Rif: AA, AG<GG	C=0.13, AA=0.14; C*=0.12; AA=0.06, C=0.05, AS=0.00; C=0.11, AA=0.00; AR=0.78	Zhang *et al.* 2001; Bosch *et al*. 2006; King *et al*. 2007; Oleson *et al*. 2009; Sookian *et al.* 2010
	10876 C>T		3'UTR			C=0.02, AA=0.00	Oleson *et al*. 2009
	11073 C>A		3'UTR			C=0.01, AA=0.00	Zhang *et al.* 2001
	11113 G>T		3'UTR			C=0.10, AA=0.00	Oleson *et al*. 2009
	11125 A>G	rs12721615	3'UTR			C=0.01, AA=0.00; C=0.01, AA=0.06	Zhang *et al.* 2001; Oleson *et al*. 2009
	11156 A>C	rs3814057	3'UTR		Intestinal Pgp protein: CC, AC< AA	C=0.16, AA=0.33; NL=0.18; IE=0.164 (IBD), IE=0.151 (Controls) AA=0.43 , C=0.09, AS=0.50 ; CN=0.46, MY=0.42, IN=0.24; CN=0.55; C=0.17, AA=0.62; ES=0.17 AR=0.17	Zhang *et al.* 2001; Op den Buijsch et al. 2005; Dring *et al.* 2006; King *et al*. 2007; Sandaranaj *et al.* 2008; Wang *et al.* 2008; Oleson *et al*. 2009; Oliver *et al.* 2010; Sookian *et al.* 2010
	11193 T>C	rs3814058	3'UTR		Intestinal Pgp protein: CC, TC< TT	C=0.16, AA=0.30; SK=0.16; AA=0.54, C=0.14, AS=0.53; CN=0.46, MY=0.42, IN=0.24; CN=0.55; C=0.17, AA=0.62; ES=0.17	Zhang *et al.* 2001; Karlsen *et al.* 2006; King *et al*. 2007; Sandaranaj *et al.* 2008; Wang *et al.* 2008; Oleson *et al.* 2009; Oliver *et al*. 2010;
	11255C>T	rs2461826	3'UTR			AA=0.04, C=0.00, AS=0.00	King *et al*. 2007
	11643G>A		3'UTR			AA=0.24, C=0.00, AS=0.06	King *et al*. 2007
	12026 A>G		3'UTR			AA=0.00, C=0.00, AS=0.04	King *et al*. 2007

Adapted from Lamba et al. 2005: Populations C=Caucasians; C*= population composed of 93% Caucasians; J=Japanese; AA=African-American; CN=Chinese; IE=Ireland; SF=Scotland; AS= Asians; PDR=Samples from a set of Polymorphism discovery resource purchased from Coriell DNA repository; ICP= Intrahepatic cholestasis of pregnancy; ZZ=Zanzibar; SK= Scandinavian; MY=Malay; IN=Indian; AR=Argentineans with "self reported" European ancestry ; FR*=French patients with renal transplant; SG*=Singapore (Patients with Breast cancer); NL=Netherland; ES=Spain

IBD=Inflammatory bowel disease

APPENDIX2: LIST OF ALL *PXR* SNPS ANALYSED BY RE-SEQUENCING IN THE VIETNAMESE POPULATION

						Allele frequency[c]		Genotypic Frequencies[c]		
N°	**Position[a]**	**Aminoacid change**	**Identifier**	**Sequence**	**n[b]**	**wt**	**mut**	**wt/wt**	**wt/mut**	**mut/mut**
1	**-25564 G>A**			GATAGA[G/A]AAGAAA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
2	**-25385 C>T**		rs3814055	CAGGTT[C/T]TCTTTT	75	0.83 (0.76-0.88)	0.17 (0.12-0.24)	0.69 (0.58-0.79)	0.28 (0.19-0.39)	0.03 (0.00-0.10)
3	**-25036 G>C**			TGGTCA[G/C]CCTTCT	73	1.00 (0.97 - 1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
4	**-25003 C>T**			GGGTCA[C/T]ATTCTC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
5	**- 24910 G>A**			TGATTG[G/A]CACCGT	75	0.99 (0.96-0.99)	0.01 (0.00-0.04)	0.99 (0.92-0.99)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
6	**-24756 G>A**		rs1523128	AAATGC[G/A]CTCAGA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
7	**-24737 A>G**			TAGACA[G/A]AGCGGA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
8	**-24719 C>G**			GCCACT[C/G]TCTTTC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
9	**-24462 G>A**			ACTTCA[G/A]TGGGAA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)

10			rs2276705 C>A	CGGCCT[C/A]AGCCTG	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
11	**-24381 A>C**		rs1523127	CCTGAA[A/C]AAGGCA	75	0.83 (0.76-0.88)	0.17 (0.12-0.24)	0.69 (0.58-0.79)	0.28 (0.19-0.39)	0.03 (0.00-0.10)
12			rs7623520 C>T	AGGCAG[C/T]GGCTCC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
13	**-23925 C>T**			TACCAC[C/T]TCCAAG	56	0.99 (0.94-1.00)	0.01 (0.00-0.05)	0.98 (0.89-1.00)	0.02 (0.00-0.10)	0.00 (0.00-0.08)
14	**-23913 T>G**		rs3814056	GGACTG[T/G]GGGAGC	56	0.71 (0.61-0.78)	0.29 (0.22-0.38)	0.48 (0.36-0.61)	0.45 (0.32-0.58)	0.07 (0.02-0.17)
15	**-23700C>T**			CTCTAC[C/T[ATTGAA	56	0.00 (0.00-0.04)	1.00 (0.96-1.00)	0.00 (0.00-0.08)	0.00 (0.00-0.08)	1.00 (0.92-1.00)
16	**23G>C**	S8T		AAGAAA[G/C]CTGGAA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
17	**34G >A**	A12T	rs1063955	AACCAT[G/A]CTGACT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
18	**52 G>A**	E18K	rs59371185	CACTGT[G/A]AGGACA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
19	**79 C>T**	P27S	rs12727613	GGAAAG[C/T]CCAGTG	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
20	**106 G>A**	G36R		GAAGTC[G/A]GAGGTC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
21	**252 A>G**		rs1464603	GGTAAC[A/G]TCTCAG	75	0.55	0.45	0.29	0.51	0.20

						(0.47-0.62)	(0.38-0.53)	(0.20-0.41)	(0.40-0.62)	(0.12-0.30)
22	**275 A>G**		rs144602	TGACCT[A/G]TCCCCC	75	0.57 (0.49-0.65)	0.43 (0.35-0.51)	0.32 (0.23-0.43)	0.51 (0.40-0.62)	0.17 (0.10-0.28)
23	**308 A>T**			TGGCCC[A/T]CCCAAA	75	0.99 (0.96-1.00)	0.01 (0.00-0.04)	0.99 (0.92-1.00)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
24	**2781C>T**			GTGCAT[C/T]CCCCCT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
25	**2904 C>T**	R98S		GCCTGC[C/T]GCCTGC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
26	**2939 G>T**	N109K	rs1140968	GAAGAA[T/G]GAGAGT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
27	**2954 G>C**			GCAGTG[G/C]GCGCGC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
28	**3015 T>G**			GGTGTG[T/G]GCATGC	61	0.84 (0.76-0.89)	0.16 (0.11-0.24)	0.74 (0.62-0.83)	0.19 (0.12-0.32)	0.07 (0.02-0.16)
29	**4321 G>A**	R122Q	rs12721608	AGAGGC[G/A]GGCCTT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
30	**4374 G>A**	V140M		CTGGGA[G/A]TGCAGG	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
31	**4399 G>A**	R148Q		AGCAGC[G/A]GATGAT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
32	**4418C>A**	Q158K		GACGCT[C/A]AGATGA	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)

33	**4444A>G**	D163G		CCTTTG[A/G]CACTAC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
34	**4448T>C**	Syn	rs12721611	TGACAC[T/C]ACCTTC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
35	**5458C>T**	Syn		CAGTGG[C/T]TGCGAG	67	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.93-1.00)	0.00 (0.00-0.07)	0.00 (0.00-0.07)
36	**5611 C>T**			CAGTGG[C/T]GGGAAA	67	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.93-1.00)	0.00 (0.00-0.07)	0.00 (0.00-0.07)
37	**5761 A>G**			GTGGCC[A/G]GGAGGT	67	0.99 (0.94-1.00)	0.01 (0.00-0.06)	0.97 (0.89-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)
38	**7635 A>G**		rs6785049	CCTCTC[A/G]CCCCCA	66	0.41 (0.33-0.49)	0.59 (0.51-0.67)	0.18 (0.11-0.29)	0.45 (0.34-0.57)	0.36 (0.26-0.48)
39	**7637 C>T**			TCTCGC[C/T]CCCAAC	67	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.93-1.00)	0.00 (0.00-0.07)	0.00 (0.00-0.07)
40	**7675C>T**		rs6797879	TGCCGG[T/C]CTGTGG	67	0.81 (0.74-0.87)	0.19 (0.13-0.26)	0.67 (0.55-0.77)	0.28 (0.19-0.40)	0.04 (0.01-013)
41	**7683C>T**			TGTGGG[C/T]TGCCTC	68	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
42	**7737C>T**		rs1140969 C>T	CTTGCC[C/T]ATCGAG	68	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
43	**7767 G>A**			GAAGGG[G/A]GCCGCT	68	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
44	**7812 T>C**		rs4058490	GTTCAA[T/C]GCGGAG	68	0.00	1.00	0.00	0.00	1.00

						(0.00-0.03)	(0.97-1.00)	(0.00-0.06)	(0.00-0.06)	(0.94-1.00)
45	**8055C>T**		rs2276707	CTCCAT[C/T]CTGTTA	73	0.60 (0.52-0.67)	0.40 (0.33-0.49)	0.44 (0.33-0.55)	0.31 (0.22-0.43)	0.25 (0.16-0.36)
46	8118 C>T	Syn (Y)	rs2229856C>T	CCACTA[C/T]ATGCTG	68	0.74 (0.66-0.80)	0.26 (0.20-0.35)	0.47 (0.36-0.59)	0.53 (0.41-0.64)	0.00 (0.00-0.06)
47	8528G>A	A370T	rs35761343	CAATTC[G/A]CCATTA	71	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
48	8555T>G	C379G		ATTGAA[T/G]GCAATC	71	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
49	8561C>T	R381W		TGCAAT[C/T]GGCCCC	71	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
50	8582T>G			CATAGG[T/G]GAGCAC	70	0.79 (0.72-0.85)	0.21 (0.15-0.28)	0.59 (0.47-0.70)	0.41 (0.31-0.53)	0.00 (0.00-0.06)
51	8592A>G			CACAGC[A/G]GGGGGT	71	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
52	8677G>A			GCATCT[G/A]GAGGTA	71	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
53	9683A>G	I403V		CGCAGC[A/G]TCAATG	60	0.99 (0.95-1.00)	0.01 (0.00-0.05)	0.98 (0.90-1.00)	0.02 (0.00-0.10)	0.00 (0.00-0.07)
54	9915T>A	F420Y		ACCCCT[T/A]TGCTAC	65	0.99 (0.95-1.00)	0.01 (0.00-0.05)	0.98 (0.91-1.00)	0.02 (0.00-0.9)	0.00 (0.00-0.07)
55	9932C>G	Q426 E	rs56162473	CTCATG[C/G]AGGAGT	65	0.98 (0.94-1.00)	0.02 (0.00-0.06)	0.97 (0.88-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)

56	9976G>A		rs3732358	CCTTGG[G/A]TGACAC	68	0.98 (0.95-0.99)	0.02 (0.00 -0.06)	0.97 (0.89-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.06)
57	9987G>A			ACCTCC[G/A]AGAGGC	68	0.99 (0.96-1.00)	0.01 (0.00-0.05)	0.98 (0.91-1.00)	0.02 (0.00-0.09)	0.00 (0.00-0.06)
58	10058C>G			AAGAGC[C/G]GACAAT	65	0.98 (0.94-1.00)	0.02 (0.00-0.06)	0.97 (0.88-1.00)	0.03 (0.00-0.11)	0.00 (0.00-0.07)
59	10098 C>T			TTCCTG[C/T]TATGAC	71	0.99 (0.96-1.00)	0.01 (0.00-0.04)	0.99 (0.92-1.00)	0.01 (0.00-0.08)	0.00 (0.00-0.06)
60	10159 C>G		rs10460826	TTCAGT[C/G]TGTAGG	66	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.93-1.00)	0.00 (0.00-0.07)	0.00 (0.00-0.07)
61	10263G>T			CCTGTG[G/T]TCTGGG	66	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.93-1.00)	0.00 (0.00-0.07)	0.00 (0.00-0.07)
62	10331A>G		rs3732359	AAGGAT[A/G]GGCCAT	68	0.36 (0.28-0.44)	0.64 (0.56-0.72)	0.22 (0.14-0.33)	0.28 (0.19-0.40)	0.50 (0.38-0.62)
63	10461 C>A		rs10511395	GGGCTC[C/A]AGGCCT	56	1.00 (0.96-1.00)	0.00 (0.00-0.04)	1.00 (0.92-1.00)	0.00 (0.00-0.08)	0.00 (0.00-0.08)
64	10477G>T		rs61760364	CTCATC[G/T]GCAGGC	46	1.00 (0.95-1.00)	0.00 (0.00-0.05)	1.00 (0.91-1.00)	0.00 (0.00-0.09)	0.00 (0.00-0.09)
65	10483 T>C		rs3732360	GGCAGG[C/C]GCATGA	66	0.36 (0.29-0.45)	0.64 (0.55-0.71)	0.23 (0.14-0.34)	0.27 (0.18-0.39)	0.50 (0.38-0.62)
66	10552 T>G			AGAAGC[T/G]TGGCAT	36	1.00 (0.94-1.00)	0.00 (0.00-0.06)	1.00 (0.88-1.00)	0.00 (0.00-0.11)	0.00 (0.00-0.11)
67	10620C>T		rs1054190	AAGCAC[C/T]GATAAT	44	1.00	0.00	1.00	0.00	0.00

						(0.95-1.00)	(0.00-0.05)	(0.90-1.00)	(0.00-0.10)	(0.00-0.10)
68	10691A>G		rs9851439	GTTTAT[A/G]GTTAAA	44	1.00 (0.95-1.00)	0.00 (0.00-0.05)	1.00 (0.90-1.00)	0.00 (0.00-0.10)	0.00 (0.00-0.10)
69	10719 A>G		rs6438550	ACAAAC[A/G]ATTTGG	64	0.82 (0.74-0.88)	0.18 (0.12-0.26)	0.75 (0.63-0.84)	0.14 (0.07-0.25)	0.11 (0.05-0.21)
70	10799 G>A		rs1054191	GATGGC[G/A]GGCACT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
71	10828 A>G		rs2472683	GTTCCC[A/G[AGGACA	75	0.00 (0.00-0.03)	1.00 (0.97-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)	1.00 (0.94-1.00)
72	10876 C>T			TGAGTG[C/T]GTGTGT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
73	10896 G>A		rs34344833	GGTGTA[G/A]GTAGGT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
74	10988 A>T		rs12107248	AATCAG[A/T]TAAACA	75	0.00 (0.00-0.03)	1.00 (0.97-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)	1.00 (0.94-1.00)
75	11073 C>A			AAAGTG[C/A]CTGCCT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
76	11113 G>T			TTTTTT[G/T]CATTTT	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
77	11125 A>G		rs12721615	TCACAA[A/G]TTATAC	75	1.00 (0.97-1.00)	0.00 (0.00-0.03)	1.00 (0.94-1.00)	0.00 (0.00-0.06)	0.00 (0.00-0.06)
78	11156 A>C		rs3814057	CACCTA[A/C]GAACTA	75	0.56 (0.48-0.64)	0.44 (0.36-0.52)	0.40 (0.30-0.51)	0.32 (022-0.43)	0.28 (0.19-0.39)

79	11193 T>C		rs3814058	TTAATG[T/C]CAAATC	75	0.56 (0.48-0.64)	0.44 (0.36-0.52)	0.40 (0.30-0.51)	0.32 (022-0.43)	0.28 (0.19-0.39)

ACKNOWLEDGEMENTS

First I would like to thank Prof. Matthias Schwab for allowing me to perform my PhD studies at the Dr. Margarete Fischer Bosch – Institute of Clinical Pharmacology, for the financial support and for reviewing this thesis.

A special thank you goes to Prof. Michael Schwarz, my co-supervisor at the University of Tubingen for all the help provided during the PhD registration process and reviewing this thesis.

Foremost I would like to express my deep and sincere gratitude to my scientific supervisors, Dr. Oliver Burk and Dr. José Pedro Gil, for all the time, ideas and funding given to this project that made this experience more productive and stimulating. Thank you for all the things you taught me during the last years.

My warm and sincere gratitude also goes to my colleagues at IKP and at the Karolinska Institutet, who contributed to my personal and professional growth. Special thank you at my colleagues at the malaria lab for making me feel completely at home, ever since the first time I went there.

My personal acknowledgements also to Prof. Paulo Martel, for helping me gather all the documents necessary for my registration.

I would also like to acknowledge the financial support provided by the IBB-Institute for Biotechnology and Bioengineering, Centre for Molecular and Structural Biomedicine, University of Algarve.

Finally, I would like also to express my thank you to all my friends for all the good times spent together during this period. Meng, Lydia and Ella thank you for all our stupid conversation and for making an office became a more pleasant place to work in. Clint, thank you for your friendship and long walks through the vineyards. Ana, thank you for the company during the first years in Stuttgart. Pedro and Isabel thank you for the friendship and

welcoming me in your home. Lena, Tiago, Bruno, thank you for always being there. A special thank you to Isa, for all the help during these years and above all, for making me decide (almost forcing, I might say) to start the PhD. And Fábio, thank you for being there, supporting me, helping me, annoying me, making me laugh.

Last but not least, I would like the express my deepest gratitude to the persons to whom this thesis is dedicated: My parents, thank you for supporting and encouraging me to follow my dreams, above all thank you for making me the person I am today.

LIST OF ACADEMIC TEACHERS

Prof. Dr. Matthias Schwab

Prof. Dr. Michael Schwarz

Prof. Anders Björkman

Dr. Oliver Burk

Dr. José Pedro Gil

CURRICULUM VITAE

Name	Rita Isabel Martins da Piedade
Date of Birth	30 October 1980
Place of Birth	S.Clemente, Loulé
Nationality	Portuguese

Academic Qualifications

1998 – 2004: Graduation course in Biochemistry, Faculty of Sciences and Technology, University of the Algarve (Final classification: 13 over 20).

Scientific/Professional activity

2002 – 2004: Internship in the Faculty of Sciences and Technology, University of the Algarve under the supervision of Prof. Dr. Vera L. Ribeiro

Diploma thesis title: "Pharmacogenetics of cytochromes P450 and the nuclear receptor PXR in colorectal cancer" (Final classification: 19 over 20).

2004 – 08.2006: Volunteer Researcher under the supervision of Prof. Dr. Vera L. Ribeiro

Area of Research: "Pharmacogenetics of the nuclear receptor PXR in both a ethnic and breast cancer context"

10.2008-12.2008/ 10.2009-12.2009: Invited PhD student at the Malaria Research Unit, Karolinska Intitutet under the supervision of Dr. José Pedro Gil.

09.2006- Present: PhD student at the Dr. Margarete Fischer-Bosch – Institute of Clinical Pharmacology (Stuttgart) and University of Tubingen under the supervision of Dr. Oliver Burk

Area of Research: "The PXR/CAR system and antimalaria chemotherapy"