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Nuclear receptor activation by antiretroviral drugs used in the treatment of HIV disease with implications for xenobiotic and endobiotic processes

A Thesis submitted to the

University of Dublin,

Trinity College

In fulfillment of the requirement

For the degree of

Doctor of Philosophy (PhD)

by

Jenny Sofia Svärd, MSc

2012

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I wish to acknowledge the technical expertise and collaboration of Dr Fernando Blanco, TCD, without whom the *in silico* predictions of nuclear receptor-antiretroviral drug interactions detailed in chapter 4 could not have been completed.

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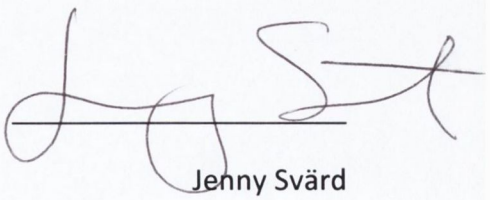

Jenny Svärd

Summary: methods and major findings

Genotyping: DNA was isolated from whole blood of 1013 HIV-patients and genotyped commercially for 37 single nucleotide polymorphisms (SNPs) and one 3-basepair insertion in genes of relevance for drug interactions: *NR1I2* (PXR), *CYP3A4*, *CYP2B6* and *ABCB1* (*MDR1*). An in-house quality control was carried out by PCR-RFLP. 22 polymorphisms (out of 29 detected) were found at significantly different ($P \leq 0.05$) allele frequencies between Caucasians and Sub-Saharan Africans (chi-square with Fisher's Exact Test). A comparison of our Sub-Saharan African population with available data from other studies of African American subjects revealed significantly different allele frequencies of four SNPs.

Antiretroviral (ARV) induction of nuclear receptor-mediated transcription of cytochrome P450 (CYP450) enzymes: The inductive ability of sixteen ARVs on *CYP3A4* and *CYP2B6* promoter activity via pregnane X receptor (PXR) or constitutive androstane receptor (CAR) was explored *in vitro* using luciferase reporter assays with HepG2 cells. Normalised results were compared to untreated cells by one-way ANOVA with Dunnett's *post hoc* analysis, $P < 0.05$ indicated significant difference. PXR-mediated *CYP3A4* promoter activity was induced by (mean fold change ± S.E.M): Fosamprenavir (13.5 ± 3.9), lopinavir (7.5 ± 2.7), nelfinavir (5.6 ± 2.3), tipranavir (9.9 ± 3.4) and efavirenz (5.7 ± 3.3). PXR-mediated *CYP2B6* promoter activity was increased by lopinavir (11.4 ± 10.0), darunavir (6.1 ± 0.4), efavirenz (4.7 ± 2.3) and abacavir (2.3 ± 0.6). CAR-mediated *CYP3A4* promoter activity was induced only by abacavir (2.5 ± 1.0), while CAR-mediated *CYP2B6* promoter activity was increased by fosamprenavir (3.4 ± 3.2), lopinavir (3.0 ± 1.3) and tipranavir (4.8 ± 2.4).

Impact of *NR1I2* (PXR) polymorphism on PXR-mediated *CYP3A4* transcription: To assess the influence of four coding *NR1I2* (PXR) polymorphisms detected in the cohort, mutation constructs of PXR were employed in reporter assays as described above. Transfection of HepG2 cells with V140M and A370T mutation constructs both resulted in lower rifampicin-, fosamprenavir- and lopinavir-stimulated *CYP3A4* promoter activity in comparison to the PXR reference sequence construct.

ARV effect on CYP450 mRNA and protein expression in primary human hepatocytes: Cells were exposed to lopinavir, efavirenz and abacavir for 48h, after which RNA and protein was isolated. Real time PCR was carried out with primers for *CYP3A4*, *CYP2B6* and internal standard β-actin (*ACTB*). Normalised results were compared to vehicle controls by one-way ANOVA with Dunnett's *post hoc* analysis, $P \leq 0.05$ indicated significant difference. *CYP3A4* mRNA expression was increased by efavirenz (mean fold change ± S.E.M 2.8 ± 0.7), while both efavirenz (30.1 ± 12.8) and abacavir (3.2 ± 0.4) increased *CYP2B6* mRNA expression. Determination of protein content by Western blot showed increased CYP3A4 by efavirenz (7.6 ± 4.7) and CYP2B6 by lopinavir (1.8 ± 0.4).

***In silico* evaluation of ARVs as nuclear receptor ligands:** Docking studies and molecular descriptor filtering parameters were used to evaluate the potential of ARVs to act as ligands of liver X receptors

(LXRα/β), estrogen receptors (ERα/β) and glucocorticoid receptor (GR). From a library of 26 ARVs, the following compounds were predicted as potential ligands of LXRα/β: darunavir, tipranavir, efavirenz, maraviroc, TAK-779 and flavopiridol. Efavirenz and flavopiridol were predicted ligands of GR, while only the former fit the criteria of an ERα/β ligand.

Direct ARV interactions with nuclear receptor ligand binding domains (LBDs): TR-FRET fluorescence LXRα and ERα co-activator assays were adopted to assess direct LBD interactions and co-activator recruitment by ARVs. The selection of drugs was guided by the *in silico* pre-screening, some additional compounds were included based on confirmed PXR induction or indication of target gene effects in the literature. LXRα agonist effects were confirmed for darunavir (EC_{50} = 21.7μM), maraviroc (EC_{50} = 16.8μM) and tipranavir (EC_{50} = 30.0μM). LXRα antagonistic effects were verified for efavirenz (IC_{50} = 45.2μM), TAK-779 (IC_{50} = 206μM, outside tested range) and flavopiridol (IC_{50} = 26.4μM).

ARV activation of LXRα/β, ERα/β and GR: Reporter assays were utilised to assess ARVs as ligands of these nuclear receptors *in vitro*, transfecting HepG2 with nuclear receptor expression plasmids as well as luciferase constructs of their respective responsive elements followed by 24h drug exposure. Normalised results were compared to vehicle controls by one-way ANOVA with Dunnett's *post hoc* analysis, $P \leq 0.05$ indicated significant difference. LXRα and LXRβ activity was increased by atazanavir (mean fold change ± S.E.M for LXRα 2.8 ± 0.5; β 2.5 ± 0.9), darunavir (LXRα 1.8 ± 0.2, β 2.0 ± 0.2) and ritonavir (LXRα 3.5 ± 1.3, β 2.7 ± 1.4). Efavirenz on the other hand reduced the activity of LXRβ to 7 ± 4% of basal levels. Transcriptional activity of ERα was increased by efavirenz (mean fold change ± S.E.M 13.6 ± 5.9) and tipranavir (5.5 ± 3.8). None of the ARV drugs tested had an effect on ERβ or GR.

Efavirenz effect on ABCA1 and ApoE gene expression using human SH-SY5Y neuroblastoma cells and primary rat cortical cultures: Cells were exposed to efavirenz and/or LXR agonist T0901317 for 24h followed by RNA isolation. Real time PCR was performed with primers for LXR target genes ABCA1 and ApoE. Efavirenz reduced *ABCA1* in SH-SY5Y (one-way ANOVA with Dunnett's *post hoc* analysis, $P \leq 0.05$) and additionally attenuated T0901317-mediated *ABCA1* induction (Mann-Whitney t-test, $P \leq 0.05$). No significant effect was seen on human *APOE* with either efavirenz or T0901317, while a trend to reduced *Abca1* and *ApoE* was noted in primary rat cortical cultures (however non-significant).

Efavirenz effect on amyloid precursor protein (APP) expression in a human neuroblastoma cell line and primary rat cortical cultures: Western blots were performed with whole cell RIPA lysates of SH-SY5Y cells and primary rat cortical cultures, however no significant effect was seen with either efavirenz or LXR agonist T0901317 on APP expression (normalised to internal standard β-actin).

Table of contents

I. Acknowledgements

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II. List of publications and presentations

Publications

Svärd J, Spiers JP, Mulcahy F, Hennessy M. **Nuclear receptor-mediated induction of CYP450 by antiretrovirals: functional consequences of *NR1I2* (PXR) polymorphisms and differential prevalence in whites and Sub-Saharan Africans**. *J Acquir Immune Defic Syndr. 2010 Dec 15;55(5):536-49 PMID: 20861742*

Presentations

3rd School of Medicine Research Day, School of Medicine, Trinity College Dublin 16 September 2010

Oral presentation: **Nuclear receptor-mediated induction of CYP450 by antiretrovirals: functional consequences of *NR1I2* (PXR) polymorphisms and differential prevalence in Caucasians and Sub-Saharan Africans**

Jenny Svard, J. Paul Spiers, Fiona Mulcahy, Martina Hennessy

WorldPharma 2010, Copenhagen 17-23 July 2010

Poster: **Activation of liver X receptors (alpha and beta) by HIV protease inhibitors atazanavir and ritonavir and inhibition by non-nucleoside reverse transcriptase inhibitor efavirenz**

Svard, Jenny; Spiers, J. Paul; Mulcahy, Fiona; Hennessy, Martina

British Pharmacological Society Winter Meeting, Brighton 16-18 Dec 2008

Oral presentation: **Nuclear receptor-mediated expression of CYP3A4 and CYP2B6 by antiretrovirals - implications for prediction of drug interaction potential**

Oral presentation: **Genetic variability in PXR, MDR-1, CYP3A4 and CYP2B6 in HIV infected Caucasian and Sub-Saharan African patients - benefits from a cohort approach**

Svard, Jenny; Spiers, Paul; Mulcahy, Fiona; Hennessy, Martina

2nd School of Medicine Research Day, School of Medicine, Trinity College Dublin 11 December 2008

Oral presentation: **Nuclear receptor-mediated expression of CYP3A4 and CYP2B6 by antiretrovirals – implications for prediction of drug interaction potential**

Poster: **Genetic variability in *PXR, MDR-1, CYP3A4* and *CYP2B6* in HIV infected Caucasian and sub-Saharan African patients – benefits from a cohort approach**

Jenny Svard, J. Paul Spiers, Fiona Mulcahy, Martina Hennessy

HIV9, Glasgow 9-13 Nov 2008

Poster: **Antivirals and nuclear receptor activation of CYP3A4 and 2B6**

Svard, Jenny; Spiers, J. Paul; Mulcahy, Fiona; Hennessy, Martina

Institute of Molecular Medicine 11th Annual Meeting 7-8 Nov 2008, Trinity College Dublin

Poster: **Nuclear receptor-mediated expression of CYP3A4 and CYP2B6 by antiretrovirals - implications for prediction of drug interaction potential**

Jenny Svard, J. Paul Spiers, Fiona Mulcahy, Martina Hennessy

Infectious Diseases Society Ireland Annual Scientific Meeting, Dublin 11-13 June 2008

Oral presentation: **Antivirals and Nuclear Receptor Activation of CYP3A4 and 2B6**

Svard, Jenny; Spiers, J. Paul; Mulcahy, Fiona; Hennessy, Martina

III. List of abbreviations

AA	African American
Aβ	β-amyloid
ABC	abacavir
ABC transporter	ATP-binding cassette transporter
ABCA1	ATP-binding cassette transporter A1
ABCB1	ATP-binding cassette transporter B1 (*MDR1*, P-glycoprotein)
ACTH	adenocorticotropic hormone
AD	Alzheimer's disease
AF-1	activation function domain 1
AF-2	activation function domain 2
AIDS	acquired immunodeficiency syndrome
AMBER	Assisted Model Building with Energy Refinement
ANOVA	analysis of variance
APP	amyloid precursor protein
ApoAI	apolipoprotein AI
ApoE	apolipoprotein E
APV	amprenavir
AR	androgen receptor
ARV	antiretroviral
ATP	adenosine triphosphate
ATV	atazanavir
AZT	zidovudine
BMD	bone mineral density
C	Caucasian
CAR	constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl)oxime
CNS	central nervous system
CYP450	cytochrome P450
CYP2B6	cytochrome P450 subfamily 2B member 6
CYP3A4	cytochrome P450 subfamily 3A member 4
CYP7A	cholesterol 7α-hydroxylase

d4T	stavudine
DBD	DNA binding domain
ddC	zalcitabine
ddI	didanosine
DLV	delavirdine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DOC	deoxycholate
DR	direct repeat
DRV	darunavir
EC_{50}	half maximal (50%) effective concentration
EFV	efavirenz
ER	estrogen receptor
ER	everted repeat
EST	expressed sequence tag
EtBr	ethidium bromide
FBS	foetal bovine serum
FB-LPDS	foetal bovine lipoprotein-deficient serum
FOS	fosamprenavir
FRED	Fast Rigid Exhaustive Docking
FRET	fluorescence resonance energy transfer
FTC	emtricitabine
FXR	farnesoid X receptor
GGPP	geranylgeranyl pyrophosphate
GR	glucocorticoid receptor
GST	glutathione-S-transferase
HAART	highly active antiretroviral therapy
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
HRP	horseradish peroxidise
Hsp	heat shock protein
HWE	Hardy-Weinberg equilibrium
IC_{50}	half maximal (50%) inhibitory concentration
IDV	indinavir

IMT	intima media thickness
IL	interleukin
IR	inverted repeat
IVDU	"intravenous drug users"
IQ	inhibitory quotient
LBD	ligand binding domain
LD	linkage disequilibrium
LDL	low-density lipoprotein
LPV	lopinavir
LXR	liver X receptor
MEC	minimum effective concentration
MDR1	multidrug resistance gene 1
MMFF94x	Merck Molecular Force Field
MOE	Molecular Operating Environment
MR	mineralocorticoid receptor
MRP	multidrug resistance-associated protein
MSM	"men who have sex with men"
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVC	maraviroc
NBM	neurobasal medium
NCoR	nuclear receptor co-repressor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NFV	nelfinavir
NFκB	nuclear factor-κB
NVP	nevirapine
OATP	organic anion transporting polypeptide
OPG	osteoprotegerin
PBS	phosphate buffered saline
PCN	pregnenolone 16α-carbonitrile
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PEPCK	phosphoenolpyruvate carboxykinase
PGC1α	peroxisome proliferator-activated receptor γ co-activator 1α

P-gp	P-glycoprotein
PI	HIV protease inhibitor
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PXR	pregnane X receptor
RAL	raltegravir
RANKL	receptor activator of NFκB ligand
RAR	retinoic acid receptor
ROC	Receiver Operating Characteristic
RTV	ritonavir
RXR	retinoid X receptor
S.D.	standard deviation
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
SHP	small heterodimer partner
SLCO	solute carrier organic anion transporter
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
SNP	single nucleotide polymorphism
SQV	saquinavir
SREBP	sterol regulatory element binding protein
SRC-1	steroid receptor co-activator -1
SSA	Sub-Saharan African
SULT	hydroxysteroid sulfotransferase
SXR	steroid and xenobiotic receptor
TCA	trichloroacetic acid
TDM	therapeutic drug monitoring
TFV	tenofovir
TIMP-3	tissue inhibitor of matrix metalloproteinase-3
TMC125	etravirine
TPV	tipranavir
TR	thyroid hormone receptor
TR-FRET	time-resolved fluorescence resonance energy transfer
TRAP220/DRIP-2	thyroid hormone receptor-associated proteins/vitamin D receptor-interacting proteins

T-20	enfuvirtide
T3	triiodothyronine
UGT	UDP-glucoronosyltransferase
UnTX	untreated
V_D	volume of distribution
VDR	vitamin D receptor
3TC	lamivudine

IV. List of Tables

V. List of Figures

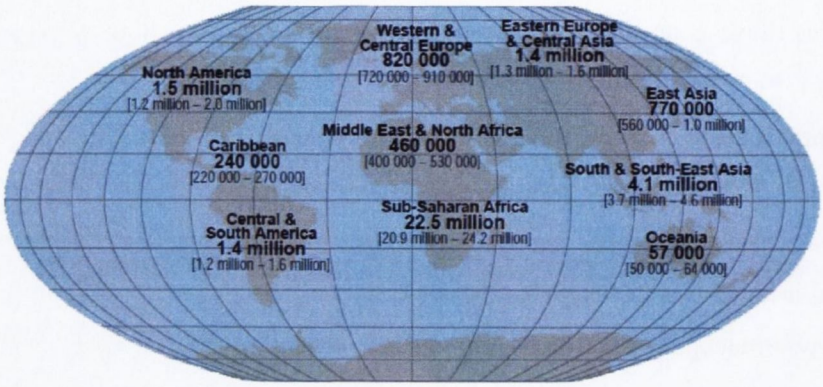
1 Introduction

The focus of this thesis is on the activation of nuclear receptors, which are important regulators of gene expression, by drugs designed to suppress replication of the human immunodeficiency virus (HIV). In this introductory chapter, the basic characteristics of HIV infection and mode of action by antiretroviral (ARV) compounds will be described and an outline given of some of the pharmacological challenges in HIV treatment; including development of drug resistance, adverse effects, the importance of achieving therapeutic drug plasma concentrations, drug interactions and the impact of pharmacogenetics. Finally, the nuclear receptor family will be introduced, and their fundamental role in the regulation of endogenous processes highlighted. In particular, the significance of pregnane X receptor (PXR) for ARV drug bioavailability and drug interactions will be discussed.

1.1 Human immunodeficiency virus (HIV)

After the emergence of acquired immunodeficiency syndrome (AIDS) disease in the United States in 1981, manifested by a high prevalence of rare opportunistic infections and Kaposi's sarcoma among young homosexual men[1], intensive research was carried out to identify the causative agent. Two years later a research group led by Montagnier at Institut Pasteur in Paris[2] published a report of isolation of a T-lymphotropic retrovirus, subsequently named HIV by an international nomenclature committee. This was later confirmed as the causative agent of AIDS by Gallo and co-workers at the National Institute of Health in Maryland, USA[3-6]. A different strain of HIV was later discovered in West Africa by Montagnier[7], leading to the distinction between HIV-1 and HIV-2. The less common HIV-2 is associated with a slower disease progression[8] and differential responses to standard HIV-1 drug regimens[9].

HIV has, since its discovery, developed into a global pandemic with two million deaths annually due to AIDS. Today more than 33 million people are living with the infection (Figure 1.1). Cases of HIV are found throughout the world but the prevalence is highest on the Sub-Saharan African continent. Swaziland is the worst affected country with 25.9% of its adult population (15-49 years) being infected. High HIV prevalence is also found in Botswana (24.8%) and Lesotho (23.6%) (UNAIDS Outlook Report 2010).



Total: 33.3 million [31.4 - 35.3 million]

Figure 1.1 Adults and children estimated to be living with HIV. UNAIDS Report on the Global AIDS Epidemic 2010 (data from 2009).

HIV is transmitted through blood and sexual contact and principally infects CD4+ T-cells, although many other cell types such as monocytes/macrophages and hematopoietic progenitor cells have also been shown to function as viral reservoirs[10]. The virus enters the cell by attaching to antigen presenting cell co-receptor CD4 and either CCR5 or CXCR4 chemokine receptors on the cell surface (Figure 1.2). In the cell cytoplasm the viral particle is uncoated and its RNA genome reverse transcribed to DNA by viral reverse transcriptase, which subsequently enters the nucleus and is incorporated in the host cell genome. This enables the virus to utilise the DNA replication and translation machinery of the host for production and release of new virus particles for propagation of infection[11, 12].

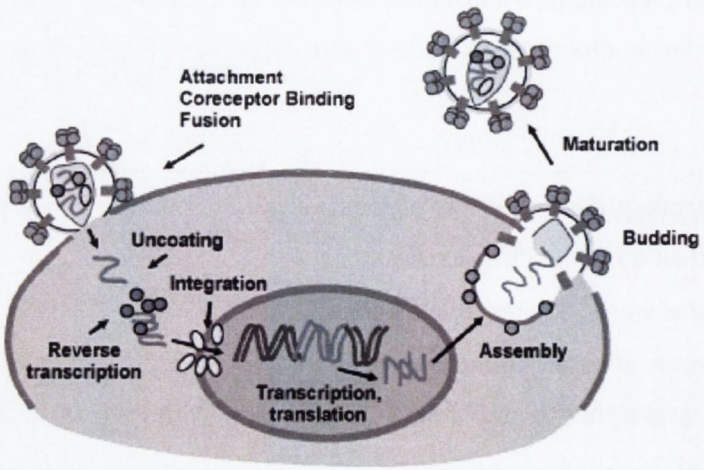


Figure 1.2 HIV life cycle (from www.clinicaloptions.com).

The early stages of HIV infection are characterised by a sharp rise in plasma viral load (≈ 5 $\log_{10}$ RNA copies/ml)[13] (Figure 1.3). After the acute phase the viral load recedes somewhat following a response from HIV-specific 'effector' CD8+ T cells[14]. Both viral RNA kinetics during the first year, as an indication of the magnitude of the initial immune response, as well as viral load, are predictive of AIDS progression[15]. Another important prognostic marker is CD4+ cell count which decreases over time as a consequence of apoptosis[16]; the rate of decline which can range between 47 cells per ml/year (non-progressors, symptom-free >5 years) to 192 cells per ml/year (progressors, mean time to AIDS 47 months)[13]. Furthermore, there is an increase in markers of immune activation such as polyclonal B cell activation, higher turnover and terminal differentiation of T cells as well as increased levels of pro-inflammatory mediators, suggesting a chronic inflammatory state[17]. In addition to depletion, activated CD4+ T cells are more susceptible to infection[18]. If HIV infection is allowed to proceed untreated, the patient becomes highly likely to develop opportunistic infections (examples are *Pneumocystis carinii*, tuberculosis, herpes etc) and other malignancies[19]. This is characteristic of AIDS and will inevitably lead to death if untreated.

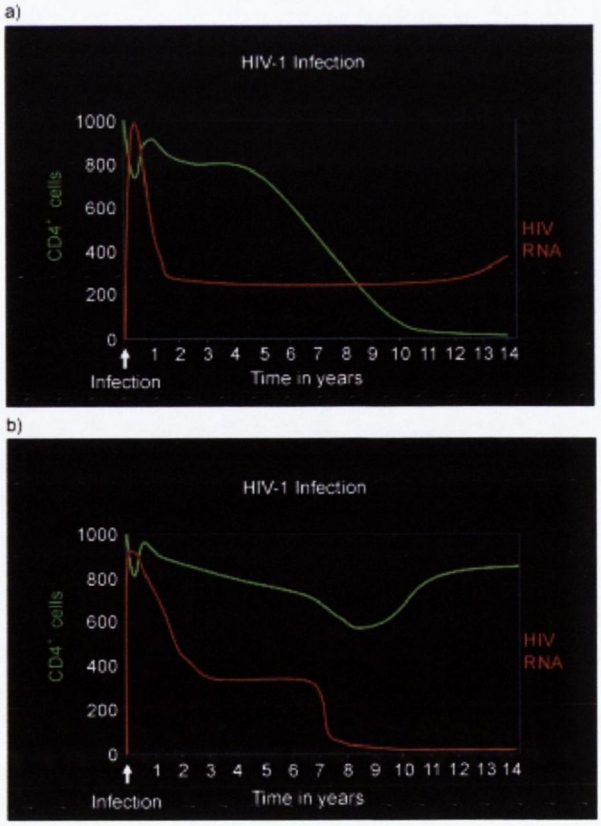


Figure 1.3 Progression of HIV-1 infection. A) Patient without access to antiretroviral treatment. B) Patient starting antiretroviral treatment at year six (from Montagnier 2009[20]).

Consequently, the prognosis for early cases was very poor: before 1997 the estimated proportion of AIDS-free survivors 10 years following seroconversion was 39% among 25-34 year-olds (data from 22 cohorts in Europe, Australia and Canada[21]). The situation changed dramatically with the development of effective ARV drugs; the corresponding number from 1999-2001 was 95%.

1.2 Antiretroviral drugs and HAART

The first anti-HIV agent to be licensed for clinical use was zidovudine (AZT) in 1987[22]. It is a thymidine analogue inhibiting the viral reverse transcriptase and preventing elongation of the DNA strand as it lacks a 3'-hydroxyl group, therefore viral replication is obstructed. Subsequently, more nucleoside reverse transcriptase inhibitors (NRTIs) were developed, such as didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC) and emtricitabine (FTC). These require intracellular phosphorylation in order to interact with the substrate-binding site of the enzyme[23]. In 1993, a nucleotide reverse transcriptase inhibitor; tenofovir (TFV), was described[24] and has since become widely used. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind allosterically to a site located closely to the catalytic site, causing a conformational change which disturbs the enzymatic activity. Examples are nevirapine (NVP), delavirdine (DLV), efavirenz (EFV) and etravirine (TMC125).
The next group of antiretrovirals (ARVs) to be developed targeted a different part of the HIV replicative cycle: the protease enzyme. This enzyme cleaves the viral precursor polyprotein into functional and structural proteins. HIV protease inhibitors (PIs) are peptidomimetic compounds (with the exception of tipranavir (TPV) which has a coumarin scaffold), designed to bind to the enzyme but cannot themselves be cleaved. The first PI saquinavir (SQV) was launched in 1995, followed by ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), fosamprenavir (FOS), tipranavir (TPV) and darunavir (DRV).

In 1997, it was established that a combination drug regimen consisting of three ARV drugs from at least two drug classes is the best way to suppress HIV viral load and restore CD4+ T cell counts: the ACTG320 study[25] as well as Gulick *et al*[26] independently demonstrated superiority of indinavir, zidovudine plus lamivudine compared to zidovudine/lamivudine alone. This was termed highly active antiretroviral therapy (HAART) and has significantly reduced morbidity and mortality for HIV-infected patients: A study by Murphy *et al*[27] demonstrated a crude death rate of 0.24 event per person and year among patients taking HAART while the corresponding number among those not taking HAART

was 0.88, The rate of non-CMV disease were 0.15 event per person and year after HAART compared to 0.45 before HAART. HAART has also proven to be a highly cost-effective medical intervention[28, 29]. European guidelines[30] currently recommend an NNRTI (efavirenz or nevirapine) or a PI with low dose ritonavir in combination with two NRTIs (tenofovir/emtricitabine or abacavir/lamivudine).

More recently, newer drug classes have been added to the list of ARV drugs: the entry inhibitor maraviroc (MVC) specifically inhibits co-receptor CCR5, utilised by the virus during the cell entry process. A limitation of this drug is that it is only active against R5 strains (macrophage-tropic, requiring co-receptor CCR5), potentially leading to selection of X4 strains (lymphocyte tropic, requiring co-receptor CXCR4) in mixed infections. To date, no CXCR4 antagonist has been licensed for ARV therapy although some promising results have been obtained *in vitro*[31]. The fusion inhibitor enfuvirtide (T-20) forms a coil-coil interaction with the viral glycoprotein gp41, blocking the fusion of the viral particle with the outer cell membrane[32]. An advantage is its effectiveness against strains which are resistant to other drug classes (TORO1[33] and TORO2[34] studies). However, being a polypeptidic compound enfuvirtide is not orally bioavailable and must be injected subcutaneously; hence it is essentially used as salvage therapy in treatment-experienced patients. Raltegravir (RAL) is the first integrase inhibitor to be approved; this drug targets and irreversibly inhibits the integration of HIV DNA into the host genome. Randomised controlled trials have demonstrated this compound to be a highly potent ARV: addition of 400mg raltegravir twice daily to optimised background treatment led to a 2 $\log_{10}$ decrease in viral load accompanied by mean CD4+ cell count increases of approximately 100 cells/μl in both the Protocol 005[35] and BENCHMRK[36] studies, with no significant adverse effects. A number of NRTIs, NNRTIs and CCR5 antagonists as well as new types of ARVs targeting viral assembly, latency and mRNA production are presently in development[37].

The time-to-start-treatment, usually described by CD4+ cell count or viral load, is often debated. Previously, the established strategy was to wait until the patient's CD4+ cell count fell below 350/μl with the objective of avoiding HAART toxicity and drug resistance development. However, recent studies have concluded that deferral of treatment initiation leads to a greater degree of immune senescence (deterioration of the immune system)[38], poorer immune recovery[39], greater risk of long-term virological failure[38] and increased mortality[40], as well as drug increased drug resistance when treatment is failing[41]. What was formerly perceived as an "asymptomatic" period may in fact be one of slow-progressing long-term damage[42]. While the efficacy of ARV treatment in reducing of mother-

to-child transmission is well established, studies have also demonstrated that HAART significantly reduces the risk of sexual transmission[43, 44]. This evidence provoked revised recommendations from the International AIDS Society-USA in 2010, advising treatment initiation for patients with CD4+ cell counts of 500/µl or less[45]. European recommendations remain at 350/µl for asymptomatic patients but are awaiting revision.

Combination ARV therapy is now standard of care. Despite a long list of approved ARV drugs and a theoretically large number of possible combinations, a number of challenges exist which will limit these possibilities in reality. First of all, HAART may be successful in suppressing viral load and restoring immune function, but it does not constitute a cure and treatment is life-long. A patient's drug regimen may work well initially but will undoubtedly require re-evaluation and alteration over time, most commonly due to intolerance, poor adherence, loss of efficacy or toxicity[46]. There are a number of pharmacological challenges which influence and encumber the choice and sustainability of a given drug regimen:

1.2.1 ARV drug resistance

The HIV reverse transcriptase is error-prone, frequently introducing mutations and recombination events during replication. As a result, the virus population in each HIV-infected individual consists of different but genetically related viral variants[47]. When subjected to drug pressure, mutants with a fitness advantage in the presence of therapy will become more predominant[48].

Within a few years of the launch of the first ARV drug zidovudine, researchers reported evidence of resistance development: viral isolates in individuals on long-term zidovudine treatment displayed lower drug susceptibility[49, 50]. After the introduction of other drug classes new mutations emerged, sometimes conferring cross-resistance within classes: for example mutation L90M in the viral protease gene (arising in 18% of patients with viral failure following nelfinavir as the first PI treatment[51]) increases resistance to all other PIs except tipranavir and darunavir[52]. NRTIs lamivudine and emtricitabine and all NNRTIs except etravirine are classified as having a "low genetic barrier" as only a single mutation is required to gain high-level phenotypic resistance, while PIs used in combination with low dose ritonavir require the accumulation of multiple mutations[53].

Although combination ARV therapy reduces the risk of emerging drug resistance[26] through enhanced viral suppression and the unlikely pre-existence of viral variants with resistance mutations to three drugs, other factors such as patient adherence[54] and host genetics[55] may influence ARV plasma drug concentrations and consequently the development of drug resistance (discussed in section 1.3).

1.2.2 Adverse effects

Although effective in inhibiting viral replication, ARV treatment is not without drawbacks. There is a high prevalence of adverse events associated with most ARV drug regimens in use that may lead to treatment switch or discontinuation (15.8% in the Swiss HIV Cohort Study[56]). The major categories of undesired effects are described below.

1.2.2.1 Hypersensitivity reactions

Several ARVs can give rise to hypersensitivity reactions. Examples are nevirapine and efavirenz hypersensitivities which can present as hepatitis, skin rash and eosinophilia[57]. Abacavir hypersensitivity is a multiorgan reaction resulting in fever, rash, malaise and gastrointestinal symptoms[58]. The latter can be fatal but has been linked to *HLA B*5701*[59]; implementation of a screening process to detect this polymorphism prior to initiating therapy has proven both cost-effective[60] and successful in reducing the incidence of abacavir hypersensitivity[61].

1.2.2.2 Mitochondrial toxicity

Although nucleoside analogues are designed to inhibit the viral reverse transcriptase, NRTIs can also be substrates for mitochondrial DNA polymerase-γ, resulting in DNA chain termination and mutations. Mitochondrial dysfunction decreases ATP (adenosine triphosphate) and increases lactate production, oxidative stress and potentially apoptosis[62]. Well-documented clinical consequences include hepatic steatosis, lactic acidosis and myopathy[63]. In addition, mitochondrial toxicity may also contribute to changes in body fat composition and nephrotoxicity[62] (see sections 1.2.2.3 and 1.2.2.6). As the tri-phosphate forms of zalcitabine, didanosine and stavudine are more easily incorporated into the mitochondrial DNA strand, these NRTIs are more prone to cause adverse effects than tenofovir, zidovudine and abacavir[64]. Lamivudine appears to be efficiently removed by the proof-reading

mechanism of DNA polymerase-γ[65] and hence falls into the category of NRTIs with lower risk of mitochondrial toxicity.

1.2.2.3 Metabolic and cardiovascular abnormalities

In 1998 – 2 years after the use of HIV PIs came into clinical practice – a comprehensive description of PI-associated fat redistribution was published by Carr *et al*[66]. This was termed HIV-associated lipodystrophy syndrome and is characterised by peripheral limb fat loss and central abdominal fat accumulation. Subsequent studies showed that NRTI therapy could also cause a form of lipodystrophy with peripheral fat wasting as its most prominent feature[67, 68]. However, a lack of consensus in defining the critical characteristics of HIV lipodystrophy resulted in substantial variation in reports of prevalence. Following a multinational case-control study[69], an objective case definition was published in the *Lancet* in 2003 and is still used today. The variables included in this model are: age, sex, duration of HIV infection, HIV disease stage, waist to hip ratio, anion gap (as a measure of metabolic acidosis), serum high-density lipoprotein (HDL) cholesterol, trunk to peripheral fat ratio, percentage leg fat, and intra-abdominal to extra-abdominal fat ratio. The syndrome may also be accompanied by other metabolic features such as insulin resistance and dyslipidemia (characterised by hypertriglyceridemia, increased low-density lipoprotein (LDL) and decreased levels of HDL cholesterol)[70]. The 2NN trial[71] compared lipid profiles of NNRTIs efavirenz and nevirapine during a 48 week follow up and found greater increases in both triglycerides and non-HDL cholesterol with efavirenz. Within the PI group differential effects have also been observed: In a prospective study by Calza *et al*[72] with a 1-year follow-up period, the incidence of hypertriglyceridemia among ritonavir and ritonavir/lopinavir-treated HIV patients was significantly higher than with other PIs (amprenavir, indinavir, nelfinavir, saquinavir). Both PIs[73] and stavudine[74] have been associated with reduced insulin sensitivity.

These metabolic abnormalities place patients at increased risk of cardiovascular disease and type 2 diabetes mellitus[75]. A case-control study by Lorenz *et al*[76] identified both long-term HIV infection and HAART as independent risk factors for atherosclerosis: the carotid bifurcation intima media thickness (IMT) was 24.4% higher for HIV-infected patients than age- and sex-matched HIV-negative controls. A comparison between HAART-naïve subjects and patients treated with HAART for more than 2 years revealed 19.7% higher carotid bifurcation IMT in the latter group. Furthermore, an association

between recent use of NRTIs abacavir or didanosine and increased risk of myocardial infarction was identified in the D:A:D study (relative rates of myocardial infarction: 1.49 for didanosine, 1.89 for abacavir) compared with no recent use[77]. The SMART study[78] supported the association between abacavir and myocardial infarction, but found no effect of didanosine use on the overall risk of cardiovascular disease. It has been suggested that increased platelet adhesiveness and reduced endothelial reactivity[79] in addition to elevated levels of pro-inflammatory cytokines[78] may be the underlying mechanisms behind this potential adverse effect by abacavir. However, in a pooled analysis of 50 clinical trials by Brothers *et al*[80] no additional link between abacavir and myocardial infarction was identified.

The use of PIs was associated with a three-fold increase in incidence of diabetes mellitus in a large cohort of HIV-positive women, while no change in incidence was noted between NRTI/NNRTI-treated and HAART-naïve patients (individual drugs were not specified)[81]. However, the CREATE2 study[81] found an association between efavirenz and incidence of metabolic syndrome, while nevirapine use was more common in the non-metabolic syndrome group.

1.2.2.4 Central nervous system (CNS) effects

Although the incidence of HIV-associated dementia (a neurological disorder associated with HIV infection) has declined in response to HAART, neurocognitive impairment may persist, probably due to viral reservoirs[82]. This is supported by the finding that better neurological recovery was associated with treatment combinations of three or more highly CNS-penetrating ARV drugs[83]. Examples of those are zidovudine, nevirapine, indinavir/ritonavir (grade 4/highest penetration on a scale of 1-4) or abacavir, emtricitabine, efavirenz, darunavir/ritonavir (grade 3 penetration)[84]. Despite the beneficial effect of viral suppression, ARV drugs may also cause CNS side effects: there have been case reports of neuropsychiatric complications in conjunction with zidovudine (psychosis), abacavir (headache, depression) and nevirapine (cognitive impairment, depression) use[85]. However, efavirenz is the ARV drug most commonly associated with CNS toxicity. More than 50% of patients initiating treatment with efavirenz experience neuropsychiatric disturbances, manifesting as dizziness, impaired concentration, insomnia, and abnormal dreams[86]. These symptoms which may persist beyond two years of treatment[87] can sometimes be the cause of efavirenz discontinuation (25.1% of patients in the TRT-5 Group[88] and 16.4% in the EuroSIDA Study[89]).

1.2.2.5 Bone abnormalities

HIV infection is associated with reduced bone mineral density (BMD)[90], however several studies indicate that loss of BMD may be worsened by HAART. Fernández-Rivera *et al*[91] showed a correlation between PI therapy and loss of BMD – although in this study osteopenia did not progress beyond 1 year of continued HAART. The SMART Body Composition substudy[92] found a more rapid and progressive decline in BMD in patients on continuous compared to intermittent HAART, with a mean follow-up time of 2.4 years. Association between HAART and lower BMD was also demonstrated in a paediatric study by Mora *et al*[93] where no difference was seen between naïve HIV-infected controls and healthy children. A meta-analysis by Brown & Qaqish[94] of 20 individual studies showed a 3-fold increased prevalence of reduced BMD among HIV-infected subjects compared to non-infected controls, as well as a 2.5-fold increased prevalence among HAART-exposed patients compared to HAART-naïve patients. Furthermore, PI-treated patients had increased odds of reduced BMD and osteoporosis compared to patients on non-PI containing regimens. However, the impact of HAART remains controversial: Cazanave *et al*[95] and García Aparicio *et al*[96] both suggest there is no relationship between the use of HAART and osteopenia or osteoporosis.

Vitamin D deficiency is a risk factor for osteopenia; both PI-[96] and NNRTI-containing[97] therapy have been associated with decreased vitamin D levels. Interestingly, *in vitro* studies have shown impairment of vitamin D bioactivation through 25-hydroxylase inhibition by both efavirenz[98] and PIs[99].

1.2.2.6 Nephropathy

HIV-associated nephropathy is common, especially among Africans[100]. While administration of effective HAART generally leads to improved renal function[101], certain ARV drugs can cause renal dysfunction: PIs indinavir[102] and atazanavir[103] are partly excreted through the kidney and may precipitate, leading to nephrolithiasis and crystaluria in general. Indinavir has however been replaced with newer PIs and atazanavir-mediated nephrotoxicity is exceedingly rare. A systematic review of renal safety of the widely used NRTI tenofovir found an association with decreased creatinine clearance, but no evidence of increased risk of severe proteinuria[104]. Disruption of mitochondrial function (see section 1.2.2.2) is the presumed cause of tubular necrosis and reduced glomerular filtration rates[105]. Abated renal function may have a significant impact on the clearance of co-administered drugs.

1.2.2.7 *Mother-to-child transmission and teratogenicity*

An estimated 370,000 children were infected with HIV in 2009 through mother-to-child transmission (UNAIDS Global Report 2010). While in high-income countries ARV drugs are used during pregnancy to prevent this, limited access to therapy in low-income settings may result in nevirapine single-dose intrapartum and neonatal treatment only[106]. The World Health Organization recommends nevirapine as first-line treatment in pregnancy because of proven safety and efficacy in reducing mother-to-child transmission[107]. Evidence from animal and cohort studies indicates potential teratogenicity of efavirenz, zidovudine and delavirdine, limiting their use in pregnant women[108]. However, zidovudine monotherapy is also commonly used[109]. A study investigating birth defects in infants born to HIV-infected mothers in UK and Ireland found no association with ARV drug exposure[110], but more research into this field is required. The prevailing opinion is however that the massive benefits of ARV prophylaxis for prevention of mother-to-child transmission far outweigh the potential for adverse effects[111]: treatment in combination with elective caesarean delivery and avoidance of breast-feeding can reduce mother-to-child-transmission from 25-40% (developing countries) to 1-2% (well-resourced health care systems)[112].

1.3 The importance of achieving adequate ARV drug plasma concentrations

Successful HAART is dependent on maintaining sufficient plasma concentrations of the ARV drugs. Minimum effective concentrations (MECs) have been established for each individual drug to achieve adequate viral suppression. The inhibitory quotient (IQ) describes the relationship between drug plasma levels and drug resistance: for a given individual, it is calculated as the ratio of the trough drug concentration (C_{trough}) over the inhibitory concentration (IC_{50}) for that individual's HIV variant. IQ has been used to improve predictions of virological response[113]. Once viral loads are reduced, immune recovery normally follows. In addition to the primary goal of viral suppression with the aim of improving patient health, there are additional long-term benefits related to avoiding development of drug resistance. By maintaining drug plasma concentrations above the MEC, the risk of resistance development is decreased through two mechanisms: both by inhibition of viral replication and hence diminishing the opportunity for mutations to arise, but also by not allowing minor genetic variants already present - which may have a fitness advantage - to proliferate. Preventing development of drug resistance and cross-resistance may help conserve treatment options for the future. Furthermore, it has been demonstrated that adequate viral suppression significantly reduces both

mother-to-child[107, 112] and sexual[43, 44] transmission, resulting in a community or even global benefit in addition to personal gain.

It is also desirable to avoid excessively high drug plasma concentrations in order to reduce the risk of toxicity. For these reasons, therapeutic drug monitoring (TDM)[114] has been recommended for optimal HIV care, in particular for PIs which show a high degree of pharmacokinetic variability: for example a 12-fold variability in saquinavir $AUC_{0\text{-}8h}$[115], a 3.5-fold variability in lopinavir $AUC_{0\text{-}12h}$[116] and 63% coefficient of variation of nelfinavir trough levels[117] have been demonstrated.

Factors influencing ARV plasma drug concentrations include adherence[118], food intake[119], weight[120, 121] and gender[122]. Poor adherence is a common issue in HIV treatment and a crucial confounding factor for any clinical ARV study. Reasons may include affordability of medication[118], side effects or a high pill burden[123], of which the latter has been addressed by the development of combination pills incorporating two or three ARV drugs, making the "one pill a day"-approach a reality. Psychosocial factors including mental illness, depression, substance abuse, or a history of sexual or physical abuse, are also barriers to ARV adherence[124].

PIs with the exception of indinavir are highly protein bound (>90%) in plasma, mainly to α_1-acid glycoprotein. Efavirenz is more than 99% bound, primarily to albumin, while NRTIs are not highly protein bound. This may affect the volume of distribution (V_D) of a drug as only the free (unbound) fraction can enter cells. Plasma protein binding is also an issue for accurately establishing drug concentrations using *in vitro* pharmacological methods; in general bovine serum is used in assay media and unbound concentrations are not directly measured[125].

Other major factors influencing ARV plasma drug concentrations are drug interactions, which are commonly encountered as part of HIV therapy, and pharmacogenetics, both of which are discussed below.

1.4 Drug interactions

ARVs have high potential for drug interactions, complicating the choice of medication. This is mainly due to either inhibition of the activity of metabolising enzymes and drug transporters, or induction of

gene expression of metabolising enzymes and drug transporters through nuclear receptor activation. All of these factors may have a significant impact on the pharmacokinetics of ARV or co-administered drugs. Reduced drug plasma levels below the MEC may lead to lack of therapeutic efficacy, while higher-than-normal drug plasma levels increase the risk of drug toxicity. The individual mechanisms of drug interactions are outlined below.

1.4.1 Cytochrome P450 inhibition

Of the PIs, ritonavir is the most potent inhibitor of cytochrome P450 (CYP450) enzymes (CYP3A4[126], CYP3A5[127], CYP3A7[127], CYP2B6[128, 129], CYP2C9[126] and CYP2D6[130]). CYP3A4 is the most abundant hepatic[131] and intestinal[132] CYP450 enzyme and a major contributor to the metabolism of more than 50% of commonly prescribed pharmaceuticals[131], including PIs and NNRTIs. Nowadays, ritonavir is only used in low dose as a pharmacoenhancer, "boosting" the bioavailability of a concomitantly administered PI. This gives the benefit of increased bioavailability of the partner PI without significant ritonavir-associated side effects[133, 134]. CYP450 enzymes which may also be inhibited by other ARVs (however with lower potency than ritonavir) include CYP3A4[126, 135, 136] (in order of decreasing potency: indinavir > nelfinavir > amprenavir > atazanavir > saquinavir), CYP3A5[127] (nelfinavir > amprenavir > saquinavir > indinavir), CYP3A7[127] (nelfinavir < amprenavir < saquinavir < indinavir), CYP2B6 [128, 129] (nelfinavir > efavirenz) and CYP2C9[126] (saquinavir > indinavir).

1.4.2 Inhibition of drug transporters

Additionally, many PIs are also inhibitors (ritonavir > nelfinavir > indinavir >> saquinavir) of P-glycoprotein (P-gp)[137]. This drug efflux pump, belonging to the ATP-binding cassette (ABC) transporter family, is highly expressed at barrier sites such as the intestine, blood-brain barrier, renal tubules and placenta[138]. There is additional evidence of PI inhibition of other ABC transporter efflux pumps; multidrug-resistance associated proteins MRP1[137] (saquinavir, ritonavir, nelfinavir and indinavir) and MRP2[139] (saquinavir, ritonavir and atazanavir). Inhibition of organic anion transporting polypeptides (OATPs; influx transporters)[140] has also been demonstrated by tipranavir, ritonavir, lopinavir, nelfinavir and atazanavir. These transmembrane carrier systems all have important roles in drug distribution and modulation of their activity can significantly affect absorption, compartment penetration and intracellular drug concentrations.

1.4.3 Induction of pregnane X receptor (PXR)

In 2001, Dussault *et al*[141] identified ritonavir as a ligand of pregnane X receptor (PXR). This nuclear receptor is an important regulator of xenobiotic metabolism-related gene expression. Target genes include several CYP450 enzymes and drug transporters, for example *CYP3A4*[142], *CYP2B6*[143], *ABCB1/MDR1* (P-gp)[144] and *MRP2*[145]. Dussault *et al*[141] could also demonstrate increased protein expression of CYP3A4, P-gp and MRP2 after ritonavir exposure in primary human hepatocytes. A subsequent study by Gupta *et al*[146] revealed a number of additional PIs (amprenavir, lopinavir, tipranavir, saquinavir, atazanavir, indinavir) to also be PXR activators (Figure 1.4) and capable of inducing transcription of *CYP3A4* and *ABCB1/MDR1*. Hariparsad *et al*[147] showed that NNRTI efavirenz similarly has PXR-inducing abilities with corresponding increases in CYP3A4 activity.

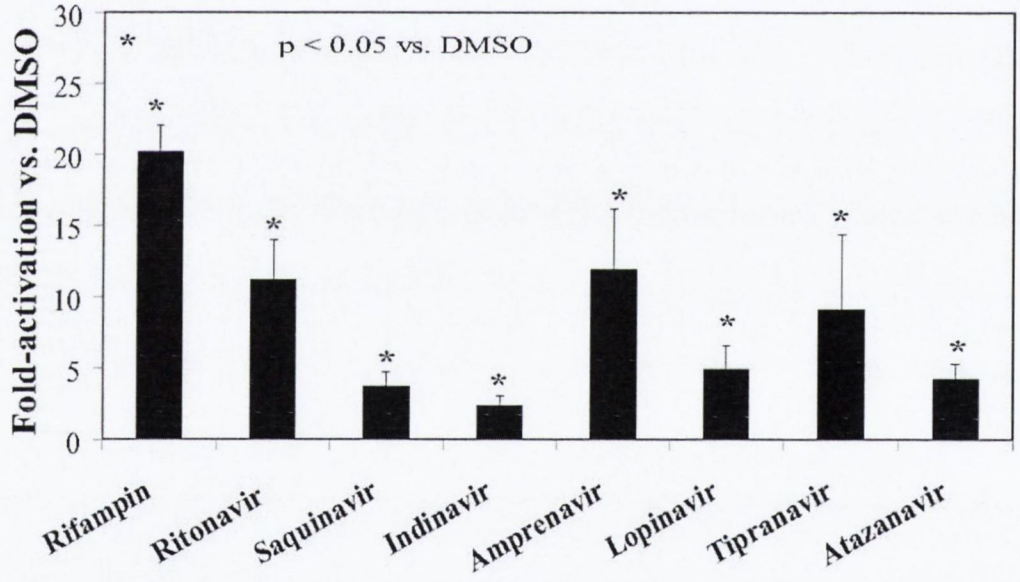


Figure 1.4 PXR activation by HIV protease inhibitors (reporter assays, from Gupta *et al* 2008[146]). LS180 intestinal human colon adenocarcinoma cells were transfected with PXR expression plasmid and CYP3A4 responsive element-luciferase construct followed by treatment with test drugs (10μM) for 48h. Luciferase activity was measured and normalised to β-galactosidase activity (mean ± standard deviation, n = 3).

These effects of metabolic enzyme or drug transporter inhibition as well as induced gene expression of the same can have a profound impact on ARV drug bioavailability. Most ARVs are metabolised primarily by CYP3A4[148] or in the case of efavirenz; CYP2B6[149]. In addition, the majority of PIs[150-152] as well as NRTI abacavir[153] and newer ARV compounds maraviroc[154] and raltegravir[155] are substrates of

P-gp. Consequently, altered activity or expression of CYP3A4, CYP2B6 and P-gp will affect plasma and intracellular concentrations of ARV drugs.

1.4.4 Non-ARV co-medications

As co-infections and co-morbidities are frequent among HIV-infected patients, concomitant medication in addition to HAART is common. In the Swiss HIV Cohort Study[156], 68% of the patients were receiving non-ARV co-medications. These were mainly central nervous system (CNS) drugs (e.g. antidepressants, anxiolytics), cardiovascular drugs (e.g. lipid-lowering agents) and methadone. The pharmacokinetics of any drug metabolised through the CYP450 system is likely to be affected by co-administration of ARVs, due to their inhibiting or inducing effects and additionally as they are competing substrates. For example, Clarke *et al*[157] showed significant reductions in methadone plasma concentrations in former intravenous drug-using HIV patients initiating treatment with efavirenz. These patients also experienced opioid withdrawal symptoms. In the Swiss HIV Cohort Study mentioned above, 40% of the patients had one or more potential drug-drug interactions. In a Kenyan cohort[158] 33.5% of patients were at risk of clinically significant drug-drug interactions; in this setting these involved mainly medication for tuberculosis and fungal infections, steroids and antimalarials.

Drugs such as macrolide antibiotics (e.g. erythromycin, clarithromycin)[159, 160] and azole antifungals (e.g. ketoconazole, fluconazole)[161] are usually avoided in HAART patients as they are themselves inhibitors of CYP3A4[162]. Rifampicin, although sometimes used for treatment of tuberculosis co-infection[163], is a highly potent PXR inducer[164] and hence rifabutin is preferred. Other therapeutics which can exacerbate the risk of drug interactions include cisplatin, an inducer of P-gp expression through PXR[165]. However, cisplatin has been included in chemotherapy of anal carcinomas[166] and non-Hodgkin's lymphoma[167] in HAART-treated patients.

Herbal remedies can also contribute to drug interactions. For example, St John's Wort has been identified as a potent inducer of CYP3A4[168] and P-gp expression[169], and patients are recommended to avoid it. Some herbal medicines commonly used among HIV patients in Africa[170-172] or in Chinese traditional medicine[173-175] have also been identified as PXR inducers or inhibitors of CYP450 and/or P-gp.

As a result of HAART, the life expectancy for HIV-infected patients in the developed world has increased substantially: according to the ATHENA study[176] conducted in the Netherlands between 1998 and 2007, the time to death for an individual diagnosed with HIV at age 25 is now approaching that of the general population. Older patients are more likely to require treatment for conditions such as cardiovascular disease, arthritis and diabetes[177, 178], leading to polypharmacy and further increasing the risk of drug interactions.

1.5 Pharmacogenetics

Considerable interindividual variability in antiretroviral drug concentrations and responses has been observed in clinical settings. In a Spanish study[179] about 70% of patients had plasma concentrations within the therapeutic range and in their analysis poor adherence only explained 35% of subtherapeutic concentrations. In an Italian cohort[180] suboptimal drug levels were found in 17% of patients and were also associated with virological failure – unfortunately as this was a retrospective study no information on adherence was available. HIV infection itself has been linked to decreased CYP3A4 activity as well as increased variability in the activity of CYP2D6 and CYP1A2, most likely due to immune activation and cytokine exposure[181]. Genetic polymorphism in metabolising enzymes and drug transporters may also account for variability in plasma drug levels. A substantial amount of research has been dedicated to this field, revealing genetic markers of ARV drug efficacy with varying prevalence across ethnic groups. Examples of identified single nucleotide polymorphisms (SNPs) with relevance for ARV pharmacokinetics are given below and summarised in Table 1.1.

1.5.1 Genetic polymorphism in CYP450 metabolising enzymes

Surprisingly few polymorphisms in *CYP3A4*, the most abundant hepatic CYP450 isoform, have been associated with altered expression or activity of the enzyme, despite substantial evidence of interindividual differences in metabolism[182]. Some examples of *CYP3A4* SNPs with impact on enzyme activity exist: promoter polymorphism -392A>G results in significantly decreased CYP3A4 activity[183]. It has been associated with decreased absorption of indinavir[184] and lower clearance of efavirenz[185], however other researchers found no effect on ARV drug levels[55, 186, 187]. *CYP3A4* 566T>C also exhibits decreased enzyme activity, whereas 878T>C increases enzyme activity[188]. Although a Japanese

study[189] showed no effect of 878T>C on efavirenz levels, only two patients included in this study carried this polymorphism.

Even though CYP2B6 is not as highly expressed as CYP3A4, polymorphism in this gene may be of greater importance to ARV pharmacokinetics as it displays large interindividual, inter-ethnic and gender differences in expression[190]. Additionally, it is involved in the metabolism of NNRTIs efavirenz and nevirapine. The *CYP2B6* 516G>T polymorphism has been associated with raised plasma concentrations of both drugs[185, 191-193] as well as increased risk of efavirenz toxicity[194, 195]. A higher proportion of homozygotes for this allele has been found in African Americans in comparison to Americans of European ancestry[194].

CYP3A5 6986A>C is a common splice defect resulting in a premature termination of the transcript and severely reduced enzyme activity, in fact the frequency of the functional wild-type allele is only 5% in Caucasians while in African Americans it is 73%[196]. CYP3A5 deficiency may not be of great clinical importance as most drugs metabolised by this enzyme are also substrates of CYP3A4. Nevertheless, lower clearance of atazanavir[197], indinavir[198] and saquinavir[199] has been reported among CYP3A5 non-expressors.

Table 1.1 Examples of genetic polymorphism in metabolising enzymes and drug transporters with effects on antiretroviral drug plasma concentration. SNP = single nucleotide polymorphism, ARV = antiretroviral, IDV = indinavir, EFV = efavirenz, NVP = nevirapine, ATV = atazanavir, SQV = saquinavir, NFV = nelfinavir, LPV = lopinavir, N.A. = not available.

Gene	SNP	SNP effect	Effect on ARV plasma conc.
CYP3A4	-392A>G	Promoter polymorphism	↓IDV[184], ↑EFV[185] (controversial)
CYP2B6	516G>T	Q172H	↑NVP, ↑EFV[185, 191, 193]
CYP3A5	6986A>C	Splice defect	↑ATV[197], ↑IDV[198], ↑SQV[199]
ABCB1/MDR1	3435C>T	I1145I (synonymous)	↓EFV, ↓NFV[186] (controversial)
OATP1B1/*SLCO1B1*	521T>C	N.A.	↑LPV[200]

1.5.2 Genetic polymorphism in drug transporters

As transmembrane transport proteins (mainly of the ABC family) are essential for the absorption, distribution and clearance of drugs, mutations in these genes can also affect ARV plasma drug levels. Furthermore, ABC transporters and OATPs can facilitate drug permeation into sanctuary sites for HIV such as brain, testis, lymphocytes and macrophages[201, 202]. With lymphocytes being the main target cells of HIV, variation in expression of P-gp (*ABCB1/MDR1*), which is naturally abundant in this cell type, can have a profound effect on intracellular ARV pharmacokinetics[203, 204].

Although the *ABCB1/MDR1* polymorphism 3435C>T is synonymous and does not result in an amino acid switch, the T allele is associated with a loss of mRNA stability[205] . There has been a considerable amount of research into the impact of this SNP on ARV therapy, however with some conflicting results: one study reported decreased efavirenz and nelfinavir plasma drug levels but greater immune recovery[186], whereas other researchers found no influence on the concentration of these drugs[189, 194] or others (atazanavir[206], indinavir[207], boosted lopinavir[208], ritonavir[209] and saquinavir[210]). In fact, experiments indicate that efavirenz is not a substrate of P-gp: uptake of efavirenz was not affected by P-gp inhibitors *in vitro*[211] and in rat and mouse brain[212]. On the other hand, Solas *et al*[213] found evidence of a higher absorption rate of indinavir in patients heterozygous for this polymorphism (3435CT genotype) compared to CC homozygotes.

Genetic polymorphism in MRPs and OATPs are less well studied in the context of ARV plasma drug concentrations. However, an inverse correlation between *MRP2/ABCC2* expression and intracellular concentration of zidovudine in peripheral blood mononuclear cells has also been observed[214] and a polymorphism in the gene encoding OATP1B1 (*SLCO1B1* 521T>C) has been associated with higher trough levels of lopinavir[200].

Despite the fact that the Sub-Saharan African continent carries the biggest burden of HIV disease with over two thirds of the world's infected population, most clinical trials investigating efficacy and toxicity of ARV drugs and likewise pharmacogenetic studies are carried out with predominantly Caucasian subjects, and Sub-Saharan Africans are underrepresented. Many American studies include African American patients (however their participation tends also not to be in proportion to the overall disease population[215, 216]) and results are often extrapolated to native Africans in spite of

potentially great genetic diversity. Further research into pharmacogenetic differences between ethnic groups in the context of ARV treatment is warranted.

1.5.3 Genetic polymorphism in nuclear receptors

Although substantial research has been conducted into the impact of polymorphisms in metabolising enzymes and drug transporters on ARV plasma drug concentrations, only one association to date has been identified with genetic variability in *NR1I2* encoding nuclear receptor PXR, a major regulator of CYP450 and ABC transporter expression: Siccardi *et al*[217] found that 63396T>C was associated with concentrations of unboosted atazanavir below the MEC and Schipani *et al*[218] confirmed higher clearance in homozygous individuals. A recent pharmacogenetics study by Lamba *et al* (2010)[219] highlights the impact of nuclear receptor polymorphism on CYP3A4 expression: along with sex and genetic variation in the *CYP3A4* promoter and *ABCB1/MDR1* sequences it can account for as much as 24.6% of the variation in hepatic CYP3A4 expression.

1.6 Nuclear receptors

The key theme of this thesis is interactions between ARV drugs and nuclear receptors. As important regulators of gene expression, nuclear receptors are implicated in a multitude of biological processes such as cell growth, inflammation, energy homeostasis, and drug metabolism. Here we outline their discovery and characterisation. With focus on pregnane X receptor (PXR), its endogenous and xenobiotic functions are described as well as cross-talk with other nuclear receptors, and finally its relevance for ARV drug bioavailability.

1.6.1 Structure, function and classification

Over the last 40 years remarkable advances have been made in the research field of nuclear receptors, revealing their importance in the regulation of various diverse processes necessary for the survival of the organism: reproduction, growth, energy metabolism, detoxification of xenobiotics and inflammation[220]. Estrogen receptor (ER) was first identified as a mediator of steroid action[221], and subsequent work indicated the involvement of transcriptional activity in the nucleus by the glucocorticoid receptor (GR)[222]. cDNA of the latter was cloned in 1985, enabling the discovery of related sequences through low stringency hybridisation[223]. In the 90s, the favoured method of

identification shifted from *in vitro* to *in silico* when new "orphan" receptors (so-called because their ligands were unknown) were identified in Expressed Sequence Tag (EST) or genome databases through sequence homology. The publication of the human genome in 2001[224, 225] surprisingly yielded only three new nuclear receptor candidates, all of which however contained multiple stop codons within their coding regions[226]. 48 human nuclear receptors have been identified to date (Table 1.2).

In 1999, a unified nuclear receptor nomenclature system was suggested by an international committee[227]. The receptors are divided into subfamilies and groups based on sequence alignment and known functional groups of receptors, designated by a number and a letter, respectively (i.e. *NR1H3* corresponds to nuclear receptor subfamily 1, group H, member 3). This system is similar to one which was developed for the cytochrome P450 enzymes[228].

Figure 1.5 Common domain structure of nuclear receptors (modified from Nagy & Schwabe 2004[229]). The N- and C-terminals are indicated. AF-1 = activation function 1 (ligand independent); AF-2 = activation function 2 (ligand dependent), DBD = DNA binding domain, LBD = ligand binding domain.

Structural characterisation revealed some distinguishing features of the newly identified nuclear receptor family: a central DNA-binding domain (DBD) accommodating two highly conserved zinc fingers; a ligand-binding domain (LBD) at the C-terminal end conferring receptor specificity and selectivity[230]; an activation function domain at the N-terminal (ligand independent, AF-1) as well as an activator function domain within the LBD (ligand dependent, AF-2)[229] (Figure 1.5). There is a high degree of conservation between mammalian species, and the presence of multiple nuclear receptor orthologs between vertebrates and invertebrates suggests early development of these receptors in evolution[231, 232]. The amino acid sequence identity between human and rodent DBDs and LBDs often exceeds 95% and 85%[233], respectively, however there are two exceptions to this rule: pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which are both xenosensors. A comparison of LBD amino acid sequences between human and mouse PXR shows only 77%

homology[234] (Figure 1.8A), while for CAR it is as low as 73%[235]. Hence, these nuclear receptors show a greater species diversity of ligand specificity. This could reflect an adaptive response to different environmental xenobiotic challenges or differences in endogenous ligands between species[236].

In the absence of a ligand, the receptors either reside in the cytoplasm and translocate to the nucleus upon ligand binding, or stay in the nucleus constitutively bound to DNA and repressing transcription until binding of a ligand. This occurs through a conformational change resulting in the dissociation from co-repressors (such as nuclear receptor co-repressor, NCoR, or silencing mediator for retinoic acid and thyroid hormone receptor, SMRT) and the association with co-activators (for example receptor-interacting proteins, RIPs)[237]. The activated receptor binds to response elements usually located distal to the promoters of target genes[238]. They consist of direct (DR), everted (ER) or inverted repeats (IR) of a core hexamer (AGGTCA, some variations exist) separated by a small number of nucleotides; the number designating receptor specificity[239, 240] (Figure 1.6). For example, the liver X receptor (LXR) response element is DR-4, i.e. the direct repeats are separated by four nucleotides[241, 242].

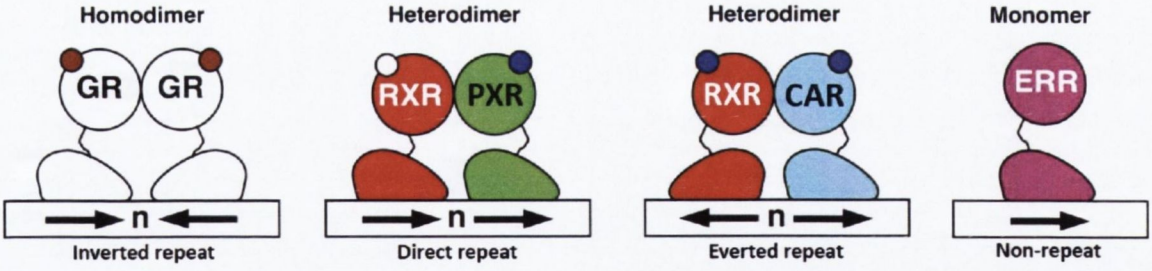


Figure 1.6 Nuclear receptor dimerisation and binding to DNA response elements (examples). Modified from Sonoda *et al* 2008[243]. GR = glucocorticoid receptor, RXR = retinoid X receptor, PXR = pregnane X receptor, CAR = constitutive androstane receptor, ERR = estrogen-related receptor, n = number of spacer nucleotides between consensus sequence.

Based on their physiological ligands and potential functions, the nuclear receptor superfamily can be broadly categorised into three subgroups (Sonoda *et al*[243], see Table 1.2): *endocrine receptors* including steroid receptors which have high affinity for lipophilic hormones, and vitamin receptors; *adopted orphan receptors* identified through sequence homology with endocrine receptors and

subsequently "adopted" as their naturally occurring ligands were determined; and *"true" orphan receptors* without known ligands, neither natural nor synthetic[243]. "True" orphan receptors appear to be activated by co-activator availability and will not be discussed in this thesis.

1.6.2 Endocrine nuclear receptors

Receptors of steroid hormones typically homodimerise and bind to inverted repeats upon activation by nanomolar concentrations of glucocorticoids (GR/*NR3C1*), mineralocorticoids (mineralocorticoid receptor, MR/*NR3C2*), progesterone (progesterone receptor, PR/*NR3C3*), androgen (androgen receptor, AR/*NR3C4*) and estrogen (ERα/*NR3A1* and ERβ/*NR3A2*). The group of endocrine nuclear receptors also includes thyroid hormone receptors (TRα/*NR1A1*, TRβ/*NR1A2*) and vitamin D receptor (VDR/*NR1I1*). TRα and TRβ mediate the activity of thyroid hormone (triiodothyronine, T3), influencing diverse metabolic pathways important in lipid and glucose metabolism, lipolysis and regulation of body weight[244]. VDR responds to calcitriol (vitamin D) and regulates calcium and phosphate homeostasis and consequently bone mineralisation. The more recent discovery that bile acids are also potent VDR ligands indicates a supplementary function in protection against their toxic effects in the gastrointestinal tract[245]. TRs and VDR are heterodimerisation partners of retinoid X receptors (RXRα/*NR2B1,* RXRβ/*NR2B2,* RXRγ/*NR2B3*), which bind to direct repeats in the DBD. The RXR heterodimer-forming receptors can be permissive or non-permissive, indicating whether or not the complex can be activated by RXR agonists alone[246].

1.6.3 Adopted orphan receptors

RXR was the first described "adopted" orphan receptor responding specifically to vitamin A metabolites[247]. However, further research also revealed its unique and essential role as a heterodimer partner to several other nuclear receptors[248, 249]. It can also form homodimers, indicating the presence of an independent RXR signalling pathway[250, 251]. In addition to promoting transcription of target genes when bound to ligands, RXRs as well as retinoic acid receptors (RARα/*NR1B1*, RARβ/*NR1B2*, RARγ/*NR1B3*) can inhibit transcription by recruitment of co-repressors in their unliganded state[252]. Another RXR heterodimerisation partner primarily activated by bile acids is FXR (*NR1H4*), which is highly expressed in liver, intestine and kidney[253]. It is the major regulator of bile salts largely through transcriptional regulation of cytochrome P450 7A1 (CYP7A1), an enzyme that performs the rate-limiting step in their synthesis[254], but also through control of bile acid export from

the liver[255]. FXR shares several target genes with the structurally related LXRs (LXRα and LXRβ, *NR1H2* and *NR1H3*) including CYP7A1 and the lipogenic transcription factor sterol regulatory element binding protein-1 (SREBP-1), however whereas the LXRs activate these genes directly FXR acts indirectly by induction of the small heterodimer partner (SHP, *NR0B2*)[253]. SHP is an atypical nuclear receptor which lacks DBD and appears to have no endogenous ligand but rather behaves as a constitutive repressor of other nuclear receptors[255]. Endogenous ligands of LXRα and LXRβ include oxysterols, derivatives of cholesterol. They form an essential part of the body's governing mechanism of absorption, transport, storage and metabolism of cholesterol, as illustrated by *Lxrα* knockout mice which are unable to tolerate dietary cholesterol and soon develop hypercholesterolemia[256]. The peroxisome-proliferator activated receptors (PPARα/*NR1C1*, PPARβ/*NR1C2*, PPARγ/*NR1C3*) respond to fatty acids and are clinically important in treatment of hyperlipidemia (fibrates, acting on PPARα/*NR1C1*) and type II diabetes (thiazolidinediones, acting on PPARγ/*NR1C3*)[257], although several therapeutics from the latter group ("glitazones") have been retracted from the market due to adverse effects. CAR (*NR1I3*) and PXR (*NR1I2*) are of clinical significance due to their activation by xenobiotics (potentially toxic foreign compounds), leading to induced metabolism and excretion from the body. Of these two, PXR has been more often implicated in mediation of drug interactions and genetic polymorphism in this gene has been associated with certain disease states.

Table 1.2 Human nuclear receptors. Modified from Germain *et al*[241]. Additional response element information and categorisation: Sonoda *et al*[243] and Wilson *et al*[258]. H = homodimer, M = monomer, RXR = RXR heterodimer, Het = heterodimer with other nuclear receptors, DR = direct repeat, IR = inverted repeat, NR = non-repeat.

Name	Gene	Ligand(s)	Dimer	Response element	Category
TRα	NR1A1	Thyroid hormones	H	AGGTCA, DR-4	Endocrine
TRβ	NR1A2	Thyroid hormones	H	AGGTCA, DR-4	Endocrine
RARα	NR1B1	Retinoic acid	RXR	AGGTCA, DR-2, DR-5	Endocrine
RARβ	NR1B2	Retinoic acid	RXR	AGGTCA, DR-2, DR-5	Endocrine
RARγ	NR1B3	Retinoic acid	RXR	AGGTCA, DR-2, DR-5	Endocrine
PPARα	NR1C1	Fatty acids, leukotriene B_4, fibrates	RXR	AGGTCA, DR-1	Adopted
PPARβ/δ	NR1C2	Fatty acids	RXR	AGGTCA, DR-1	Adopted
PPARγ	NR1C3	Fatty acids, PG J_2, thiazolidinediones	RXR	AGGTCA, DR-1	Adopted
Rev-erbα	NR1D1	?	M	AGGTCA, NR, DR-2	Orphan
Rev-erbβ	NR1D2	?	M	AGGTCA, NR, DR-2	Orphan
RORα	NR1F1	Cholesterol, cholesteryl sulphate	M	WWCWRGGTCA, NR	Adopted
RORβ	NR1F2	Retinoic acid	M	WWCWRGGTCA, NR	Adopted
RORγ	NR1F3	?	?	WWCWRGGTCA, NR	Orphan
LXRα	NR1H3	Oxysterols, T0901317, GW3965	RXR	RGKTCA, DR-4	Adopted
LXRβ	NR1H2	Oxysterols, T0901317, GW3965	RXR	RGKTCA, DR-4	Adopted
FXRα	NR1H4	Bile acids, fexaramine	RXR	AGGTCA, IR-1, DR-5	Adopted
VDR	NR1I1	Vitamin D, 1,25-dihydroxyvitamin D_3	RXR	AGGTCA, DR-3	Endocrine
PXR	NR1I2	Xenobiotics, 16α-cyanopregnenolone	RXR	RGKTCA, DR-4	Adopted
CAR	NR1I3	Xenobiotics, phenobarbital	RXR	RGKTCA, DR-5	Adopted
HNF4α	NR2A1	?	?	AGGTCA, DR-1	Orphan
HNF4γ	NR2A2	?	?	AGGTCA, DR-1	Orphan
RXRα	NR2B1	Retinoic acid	RXR/Het	AGGTCA, DR-1	Adopted
RXRβ	NR2B2	Retinoic acid	RXR/Het	AGGTCA, DR-1	Adopted
RXRγ	NR2B3	Retinoic acid	RXR/Het	AGGTCA, DR-1	Adopted
TR2	NR2C1	?	?	AGGTCA, DR-1	Orphan
TR4	NR2C2	?	?	AGGTCA, DR-1	Orphan
TLL	NR2E2	?	?	AGGTCA, NR	Orphan
PNR	NR2E3	?	?	?	Orphan
COUP-TFI	NR2F1	?	?	RGGTCA, DRs, IRs	Orphan
COUP-TFII	NR2F2	?	?	RGGTCA, DRs, IRs	Orphan
EAR2	NR2F6	?	?	?	Orphan
ERα	NR3A1	Estradiol-17β, tamoxifen, raloxifene	H	AGGTCA, IR-3	Endocrine
ERβ	NR3A2	Estradiol-17β	H	AGGTCA, IR-3	Endocrine
ERRα	NR3B1	?	M	TCAGGTCA, NR	Orphan
ERRβ	NR3B2	DES, 4-OH tamoxifen	M	TCAGGTCA, NR	Adopted
ERRγ	NR3B3	DES, 4-OH tamoxifen	M	TCAGGTCA, NR	Adopted
GR	NR3C1	Cortisol, dexamethasone, RU486	H	AGAACA, IR-3	Endocrine
MR	NR3C2	Aldosterone, spironolactone	H	AGAACA, IR-3	Endocrine
PR	NR3C3	Progesterone, MPA, RU486	H	AGAACA, IR-3	Endocrine
AR	NR3C4	Testosterone, flutamide	H	AGAACA, IR-3	Endocrine
NGFI-B	NR4A1	?	M	AAAGGTCA, NR	Orphan
NURR1	NR4A2	?	?	?	Orphan
NOR1	NR4A3	?	?	?	Orphan
SF1	NR5A1	?	M	?	Orphan
LRH-1	NR5A2	?	?	?	Orphan
GCNF	NR6A1	?	?	?	Orphan
DAX-1	NR0B1	?	?	?	Orphan
SHP	NR0B2	?	M	?	Orphan

1.7 Pregnane X receptor: a guardian against toxicity

Pregnane X receptor (PXR) was discovered through searching public mouse EST databases, on the basis of its homology with other nuclear receptors. Its name stems from the initial finding that it could be activated by pregnanes (natural steroid derivatives)[259], however some researchers maintain it should be called SXR (steroid and xenobiotic receptor) to better describe its function[260]. Among all the members of the nuclear receptor family PXR is the most promiscuous, binding a considerable number of structurally diverse compounds. This is reflected by its large and flexible ligand-binding pocket allowing interactions with a wide range of hydrophobic molecules[261, 262]. Despite this flexibility, the ligand specificity is remarkably divergent across species, owing to lower sequence similarities in the LBD compared to most other nuclear receptors (as described in section 1.6.1). For example, the antibiotic rifampicin is a potent inducer of human and rabbit PXR, but has a negligible effect on mouse or rat PXR. By contrast, rabbit and rat PXR can be effectively induced by pregnenolone 16α-carbonitrile (PCN) whereas induction of the mouse receptor is more modest and the human receptor nearly unaffected (Figure 1.8B)[236, 263]. The difference between human and mouse PXR ligand specificity has been isolated to four amino acids in the LBD, elegantly illustrated through a quadruple mutation of the mouse PXR-LBD conveying a human-like response[261].

1.7.1 Xenobiotic function of human PXR

A large number of genes, mainly expressed at important physiological barriers such as the liver, intestine, placenta and blood-brain-barrier are subjected to transcriptional regulation by PXR (Figure 1.7). Many of these are also co-regulated by CAR, and considerable cross-talk exists between the two xenobiotic receptors[264].

1.7.1.1 PXR target genes: metabolising enzymes

Target genes of PXR include both phase I and II metabolic enzymes. The CYP450 family is responsible for both chemical modification of endogenous substrates as well as the detoxification of many xenobiotics, predominantly through oxidation of organic substances. CYP3A4 is highly expressed in the liver and small intestine, and is the subtype implicated in the metabolism of most (>50%) commonly used pharmaceuticals[131]. The CYP2B family is responsible for another 25-30% of drug and xenobiotic metabolism, and is likewise highly regulated by PXR and CAR[265]. The combined versatility

of the CYP3A and CYP2B subfamilies are of particular medical significance as modulation of these enzymes constitutes the basis of many drug-drug interactions: CYP3A4 inducers rifampicin[266], phenytoin[267, 268] and CYP3A4 inhibitor ritonavir[269] as well as CYP2B6/CYP3A4 inducer phenobarbital[270-272] have all been shown to affect the clearance of co-medication also metabolised by these enzymes. In addition to the phase I metabolising enzymes, PXR is also a regulator of phase II conjugation enzymes such as glutathione-S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs) and hydroxysteroid sulfotransferases (SULTs)[273].

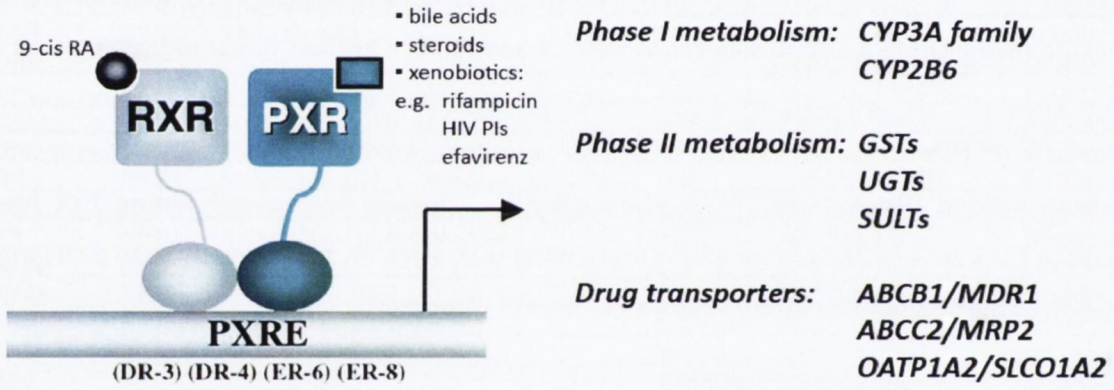


Figure 1.7 PXR-induced gene expression of metabolising enzymes and drug transporters (modified from Edwards *et al* 2002[274]). PXR = pregnane X receptor, RXR = retinoid X receptor, 9-cis RA = 9-cis retinoic acid, PIs = protease inhibitors, CYP3A = cytochrome P450 3A, CYP2B6 = cytochrome P450 2B6, GSTs = glutathione-S-transferases, UGTs = UDP-glucuronosyltransferases, SULTs = hydroxysteroid sulfotransferases, ABCB1 = ABC transporter B1, MDR1 = multidrug-resistance gene 1, ABCC2 = ABC transporter C2, MRP2 = multidrug resistance-associated protein 2, OATP1A2 = organic anion-transporting polypeptide 1A2, SLCO1A2 = solute carrier organic anion transporter 1A2.

1.7.1.2 PXR target genes: drug transporters

In addition to governing the expression of metabolising enzymes, PXR (and to some extent CAR) play a central role in regulation of drug transporters: Membrane efflux transporter P-gp (*MDR1/ABCB1*) has a pivotal function in absorption, renal secretion, biliary excretion and CNS delivery of a wide range of hydrophobic substrates such as HIV PIs[150]. *MRP2/ABCC2* facilitates excretion of conjugated anions (glucuronide, glutathione, sulphate) from hepatocytes into the bile[275]. OATP1A2/*SLCO1A2*[276] is

a bile acid transporter primarily expressed in the brain but also in liver and other organs. Similarly to P-gp, substrates of MRP2 and OATP1A2 include PIs[200, 277] and NRTIs[278]. Drug efflux by P-gp and MRPs, along with drug influx by OATPs, modulate intracellular concentrations. Consequently, PXR activity could have a significant effect on drug transporter-mediated ARV bioavailability.

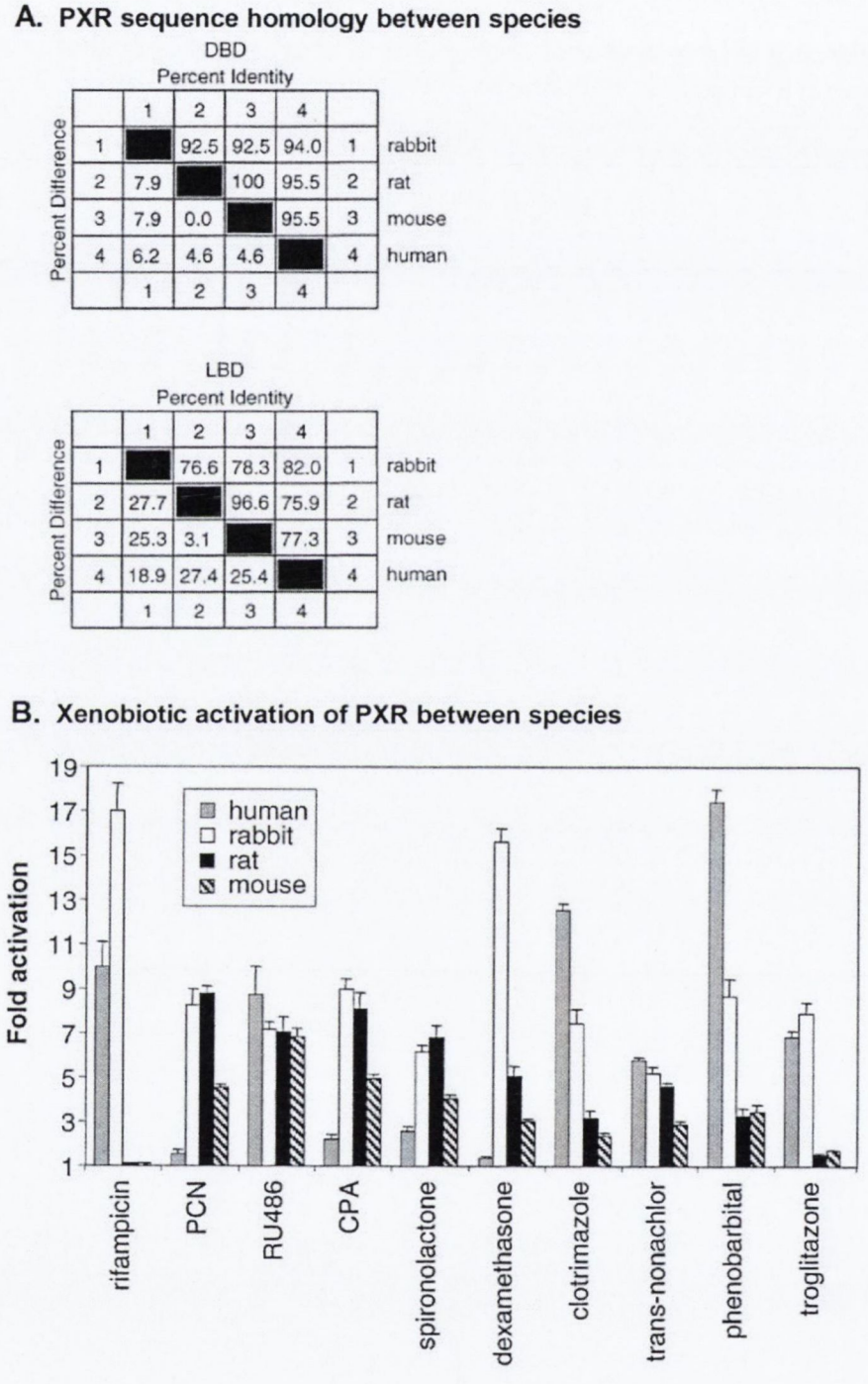


Figure 1.8 PXR species differences: comparison between human, rabbit, rat and mouse PXR (from Jones *et al* 2000[236]). A) Comparison of sequence identity in DNA binding domains (DBDs) and ligand binding domains (LBDs). B) Assessment of xenobiotic activation of PXR by reporter assays in CV-1 cells. PXR = pregnane X receptor, PCN = pregnenolone 16 α-carbonitrile, RU486 = synthetic steroid, CPA = cyproterone acetate.

1.7.2 Endobiotic function of human PXR and crosstalk with other nuclear receptors

Despite the early hypothesis that PXR functioned merely as a sensor of foreign and potentially harmful chemicals, it is now clear that it has an equally important role as an "endobiotic" receptor and often acts in orchestration with other nuclear receptors to regulate transcription of various physiological processes. Endogenous ligands include bile acids such as litocholic acid, which normally activate FXR leading to blocked catabolism of cholesterol, and bilirubin. When concentrations of these rise beyond normal levels - threatening to cause cholestasis and hyperbilirubinemia – PXR is activated. It promotes transcription of genes central to the conjugation and clearance of these potentially toxic products, forming a hepatoprotective pathway[279, 280]. LXRs on the other hand promote cholesterol breakdown and fatty acid synthesis in the liver upon activation by oxysterols and upregulate cholesterol efflux in other peripheral tissues. Their action is mediated mainly via expression of the transcription factor SREBP-1c[254]. PXR can modulate this pathway as well as induce lipogenesis independently of SREBP-1c involving upregulation of the free fatty acid uptake transporter CD36[281]. CD36 has also been associated with secretion of the anti-inflammatory cytokine interleukin-10 (IL-10)[282] and is under transcriptional control by PPARγ[283]. This nuclear receptor plays a role in adipogenesis and insulin sensitisation and as such is an important drug target for treatment of diabetes by thiazolidinediones. PPARγ additionally antagonises the pro-inflammatory effect of NF-κB through transrepression, an effect also exerted by PXR. This PXR/NF-κB crosstalk provides an explanation for immunosuppressing effects by certain PXR activators and likewise the suppression of CYP450 expression by inflammatory stimuli[284]. Impaired hepatic drug metabolism during inflammation and infection is a well-known phenomenon, and has been linked to repression of *NR1I2* (PXR) and *NR1I3* (CAR) expression by interleukin-6 (IL-6)[285]. Furthermore, polymorphisms in *NR1I2* (PXR) have been associated with susceptibility to inflammatory bowel disease[286].

PXR plays a role in homeostasis of adrenal steroid hormones as demonstrated by increased levels of corticosterone and aldosterone following rifampicin treatment in humanised mice[287], and conversely activation of GR can induce expression of CYP3A4[288]. Furthermore, PXR has been suggested to have an impact on bone metabolism through indirect binding to VDR response elements in the promoter sequence of CYP24, a major vitamin D degradation enzyme[289, 290]. There is also evidence of PXR regulation of genes involved in osteoblastic differentiation following activation by vitamin K[291].

In summary, PXR forms part of the regulation of many biological processes in concert with other nuclear receptors. PXR agonists could potentially be used in treatment of bile acid-associated cholestasis, hyperbilirubinemia and inflammatory bowel disease while PXR antagonists may be useful for preventing drug-drug interactions. Its broad role in both xenobiotic metabolism and endogenous pathways may however limit their application as therapeutic candidates[273, 292].

1.7.3 Implications of genetic variation of human PXR

The genomic structure of the *NR1I2* (PXR) gene, expressed in liver, colon and small intestine[164, 263] was explored in 2001 by Zhang *et al*[293] who deposited its sequence in GenBank under accession number AF364606: it is located on chromosome 3q13-21 and consists of nine exons. The researchers hypothesised that there may be genetic variation in this gene of importance for CYP3A4 expression and activity based on the following: I) there is not enough polymorphism in the coding sequence of *CYP3A4* nor in PXR binding sites in distal and proximal 5' regions of *CYP3A4* to explain interindividual differences in CYP3A4 activity[294], and II) significant variation of rifampicin inducibility of human hepatic and intestinal CYP3A4 and P-gp has been demonstrated[295]. 38 SNPs were identified and indeed several of these were correlated to changes in CYP3A4 expression or activity. Examples include -25564G>A located in a putative transcription binding site in the promoter sequence which was found in two individuals with the lowest hepatic CYP3A4 expression and nifedipine clearance, respectively. Subjects with -25385C>T had significantly higher erythromycin breath test values after rifampicin treatment. 7635A>G and 8055C>T in introns were associated with increased intestinal rifampicin inducibility. Individuals with at least one 11156A>G allele had lower P-gp levels in gut biopsies. Three exonic SNPs were also identified, P27S and G36R which increased PXR-RXR complex formation with responsive elements, and R122Q which drastically reduced the same.

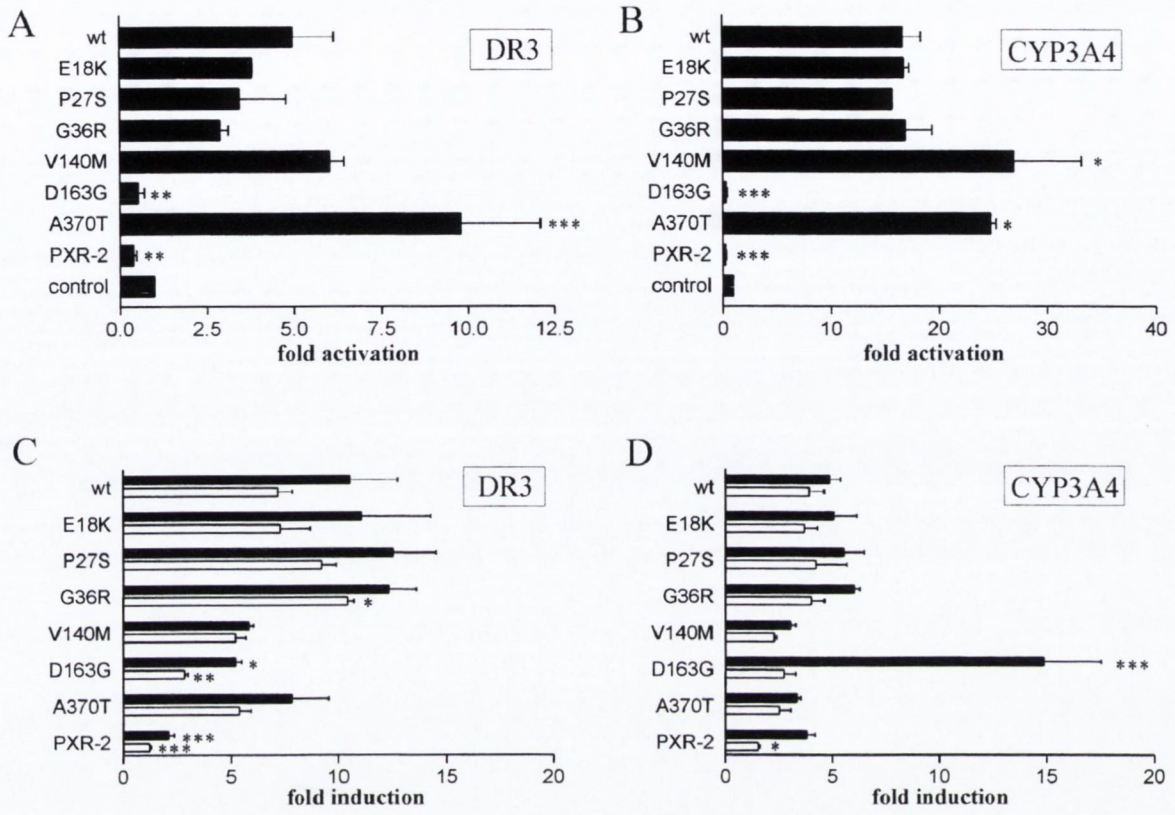


Figure 1.9 Transactivation of *NR1I2* (PXR) variants (from Hustert *et al* 2001[296]). Effects on basal (A and B) or induced (C and D) transactivation in reporter assays in LS174T cells transfected with either a DR3 motif construct or the *CYP3A4* promoter region. C and D: treatment with 10μM rifampicin (black bars) or 10μM corticosterone (open bars). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. PXR = pregnane X receptor, PXR-2 = splice variant.

A subsequent study by Hustert *et al*[296] revealed additional coding SNPs of which V140M, D163G and A370T displayed altered basal or induced CYP3A transactivation as well as an alternatively spliced form (PXR-2) with near negligible basal and inducible activity (Figure 1.9). Lamba *et al*[297] further examined sequence diversity in *NR1I2* cis-regulatory regions and identified some novel promoter and intron polymorphisms which likewise had effects on CYP3A4. These included 63396T>C, which has subsequently been associated with unboosted atazanavir plasma concentrations[217], as described in section 1.5.3. However, no other studies to date have been published investigating the impact of *NR1I2* (PXR) polymorphism on ARV pharmacokinetics. Furthermore, a more comprehensive investigation into the implications of nuclear receptor activation by ARV drugs is warranted.

1.8 HIV in Ireland and the Dublin HIV Cohort

Prior to the year 2000, HIV patients in Ireland were primarily of Irish origin and equally distributed across the main risk categories: intravenous drug users (IVDU), men who have sex with men (MSM) and heterosexuals. Since then there has been considerable immigration to Ireland, especially from Sub-Saharan Africa. Between 2000 and 2002 the number of annual newly diagnosed cases of HIV tripled, almost exclusively within the heterosexual category. More recently, however, there has been a decrease in heterosexual newly diagnosed HIV cases from nearly 200 in 2008 to around 150 in 2009. On the contrary, the MSM category has increased to nearly the same levels as heterosexuals while only 30 IVDUs were diagnosed with HIV in 2009.

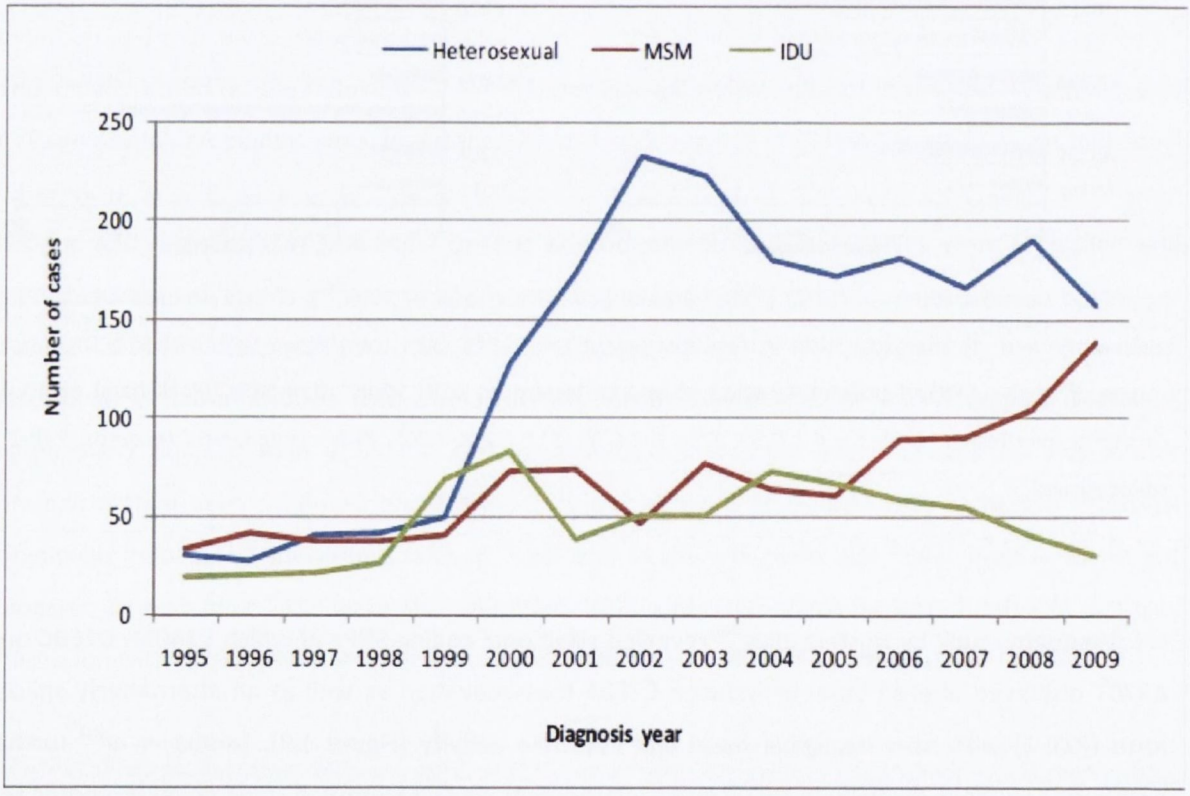


Figure 1.10 Newly diagnosed HIV infections in Ireland 1995-2009 by exposure category. "HIV and AIDS in Ireland 2009", Health Protection Surveillance Centre, Health Services Executive, May 2010.

In order to improve understanding of the HIV epidemic in Ireland, the Dublin HIV Cohort was established in 2005 through a research grant from the Irish Health Research Board. Development of the cohort represents a collaboration between all the Dublin Hospitals involved in treating patients

with HIV disease; including St James's Hospital, The Mater Misericordiae and Beaumont Hospital. The aim of the cohort is to collate clinical and epidemiological information regarding the care of HIV infected individuals within the Irish health care system for the purpose of improving the standard of care provided, enhancing knowledge and delivery of a cohesive national HIV care strategy.

A database was set up incorporating both clinical and epidemiological information such as demographics, HIV exposure history, social and medical history as well as laboratory data. To date, a total of 1748 patients have been enrolled. Of these, about a third originated from Sub-Saharan Africa while the remainder are mostly Irish Caucasians. The total gender distribution is 59% male, 41% female. The cohort also contains a biobank stored at the Mater Misericordiae Hospital in Dublin and at the National Virus Reference Laboratory in University College Dublin.

The cohort data provides a rich opportunity to study the natural history of the disease and the effects of treatment, indicators of quality of care as well as the prevalence of co-morbid conditions such as cardiovascular disease associated with HIV infection. From a pharmacological perspective cohort data facilitates investigation of treatment-related adverse effects, the effects of ethnicity on viral evolution, drug resistance and drug metabolism. The Dublin HIV Cohort provides a unique platform for pharmacogenetic studies to investigate differences of potential relevance for antiretroviral treatment response between these ethnic groups. In the first study of this thesis, we had the benefit of gaining access to biological samples from the cohort for genotypic analysis.

2 Aims and objectives

Despite considerable advances in HIV therapy, major challenges remain in maintaining patients on life-long antiretroviral (ARV) treatment. Drug interactions and adverse effects are common; the former may have a substantial influence on drug plasma concentrations and the latter is a consequence of elevated drug concentrations and off-target effects. The predisposition for drug interactions[120, 298] and adverse effects[299] may vary across ethnic groups, indicating an important role of pharmacogenetics. It is notable that ethnic subpopulations, specifically African and Asian patients, are underrepresented in ARV clinical trials and likewise in pharmacogenetics studies despite constituting the largest HIV infected groups globally.

The theme of this thesis is an investigation of interactions between ARV drugs and nuclear receptors. This superfamily of ligand-dependent transcription factors are important regulators of gene expression, with both xenobiotic and endobiotic functions. Induction of pregnane X receptor (PXR) is a well-known cause of drug interactions, as PXR governs the expression of cytochrome P450 (CYP450) metabolising enzymes and drug transporters such as P-glycoprotein (P-gp), of which many ARV drugs are substrates. Some previous evidence exists of activation of PXR by HIV protease inhibitors (PIs)[141, 146, 300] and non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz[147]. A closely related nuclear receptor, constitutive androstane receptor (CAR), is also involved in the regulation of xenobiotic metabolism and cross-talk occurs between PXR and CAR. The aim of the first study had three components: Firstly, to compare the prevalence of polymorphisms in genes of relevance for drug interactions (*NR1I2* (PXR), *CYP3A4*, *CYP2B6*, *ABCB1/MDR1*) between Caucasians and Sub-Saharan Africans from the Dublin HIV Cohort. Secondly, a comprehensive investigation of the ability of a wide range of ARV drugs to activate PXR and CAR and induce transcription of *CYP3A4* and *CYP2B6*, the two principal ARV metabolising enzymes, using luciferase reporter assays and also by quantifying mRNA and protein expression. Thirdly, an analysis of the impact of genetic polymorphism in the *NR1I2* (PXR) gene detected in the cohort, on nuclear receptor activation by ARVs.

Given the confirmed activation by several ARV drugs of PXR and CAR, and the well-documented ligand overlap and cross-regulation between nuclear receptors, it is plausible that ARVs could activate other related receptors. Metabolic abnormalities, for example lipid disturbances, fat redistribution, insulin resistance, atherosclerosis, osteopenia and neuropsychiatric symptoms are frequently experienced by

HIV patients on combination ARV therapy[70]. These observations suggest off-target effects by the ARV compounds. Nuclear receptors such as liver X receptors (LXRs), estrogen receptors (ERs) and glucocorticoid receptor (GR) are likely candidate targets due to their involvement in lipid and cholesterol homeostasis[301], adipocyte differentiation, bone remodelling[302, 303], glucose sensitisation[304-307] and central nervous system (CNS) function[308-310]. Very few studies have investigated this possibility, and most investigations of direct nuclear receptor interactions have only included a small number of drugs. The aim of the second study was therefore to undertake an extensive evaluation of the ability of a wide range of ARV compounds from different drug classes to act as ligands of nuclear receptors LXRα/β, ERα/β and GR, by a combined *in silico* and *in vitro* approach: Screening of a library of ARV compounds to identify possible ligands through docking and molecular descriptor analysis, followed by detection of direct interactions with receptor ligand binding domains (LBDs) in a cell-free TR-FRET system, and finally assessment of nuclear receptor activation in cell-based reporter assays.

Efavirenz displayed a potent antagonistic effect on both LXRα and LXRβ. Being regulators of cholesterol homeostasis, these nuclear receptors have been implicated in neuronal function: LXR double knockout mice suffer from neurodegeneration[311] while LXR agonists have been demonstrated to reduce levels of β-amyloid[312-314], a suggested marker for Alzheimer's disease (AD) progression[315]. A proposed mechanism for this neuroprotective effect of LXR is upregulation of ATP-binding cassette transporter A1 (*ABCA1*), a cholesterol efflux transporter[316]. Cholesterol homeostasis is required for the integrity of the neuronal cell membrane and optimal neuronal function. A genetic variant of apolipoprotein E (*APOE*), another LXR target gene and a facilitator of cholesterol efflux, is associated with an increased risk of late-onset AD[317, 318]. Efavirenz is recognised to cause CNS toxicity [319, 320], characterised by depression, sleep disturbances, memory deficits and neurocognitive impairment. This efavirenz-LXR link informed the aim of the third study: to investigate the effect of efavirenz exposure on *ABCA1/Abca1* and *APOE/Apoe* mRNA expression in a human neuroblastoma cell line and primary rat cortical cells. In addition, to examine the impact of efavirenz on β-amyloid processing in the same *in vitro* systems by Western blots.

3 Nuclear receptor-mediated induction of CYP450 by ARVs: functional consequences of *NR1I2* (PXR) polymorphisms and differential prevalence in Caucasians and Sub-Saharan Africans

3.1 Introduction

A number of pharmacological factors influence drug metabolism, including genetic variability in metabolising enzymes and their regulators as well as exposure to various xenobiotic compounds which possess the capacity to modulate enzyme activity and/or expression. These factors become particularly important when complex drug regimens are used as is the case in HIV treatment. Knowledge of these risk factors for drug interactions is essential, especially in resource-poor settings where the infection is widespread but treatment options are limited[321].

Nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have in recent years emerged as coordinators of cholesterol[322], glucose and lipid homeostasis, as well as inflammatory response[323]. However, their roles as xenosensors and regulators of cytochrome P450 (CYP450) metabolising enzymes remain of importance in pharmacokinetics, as several pharmaceuticals have been reported as activators of nuclear receptors (mainly PXR) with implications for drug interactions. Some HIV protease inhibitors (PIs), fall into this category: Ritonavir is a confirmed ligand of PXR[324, 325], and increased hepatic expression of PXR target genes of the CYP3A subclass has been demonstrated in amprenavir- and nelfinavir-treated rats[326]. Gupta *et al*[327] employed a reporter assay-based approach in an intestinal cell line for a number of single-concentration (10μM) PIs, which all gave rise to significantly increased *CYP3A4* promoter activation when co-transfected with PXR. Hariparsad *et al*[328] demonstrated also that the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz can induce *CYP3A4* promoter activity via PXR, and indeed an increase in CYP3A4 activity is seen in patients on efavirenz treatment[329].

Studies of naturally occurring polymorphic variants of the PXR encoding gene (*NR1I2*) have revealed not only changes in PXR expression and activity, but also effects on CYP3A4 expression and inducibility as demonstrated by Zhang *et al* (2001)[330], King *et al* (2007)[331] and Lamba *et al* (2008)[332]. Hustert *et al* (2001)[333] assessed the impact of six non-synonymous coding polymorphisms and found significant changes in basal and/or induced transcriptional activity after treatment with rifampicin or

corticosterone in four of them; G36R (106G>A), V140M (4374G>A), D163G (4444A>G) and A370T (8528A>G). Only one single nucleotide polymorphism (SNP) in *NR1I2* has been associated with alterations in antiretroviral (ARV) drug plasma concentrations; patients homozygous for -6994T (position 63396 relative to GenBank Accession AF364606 origin) had atazanavir trough levels below the minimum effective concentration[334]. These *NR1I2* polymorphisms, in combination with polymorphisms in target CYP450 and drug transporter genes could have a great influence on interindividual variation in ARV drug metabolism: several SNPs in the coding regions of C*YP3A4* have shown association with altered enzyme activity and/or expression levels. Furthermore, some researchers have demonstrated changes in efavirenz plasma levels among subjects with the -392A>G polymorphism[335, 336], whereas other studies reported no effect on either efavirenz or nelfinavir[337, 338]. In the *CYP2B6* gene, a number of SNPs such as the well studied 516G>T (Q172H)[335, 339, 340] have been correlated to changes in plasma drug concentrations of efavirenz and/or nevirapine in patients. In the *ABCB1* (*MDR1*) gene encoding drug efflux pump P-glycoprotein (P-gp), a synonymous SNP (3435C>T) has been the subject of many ARV pharmacokinetic investigations. However, although this polymorphism has been associated with reduced efflux activity[341] and in some studies correlated with changes in nelfinavir[342-345] and efavirenz[343] concentrations, other contradicting results[344, 346, 347] render these findings controversial. Few studies have focussed on polymorphism in the gene encoding CAR, *NR1I3*, and only a small number of rare SNPs in this gene have been correlated to significant changes in nuclear receptor activity or expression[348].

Africans are underrepresented in clinical trials in general and likewise in genetic screenings of the above mentioned genes, and most ARV dosage recommendations are based on results from studies with Caucasian subjects. The functional consequence of SNPs in nuclear receptors and their target genes in the context of activation potential by ARV drugs has not been investigated. It is not clear whether all PIs are inducers of PXR-mediated CYP3A4 expression, if this ability is shared by more NNRTIs other than efavirenz, by nucleoside reverse transcriptase inhibitors (NRTIs; generally not metabolised by CYP450 enzymes and hence not expected to influence their transcriptional regulation) or by newer classes such as entry inhibitors. Furthermore, it is not known if this effect is changed in any way in combination with low-dose ritonavir, used to "boost" the bioavailability of the partner PI. Additionally, many previous studies have used uniform concentrations although these may not reflect clinical plasma concentrations. ARV induction of CYP2B6 (metaboliser of NNRTIs) has not been explored, and neither has the importance of CAR as a CYP3A4/CYP2B6 induction pathway by ARVs.

3.2 Aims

The aims of this study were to 1) investigate the frequency distributions of a wide range of SNPs in the *NR1I2* (PXR), *CYP3A4*, *CYP2B6* and *ABCB1* (*MDR1*) genes in Caucasian and Sub-Saharan African HIV patients as well as comparing Sub-Saharan African SNP frequencies with published results from studies of African-American groups; 2) examine the ability of a wide range of ARVs across classes, both new and established and at concentrations derived from c_{max} values from clinical studies, to induce promoter activity of *CYP3A4* and *CYP2B6* via nuclear receptors PXR or CAR pathways and to determine if the presence of low-dose ritonavir alters the response, and additionally validate these results in primary human hepatocytes by measuring mRNA and protein expression of *CYP3A4* and *CYP2B6* after exposure to ARVs; 3) assess the impact of coding polymorphisms in *NR1I2* (PXR) detected in the genotype screening on ARV induction of *CYP3A4* promoter activity.

3.3 Materials and methods

3.3.1 Study population and genotyping

1013 subjects from the Dublin HIV Cohort (established in 2005 after obtaining ethical approval and encompassing HIV-positive patients from three major Dublin hospitals: St James's Hospital, Mater Misericordiae University Hospital and Beaumont Hospital) were included in the study (Table 3.2). The ethnicity distribution was approximately 65% Caucasians and 35% Sub-Saharan Africans. DNA was isolated from whole blood using QIAamp DNA Blood Midi Kit (Qiagen, West Sussex, UK).

37 SNPs and one 3-basepair insertion in *NR1I2* (PXR), *CYP3A4*, *CYP2B6* and *ABCB1* (*MDR1*) were selected for screening based on previous association with altered expression levels or activity of the respective proteins, or potential to affect the same by virtue of its location in regulatory regions, transcription factor binding sites or coding regions (see Table 3.3 and Table 3.4 in section 3.4). Genotyping was performed by KBioscience (Herts, UK) using patented KASPar technology (homogenous FRET-based system coupled with competitive allele specific PCR, see http://www.kbioscience.co.uk/reagents/KASP.html).

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was carried out in-house with 9% of the DNA samples as a quality control. Previously described methods were used for

one SNP per gene: *NR1I2* 7635A>G[349], *ABCB1* (*MDR1*) 3435C>T[350], *CYP3A4* 1221C>T[351] and *CYP2B6* 516G>T[352]. PCR reactions were performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, MA, USA) in 25µl volumes with 0.625U Thermo-Start *Taq* DNA polymerase, 1x High Performance Buffer, 1.5mM $MgCl_2$ (Thermo Fisher Scientific, Dublin, Ireland), 400µM dNTPs and 0.5µM each forward and reverse primers (Sigma-Aldrich, Wicklow, Ireland). The PCR programme was as follows: 95°C 15min (hot start) followed by 30 cycles of 95°C 20sec (denaturation), 60°C 30sec (annealing), 72°C 50sec (extension) and a final extension step of 72°C for 5min. The PCR products were subjected to 3h restriction digestion in 25µl volumes (with 1µl enzyme and 1x buffers from New England Biolabs, Herts, UK) followed by gel electrophoresis (2% Agarose For Routine Use, Sigma-Aldrich) with 1x TAE buffer (40mM Tris-acetate, 1mM EDTA, Sigma-Aldrich) and visualisation by Syngene gel imaging system (Synoptics Ltd, Cambridge, UK). Primer sequences and restriction digestion conditions for each SNP are found in Table 3.1 (section 3.4). Representative agarose gel images of restriction fragments are shown in Figure 3.1 (section 3.4).

3.3.2 Plasmids

The XREM-CYP3A4 luciferase construct was a gift from Professor Chris Liddle (University of Sydney, New South Wales, Australia) and the CYP2B6-PBREM/XREM luciferase construct was kindly donated by Professor Hongbing Wang (University of Maryland, College Park, USA). Dr Steven Kliewer (University of Texas, Dallas, USA) provided the pSG5-hCAR and pSG5-hPXR plasmids, while Dr Oliver Burk (Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany) supplied the PXR variant constructs in pcDNA3 ("PXRwt" i.e. the reference sequence, P27S, G36R, V140M, A370T). An internal standard, pRL-TK (expressing *Renilla* luciferase), was obtained from Promega (Madison, WI, USA).

3.3.3 ARV drugs and controls

Abacavir and fosamprenavir were gifts from GlaxoSmithKline (Hertfordshire, UK). Lopinavir, nelfinavir, nevirapine and tenofovir were provided by Abbott (Abbott Park, IL, USA), Pfizer (Groton, CT, USA), Boehringer Ingelheim (Dublin, Ireland) and Gilead (Foster City, CA, USA), respectively. Efavirenz was purchased from LGM Pharmaceuticals (Boca Raton, FL, USA) and indinavir, ritonavir and saquinavir from USP Reference Standards (Rockville, MD, USA). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Atazanavir

sulphate, maraviroc, lamivudine, tipranavir and zidovudine. Darunavir was provided by Tibotec, Inc. through the same program. Positive controls rifampicin and CITCO were purchased from Sigma-Aldrich.

3.3.4 HepG2 cell culture and MTT cytotoxicity assays

HepG2 cells (kindly donated by Dr Stephen Gray, TCD) were cultured in Minimum Essential Medium Eagle supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and 100units + 0.1mg/ml penicillin-streptomycin (all from Sigma-Aldrich). To assess cytotoxicity of the ARV drugs used in subsequent assays, HepG2 cells were seeded into 96-well plates (8,000 cells per well). The following day dilution series were made of drugs for final concentrations of 0.1μM, 1μM, 10μM, 25μM and 50μM and cells were exposed for 48h. Concentration curves of vehicles; dimethylsulphoxide (DMSO) and ethanol, were also included (0.001%, 0.01%, 0.1%, 0.25% and 0.05%) as well as fixed concentrations of either ethidium bromide (EtBr) or doxorubicin (both from Sigma-Aldrich) as cytotoxic controls. 2h prior to the end of the experiments, 10μl MTT (thiazolyl blue tetrazolium bromide 5μg/ml in medium, Sigma-Aldrich) was added to each well. The culture medium was removed after 48h drug exposure and the purple formazan complexes produced by mitochondrial reductase were dissolved in DMSO. A Bio-Tek ELx808 Absorbance Microplate Reader (Vermont, US) was utilised for absorbance measurement at 540 nm, and values from drug-treated wells normalised to untreated controls.

3.3.5 Transfections, drug exposure and luciferase reporter assays

Cells were seeded into 24-well plates (40,000 cells per well) the day before transient transfection using Lipofectamine LTX (Invitrogen, Paisley, UK) according to manufacturer's instructions with the following DNA quantities (ng nuclear receptor / reporter construct / internal standard, optimised for maximal positive control induction): PXR/CYP3A4/pRL-TK 2/400/10ng, PXR/CYP2B6/pRL-TK 10/400/25ng, CAR/CYP3A4/pRL-TK 10/400/50ng or CAR/CYP2B6/pRL-TK 2/400/10ng. The transfections were allowed to proceed for 8-9h in serum- and antibiotic-free medium. The cells were then washed with phosphate buffered saline (PBS, Sigma-Aldrich) and treatment initiated with drugs diluted in phenol red-free Minimum Essential Medium (Gibco/Invitrogen) with 10% charcoal-stripped FBS, 2mM L-glutamine and 100units + 0.1mg/ml penicillin-streptomycin (Sigma-Aldrich). Drug concentrations used correspond to reported mean (median for nelfinavir, ritonavir and tipranavir)

plasma c_{max} values from clinical studies (PI concentrations were ritonavir "boosted" as this is how they are usually administered): PIs atazanavir 4μM (3.211μg/ml[353]), darunavir 10μM (5.834μg/ml[354]), fosamprenavir 13μM (8.039μg/ml[355]), indinavir 15μM (10.65μg/ml[356]), lopinavir 16μM (9.69μg/ml[357]), nelfinavir 6μM (3.614μg/ml[358]), ritonavir 1μM (1.08μg/ml[359]), saquinavir 4μM (3.064μg/ml)[360], tipranavir 20μM (22.5μM[361]); NRTIs abacavir 5μM (3.19μg/ml[362]), lamivudine 7μM (1.567μg/ml[363]), tenofovir 1μM (360ng/ml[364]) and zidovudine 4μM (1.067μg/ml[365]); NNRTIs efavirenz 10μM (3.28μg/ml[366]) and nevirapine 7.5μM (1.93μg/ml[367]); and entry inhibitor maraviroc 0.5μM (144ng/ml[368]). Additionally, the cells were exposed to a range of concentrations (0.1μM, 1μM, 5μM, 10μM, 20μM) of selected CYP3A4 and/or CYP2B6 inducers (lopinavir, efavirenz and abacavir), for construction of dose-response curves. Drugs were either dissolved in ethanol, DMSO or H_2O. The following PIs were also tested in combination with low-dose (1μM) ritonavir: atazanavir, darunavir, fosamprenavir, lopinavir and saquinavir. Rifampicin (10μM) and CITCO (100nM) were included as positive controls for PXR and CAR respectively, as well as vehicle controls representing the highest ethanol (0.17%) and DMSO (0.1%) final concentrations. After 48h, the cells were harvested and the Dual-Luciferase Reporter Assay System (Promega) utilised to measure transcription levels with the aid of a luminometer (Thermo Fisher Scientific). Reporter construct responses (firefly) were normalised to internal standard (*Renilla*) and fold increases calculated relative to untreated controls.

3.3.6 Assessing the effect of *NR1I2* (PXR) polymorphisms on ARV induction of *CYP3A4*

Four *NR1I2* (PXR) polymorphisms, P27S (79C>T), G36R (106G>A), V140M (4374G>A) and A370T (8528A>G), were selected for *in vitro* assessment of their functional impact on ARV induction of *CYP3A4* based on the following criteria: they were coding polymorphisms (resulting in an amino acid change) detected among the patients in the cohort; and have previously been associated with altered activity. Hustert *et al*[333] found increased corticosterone-induced PXR activity with the G36R variant and increased basal promoter activity of CYP3A4 with V140M and A370T, as well as a trend towards decreased rifampicin-activated expression with the latter two PXR variants and increased corticosterone activation with P27S. This SNP has also been reported in a patient with reduced nifedipine clearance[330].

The P27S, G36R, V140M and A370T constructs along with a PXR reference sequence plasmid were subsequently used for co-transfections with the XREM-CYP3A4 luciferase construct. Cells were

exposed to rifampicin, lopinavir, fosamprenavir, nelfinavir, efavirenz and tenofovir (to represent PIs, NNRTIs and NRTIs) at the same concentrations as in previous experiments, and Dual-Luciferase Reporter assays were performed as described above.

3.3.7 Primary human hepatocytes: mRNA and protein expression of CYP3A4 and CYP2B6

Fresh primary human hepatocytes (from two male Caucasian donors, supplied by Biopredic International, Rennes, France) in 24-well plates (350,000 cells/well) were exposed in duplicate to 0.1% DMSO, 10μM rifampicin, 100nM CITCO, or varying concentrations of lopinavir, efavirenz or abacavir (0.1μM, 1μM and 10μM), for 48h in phenol red-free William's E incubation medium (Biopredic). Total RNA and protein was isolated using TRIsure (Bioline, London, UK) according to the manufacturer's instructions. 1μg RNA from each sample was first treated with DNaseI (Sigma-Aldrich) to remove genomic DNA, and then reverse transcribed to cDNA using random hexamers (Bioline) and M-MLV RT (Sigma-Aldrich). Real time PCR was subsequently performed with QuantiTect SYBR Green PCR Kit and Primer Assays (Qiagen, West Sussex, UK) for human *CYP3A4*, *CYP2B6* and *ACTB* (β-actin, housekeeping gene) with Applied Biosystems 7900HT.

Isolated protein was denatured by boiling in sample buffer (6% sodium dodecyl sulphate (SDS), 100mM Tris-HCl pH 6.8, 20% glycerol, 0.4% bromophenol blue) with protease inhibitor cocktail (Sigma-Aldrich) and loaded onto 10% SDS-polyacrylamide gels. Western blots were carried out by semi-dry transfer (100mA, 1 hour) onto Amersham Hybond-P membranes (GE Healthcare, Buckinghamshire, UK) which were subsequently blocked with 5% milk powder (Marvel, PremierFoods, Ireland) in TBST (pH 7.5 10mM Trizma base, 100mM NaCl, 1M HCl, 0.1% Tween-20) for 1 hour at room temperature. Probing with primary antibody was performed at 4°C overnight, followed by TBST washing and probing with secondary antibody for 1h at room temperature. Primary rabbit anti-human CYP3A4 (CR3340, 1:1000 dilution) and CYP2B6 (CR3290, 1:500 dilution) were from Biomol/Enzo Life Sciences (Exeter, UK), whereas horseradish peroxidise (HRP)-conjugated swine anti-rabbit secondary antibody was purchased from Dako Denmark A/S (Glostrup, Denmark). Blots were visualised by enhanced chemiluminescence detection as described by Haan and Behrmann[369] using a Fuji LAS 4000 chemiluminescent imager. Membranes were subsequently blocked once again with 5% milk powder in TBST, followed by re-probing with internal standard β-actin (HRP-conjugated, Santa

Cruz Biotechnology Inc., CA, USA). Band intensities were measured by densitometry using GeneTools software (Syngene, Cambridge, UK).

3.3.8 Statistics

Differences in allele frequencies between Caucasians and Sub-Saharan Africans were compared by the chi-square test (SPSS version 15.0) and P-values were calculated by Fisher's Exact Test (2-sided). Allele frequencies of Sub-Saharan Africans were also compared to available frequency data from African Americans using the same method. Haploview version 4.2 (http://www.broadinstitute.org/mpg/haploview)[370] was utilised for construction of linkage disequilibrium (LD) plots and Hardy-Weinberg Exact Tests with Bonferroni correction, performed within both ethnic groups. *NR1I2* (PXR) haplotype analysis was performed using HAP (http://research.calit2.net/hap/)[371].

Data from reporter assays, real time PCR and Western blots were normalised to internal standards and analysed by one-way analysis of variance (ANOVA) with Dunnett's *post hoc* analysis, while results from reporter assays with ritonavir combinations were compared to single PIs by Mann-Whitney t-tests (GraphPad Prism version 5). EC_{50} values from dose-response experiments were calculated using the same software (non-linear fit, sigmoidal dose-response curves). All reporter assay experiments were performed in duplicates at least three independent times and presented as means ± S.E.M. P-values of ≤0.05 were regarded as indication of significant difference for all experiments.

3.4 Results

3.4.1 Patient demographics

The demographics of the Caucasian and Sub-Saharan African subgroups in the cohort differ in terms of gender and probable route of transmission (Table 3.2). Three quarters of the Caucasian population are male, and the risk groups "men who have sex with men" (MSM) and "intravenous drug users" (IVDU) are well represented. Among the Sub-Saharan Africans - consisting mainly of first-generation immigrants - the gender distribution is the opposite, and the route of transmission is primarily

through heterosexual contact. The high proportion of women in this group reflects detection of HIV infection through the national antenatal screening programme.

3.4.2 *NR1I2* (PXR), *CYP3A4*, *CYP2B6* and *ABCB1* (*MDR1*) polymorphism frequencies

Out of 38 polymorphisms examined, 22 were found at significantly different ($P < 0.05$) allelic frequencies in Caucasians compared to Sub-Saharan Africans: in *NR1I2*; -25564G>A, -25385C>T, -24756G>A and -24381A>C in the promoter region; 79C>T and 106G>A in exons; -6994C>T, 7635A>G and 8055C>T in introns as well as position 11156A>C in the 3' untranslated region (Table 3.3). P-values below 0.05 were also reached with *CYP3A4* polymorphisms -11128insTGT (3 base-pair insertion), -392A>G and 683C>T, and similarly with *CYP2B6* SNPs 516G>T, 785A>G, 983T>C and 1459C>T (Table 3.4). All *ABCB1/MDR1* SNPs screened for (-129T>C, 61A>G, 1199G>A, 2677T>G, 3435C>T) were present at significantly different allele frequencies between the two ethnic groups (Table 3.3). For all of these SNPs, homozygotes of the minor alleles were present (albeit often in small numbers) with the exception of *NR1I2* 106G>A, *CYP3A4* 683C>T and *ABCB1* (*MDR1*) 1199G>A. Remaining SNPs were either absent in the populations or found at a very low prevalence and any difference between Caucasians and Sub-Saharan Africans could not be determined.

Genotype distributions were in Hardy-Weinberg equilibrium (HWE) with the exception of 7635A>G (*NR1I2*, PXR) and 2677T>G (*ABCB1/MDR1*) among the Sub-Saharan Africans. In *CYP2B6*, positions 516 and 785 were in LD among both ethnicities (r^2 = 0.82 and 0.91 for Caucasians and Sub-Saharan Africans, respectively) which is in accordance with other studies (Leger *et al* 2009[372], Haas *et al* 2009[373]) and shown in Figure 3.2. As were *NR1I2* SNP pairs -25385/-24381 (r^2 = 0.96) and 8055/11156 (r^2 = 0.96) in the Caucasian population, consistent with HapMap data as well as Dring *et al*[349]. Sub-Saharan Africans exhibited different LD patterns for the same gene; 52/8528 showed moderate LD (r^2 = 0.70), however as HapMap data is not available for position 8528 (rs59152710) this could not be confirmed. In *ABCB1* (*MDR1*), 2677G>T and 3435C>T displayed weak LD (r^2 = 0.50) among Caucasians in the cohort while the same was not seen in the Sub-Saharan African population (r^2 = 0.17), despite HapMap data indicating a similar degree of LD for both Caucasians and Africans (r^2 around 0.5). However, similar to our results, a larger (111 subjects) West African study did also not find LD between these SNPs[374]. These ethnicity-specific patterns are reflected in their contrasting block structures of the haplotype reconstruction, shown in Table 3.6. None of the *CYP3A4* polymorphisms

detected displayed LD. Genotyping results were deemed reliable by absolute correlation with in-house PCR-RFLP controls.

3.4.3 Comparison of Sub-Saharan African allelic frequencies with African Americans

Four allelic frequencies of Sub-Saharan Africans observed in this study were found to be significantly different to those of African American subjects in other studies: -24756G>A and 8055C>T in *NR1I2* (PXR) compared with a study by Zhang *et al*[330], as well as -392A>G in *CYP3A4* and 3435C>T in *ABCB1* (*MDR1*) compared to data from a publication by Haas *et al*[335] (Table 3.5). The three minor allele frequencies of *NR1I2* and *CYP3A4* SNPs were higher among Sub-Saharan Africans than African Americans whereas *ABCB1* (*MDR1*) 3435T was less frequent in the former population. Of the remaining SNPs with available data on African American populations, four SNPs were not significantly different between the two groups whereas six SNPs failed to reach statistical significance due to low numbers of the minor allele.

3.4.4 MTT cytotoxicity assays

Ritonavir, lopinavir, saquinavir and efavirenz had cytotoxic effects (defined as cell survival less than 80%) at 25μM, while nelfinavir was cytotoxic already at 10μM (Figure 3.3). In subsequent experiments concentrations did not however exceed these limits. None of the other ARVs affected cell survival, and neither did vehicle controls (up to 0.5%).

3.4.5 ARV-induced PXR-mediated *CYP3A4* and *CYP2B6* promoter activity

Fosamprenavir, lopinavir, nelfinavir and tipranavir showed the ability to induce PXR-mediated *CYP3A4* promoter activity significantly in reporter assays, producing fold increases of the following magnitudes compared to untreated: 13.5 ± 3.9, 7.5 ± 2.7, 5.6 ± 2.3 and 9.9 ± 3.4 (Figure 3.4). *CYP2B6* promoter activity was also increased by lopinavir (11.4 ± 10.0) as well as by darunavir (6.1 ± 0.4). Efavirenz increased both *CYP3A4* and *CYP2B6* by 5.7 ± 3.3 and 4.7 ± 2.3 fold, respectively, whereas abacavir increased only *CYP2B6* by 2.3 ± 0.6 fold. The EC_{50} value of lopinavir for induction of *CYP3A4* promoter activity was calculated to 3.7μM (95% CI: 689nM, 19.8μM). However, lopinavir-induced *CYP2B6* promoter activity as well as efavirenz-induced *CYP3A4* and *CYP2B6* promoter activity did not

reach maximal response for *CYP2B6* promoter activity; hence EC_{50} values were estimated to >20μM (maximum concentration tested) in all three cases (Figure 3.6).

3.4.6 ARV-induced CAR-mediated *CYP3A4* and *CYP2B6* promoter activity

In reporter assays using CAR-transfected HepG2, *CYP2B6* promoter activity was increased by fosamprenavir, lopinavir and tipranavir with fold increases of 3.4 ± 3.2, 3.0 ± 1.3 and 4.8 ± 2.4 compared to untreated controls, but unchanged by non-PI ARVs (Figure 3.5). None of the PIs tested had an effect on CAR-mediated *CYP3A4* transcriptional activity. However, it was induced after treatment with abacavir (2.5 ± 1.0). EC_{50} of abacavir was 1.7μM (95% CI: 200nM, 13.9 μM) for *CYP3A4* induction and estimated to >20μM for lopinavir-induced *CYP2B6* promoter activity, as maximal induction was not reached with this PI (Figure 3.6). No change in promoter activity was seen with vehicle controls (data not shown).

3.4.7 Effect of co-administration with ritonavir

Promoter activity of *CYP3A4* by lopinavir and saquinavir was increased ($P < 0.05$) when these PIs were combined with 1μM ritonavir (fold increases compared to single drugs with PXR were 1.7 ± 0.5 and 2.7 ± 1.0 respectively, and with CAR 2.5 ± 0.9 and 2.4 ± 1.1 respectively), as well as fosamprenavir/ritonavir in the CAR-mediated assay (2.4 ± 0.5). Saquinavir was the only drug tested which increased *CYP2B6* promoter activity when low-dose ritonavir was added, through PXR: fold increase 5.7 ± 1.4 (Table 3.7).

3.4.8 Impact of *NR1I2* (PXR) polymorphism on *CYP3A4* induction

135 patients within the cohort were found to have SNPs in the *NR1I2* gene leading to PXR amino acid substitutions P27S, G36R, V140M and A370T. Interestingly, none of these patients carried more than one. The effect of these exonic polymorphisms on ARV induction of *CYP3A4* was assessed. Transfection of HepG2 cells with V140M or A370T mutation constructs both resulted in lower *CYP3A4* promoter activity after rifampicin-stimulation in comparison to the reference PXR sequence. A comparable effect was seen with the same PXR variants in the presence of fosamprenavir and lopinavir, where induction was significantly ($P < 0.05$) lower. Variants P27S and G36R showed trends towards reduced rifampicin and fosamprenavir induction, however did not reach statistical

significance. Nelfinavir and efavirenz-induced *CYP3A4* promoter activity was not altered by PXR variants P27S, G36R, V140M or A370T. Tenofovir, included as a negative control because it did not increase *CYP3A4* promoter activity using the PXR reference sequence construct, similarly had no effect in experiments with PXR variants (Figure 3.7).

3.4.9 Primary human hepatocytes: mRNA and protein expression of CYP3A4 and CYP2B6

The effect of lopinavir, efavirenz and abacavir on mRNA and protein expression in primary human hepatocytes was determined. *CYP3A4* mRNA levels were increased significantly only by 10μM efavirenz (fold increase 2.8 ± 0.7), although a trend towards increased expression was seen with increasing concentrations of abacavir - however the lowest concentration 0.1μM gave rise to significantly lower *CYP3A4* mRNA expression compared to vehicle control (Figure 3.8A). *CYP2B6* mRNA was increased by 10μM efavirenz (30.1 ± 12.8) and 10μM abacavir (3.2 ± 0.4, Figure 3.8B). Determination of protein content by Western blot showed increased CYP3A4 by 10μM efavirenz (7.6 ± 4.7) and CYP2B6 by 10μM lopinavir (1.8 ± 0.4) (Figure 3.8C and D). Representative Western blots are also shown in Figure 3.8 (E and F).

Table 3.1 PCR-RFLP primer sequences and restriction digest conditions. Primers were designed by other researchers, see references in table. Buffers (with or without BSA) were used according to New England Biolabs recommendations. PCR-RFLP = polymerase chain reaction restriction fragment length polymorphism, bp = base pairs.

Gene/SNP	Primer sequences	PCR product size	Restriction enzyme (temp)	Fragment sizes (allele allowing enzyme cleavage)
***NR1I2* (PXR) 7635(A/G)**[286]	fw 5'-TGG ATG CCA AGC TCA GTGG-3' rev 5'-CAG CAG CCA TCC CAT AAT CC-3'	194 bp	*Hph*I (37°C)	43 + 151 bp (A allele)
***CYP3A4* 1221(C/T)**[375]	fw 5'-ATC CAA ATC TGT TTC GTT CTT TC-3' rev 5'-CCA CAT GAC TGT CCT GTA GAT TAA-3'	341 bp	*Bsm*AI (55°C)	235 + 106 bp (C allele)
***CYP2B6* 516(G/T)**[376]	fw 5'-GGTCTGCCCATCTATAAAC-3' rev 5'-CTGATTCTTCACATGTCTGCG-3'	526 bp	*Bsr*I (65°C)	241 + 268 + 17 bp (G allele), 509 + 17 bp (T allele)
***ABCB1/MDR1* 3435(C/T)**[350]	fw 5'-TGT TTT CAG CTG CTT GAT GG-3' rev 5'-AAG GCA TGT ATG TTG GCC TC-3'	197 bp	*Bfu*CI (37°C)	158 + 39 bp (C allele)

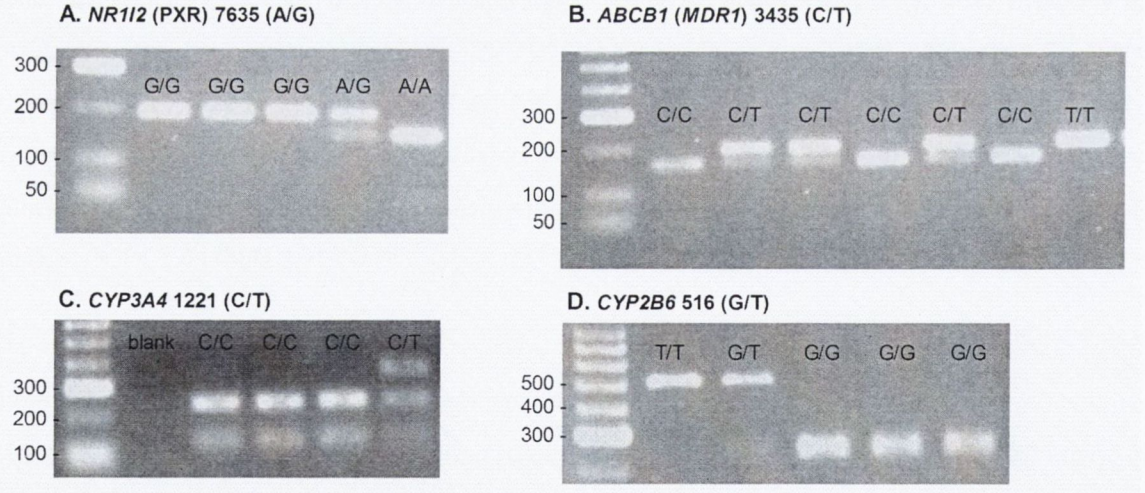


Figure 3.1 Representative PCR-RFLP agarose gels (2% agarose, 1xTris-acetate EDTA buffer). Molecular weight marker: Hyperladder II, Bioline. PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism.

Table 3.2 Baseline demographics of study subjects: Caucasian and Sub-Saharan African patients from the Dublin HIV Cohort. S.D. = standard deviation, MSM = "men who have sex with men", IVDU = intravenous drug users, IQR = interquartile range, ARV = antiretroviral.

Characteristic	Overall (n = 1013)	Caucasians (n = 656)	Sub-Saharan Africans (n = 357)
Mean age, years ± S.D.	40.3 ± 8.5	42.2 ± 8.9	36.8 ± 6.3
Sex, n (%)			
Male	586 (58%)	488 (74%)	98 (27%)
Female	427 (42%)	168 (26%)	259 (73%)
Probable route of transmission, n (%)			
MSM	228 (23%)	222 (34%)	6 (2%)
IVDU	239 (24%)	238 (36%)	1 (<1%)
Heterosexual contact	451 (45%)	149 (23%)	302 (85%)
Mother-to-child transmission	2 (<1%)	1 (<1%)	1 (<1%)
Blood transfusion/Occupational exposure	22 (2%)	9 (1%)	13 (4%)
Other/unknown	71 (7%)	37 (6%)	34 (9%)
CD4 at enrolment x 10^6/l, median (IQR)	386 (250 to 549)	397 (250 to 578)	371 (250 to 529)
HIV RNA (log_{10} copies/ml) at enrolment, mean ± S.D.	2.6 ± 1.2	2.7 ± 1.3	2.5 ± 1.1
Patients on ARV treatment at enrolment, n (%)	627 (62%)	401 (61%)	226 (63%)

Table 3.3 Allele frequencies for single nucleotide polymorphisms (SNPs) in *NR1I2* (PXR) and *ABCB1* (*MDR1*).

Frequencies of Caucasians (C) and Sub-Saharan Africans (SSA) were compared by the chi-square test, *indicates significant difference ($P \leq 0.05$, Fisher's Exact Test, 2-sided). [a]Position in relation to translation start site (*NR1I2*: GenBank Accession AF364606, *ABCB1*: NCBI Reference sequence: NM_000927) [b]Too few minor alleles present.

Position[a]	Region	Effect	dbSNP rs#	Frequency		P-value
NR1I2 (PXR)				C	SSA	C vs. SSA
-25564G>A	upstream	alters TF binding site[330]	rs12721602	0.015	0.002	0.003*
-25385C>T	upstream	↑ rifampicin activation of CYP3A4[330]	rs3814055	0.376	0.246	<0.001*
-24756G>A	upstream	alters TF binding site[330]	rs1523128	0.008	0.371	<0.001*
-24381A>C	5'UTR	↑ CYP3A4 and P-pg expression[331]	rs1523127	0.384	0.889	<0.001*
-6994C>T	intron 1b	↑ CYP3A4[332], ↓ATV trough[334]	rs2472677	0.594	0.381	<0.001*
52G>A	exon 2: E18K	alters DNA binding domain[333]	rs59371185	0.000	0.011	Undefined[b]
79C>T	exon 2: P27S	↓ PXR expression, ↑ PXR-RXR-PXRE complex[330]	rs12721613	0.002	0.126	<0.001*
106G>A	exon 2: G36R	↓ PXR expression, ↑ PXR-RXR-PXRE complex[330]	rs12721607	0.028	0.001	<0.001*
4321G>A	exon 4: R122Q	↓ PXR-RXR-PXRE complex[330]	rs12721608	0.000	0.000	-
4374G>A	exon 4: V140M	↑ basal CYP3A4 expression[330]	rs72551372	0.001	0.002	Undefined[b]
4444A>G	exon 4: D163G	↓ basal CYP3A4, ↑rifampicin CYP3A4[330]	rs72551374	0.000	0.000	-
7635A>G	intron 5	↑ rifampicin activation of CYP3A4[330]	rs6785049	0.396	0.965	<0.001*
8055C>T	intron 6	↑ rifampicin activation of CYP3A4[330]	rs2276707	0.179	0.425	<0.001*
8528G>A	exon 8: A370T	↑ basal CYP3A4 expression[330]	rs59152710	0.000	0.011	Undefined[b]
8555T>G	exon 9: C379G	alters ligand binding domain[377]	n/a	0.000	0.002	Undefined[b]
11156A>C	3'UTR	↓ P-pg expression[330]	rs3814057	0.180	0.525	<0.001*
ABCB1 (MDR1)						
-129T>C	promoter	change in promoter activity[378]	rs3213619	0.033	0.121	<0.001*
61A>G	exon 3	↑intracellular NFV concentration[379]	rs9282564	0.097	0.004	<0.001*
1199G>A	exon 12	(↓intracellular NFV concentration)[379]	rs2229109	0.021	0.000	<0.001*
2677G>T	exon 22	↑RTV and ATV clearance[197]	rs2032582	0.460	0.040	<0.001*
3435C>T	exon 27	↓EFV plasma conc[186]	rs1045642	0.551	0.118	<0.001*

Table 3.4 Allele frequencies for single nucleotide polymorphisms (SNPs) in *CYP3A4* and *CYP2B6*. Frequencies of Caucasians (C) and Sub-Saharan Africans (SSA) were compared by the chi-square test, *indicates significant difference ($P \leq 0.05$, Fisher's Exact Test, 2-sided). [a]Position in relation to translation start site (*CYP3A4*: GenBank Accession M18907, *CYP2B6*: NCBI Reference sequence: NM_000767.4) [b]Too few minor alleles present.

Position[a]	Region	Effect	dbSNP rs#	Frequency		P-value
CYP3A4				**C**	**SSA**	**C vs. SSA**
ins11128TGT	promoter	↓ enhancer activity[380]	n/a	0.038	0.000	<0.001*
-392A>G	Promoter	trend to higher/lower EFV AUC[335, 336], ↓IDV cmax[381]	rs2740574	0.034	0.745	<0.001*
658C>G	exon 7: T185S	↓enzyme activity[382, 383]	rs12721627	0.000	0.000	-
670T>C	exon 7: F189S	↓enzyme activity[384]	rs4987161	0.000	0.000	-
683C>T	exon 7: I193I	exonic splicing enhancer[385]	rs4987159	0.001	0.045	<0.001*
982T>C	exon 10: L293P	↓*in vitro* activity, ↑*in vivo* activity[384, 386]	rs28371759	0.000	0.001	Undefined[b]
1088C>T	exon 11: T363M	↓ expression levels in bacterial expression system[387]	rs67784355	0.000	0.000	-
1221C>T	exon 11: L373F	↓ enzyme activity[387]	rs12721629	0.000	0.013	Undefined[b]
1351C>T	exon 11: P416L	↓ enzyme expression[387]	rs4986909	0.000	0.000	-
CYP2B6						
136A>G	exon 1: M46V	↑EFV AUC[388, 389]	rs35303484	0.004	0.000	Undefined[b]
499C>G	exon 4: P167A	↑EFV AUC[390]	rs3826711	0.000	0.000	-
516G>T	exon 4: Q172H	↑EFV + NVP AUC[335, 339, 340]	rs3745274	0.236	0.401	<0.001*
593T>C	exon 4: M198T	↓ enzyme activity, ↑EFV AUC[388, 389]	rs36079186	0.000	0.000	-
785A>G	exon 5: K262R	↑EFV AUC[390]	rs2279343	0.270	0.421	<0.001*
983T>C	exon 7: I328T	plasma conc of EFV + NVP[191]	rs2899499	0.134	0.254	<0.001*
1132C>T	exon 7: R378stop	↑EFV AUC[191]	rs34097093	0.000	0.000	-
1459C>T	exon 9: R487C	no effect on EFV and NFV conc[55, 187, 391]	rs3211371	0.142	0.007	<0.001*

Table 3.5 Comparison of allele frequencies of Sub-Saharan Africans (SSA) vs. African Americans (AA). Comparison was performed by the chi-square test using available data of AA from other studies, *indicates significant difference (P ≤0.05, Fisher's Exact Test, 2-sided). [a]Position in relation to translation start site (*CYP3A4*: GenBank Accession M18907, *CYP2B6*: NCBI Reference sequence: NM_000767.4) [b]Too few minor alleles present.

Position[a]	dbSNP rs#	Frequency		P-value
NR1I2 (PXR)		**SSA**	**AA**	**SSA vs. AA**
-25564G>A	rs12721602	0.002	0.09 [330]	Undefined[b]
-25385C>T	rs3814055	0.246	0.32 [330]	0.455
-24756G>A	rs1523128	0.371	0.14 [330]	0.024*
-24381A>C	rs1523127	0.889	0.27 [330]	Undefined[b]
79C>T	rs12721613	0.126	0.20 [330]	0.126
106G>A	rs12721607	0.001	0.03 [330]	Undefined[b]
4321G>A	rs12721608	0.000	0.00 [330]	-
7635A>G	rs6785049	0.965	0.77 [330]	Undefined[b]
8055C>T	rs2276707	0.425	0.18 [330]	0.027*
11156A>C	rs3814057	0.525	0.33 [330]	0.151
CYP3A4				
-392A>G	rs2740574	0.745	0.63 [335]	0.021*
658C>G	rs12721627	0.000	0.00 [382]	-
683C>T	rs4987159	0.045	0.05 [382]	Undefined[b]
1088C>T	rs67784355	0.000	0.00 [382]	-
1351C>T	rs4986909	0.000	0.00 [382]	-
CYP2B6				
516G>T	rs3745274	0.401	0.38 [335]	0.743
1459C>T	rs3211371	0.007	0.01 [335]	Undefined[b]
ABCB1 (MDR1)				
2677G>T	rs2032582	0.040	0.11 [194]	Undefined[b]
3435C>T	rs1045642	0.118	0.22 [194]	0.007*

Table 3.6 *NR1I2* (PXR) haplotype predictions. A) Caucasians, B) Sub-Saharan Africans. Predictions based on single nucleotide polymorphisms (SNPs) found at significantly different allele frequencies between the two subpopulations were computed by HAP (**http://research.calit2.net/hap/**), only haplotypes >5% frequencies are shown. Note that block predictions differ between the two ethnic groups. Freq. = frequency.

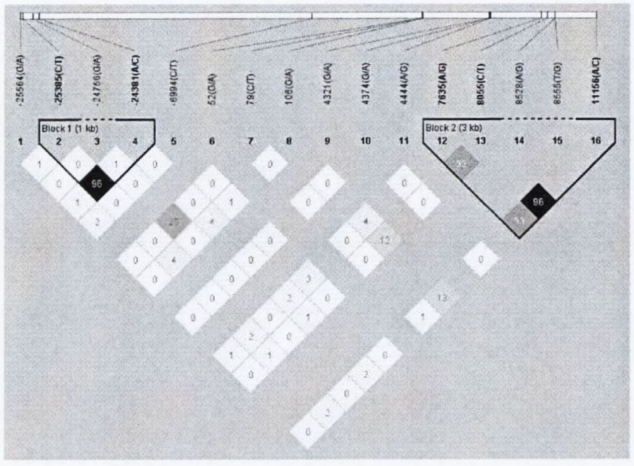
A. Caucasians *NR1I2* (PXR) haplotypes

Block 1							Freq.	Block 2			Freq.
-25564	-25385	-24756	-24381	-6994	79	106		7635	8055	11156	
G	C	G	A	T	C	G	0.42	A	C	A	0.61
G	C	G	A	C	C	G	0.20	G	C	A	0.21
G	T	G	C	C	C	G	0.18	G	T	C	0.18
G	T	G	C	T	C	G	0.15				

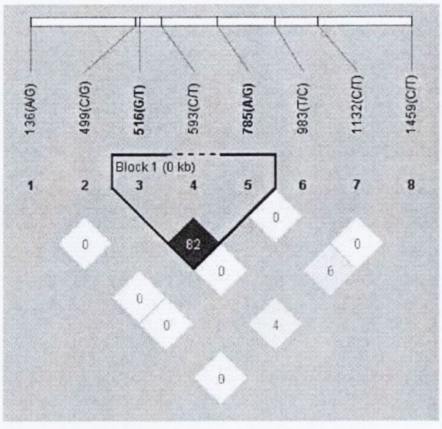
B. Sub-Saharan Africans *NR1I2* (PXR) haplotypes

Block 1				Freq.	Block 2					Freq.	Block 3	Freq.
-25564	-25385	-24756	-24381		-6994	79	106	7635	8055		11156	
G	C	A	C	0.37	C	C	G	G	C	0.41	C	0.52
G	C	G	C	0.27	T	C	G	G	T	0.23	A	0.48
G	T	G	C	0.25	C	C	G	G	T	0.11		
G	C	G	A	0.11	T	C	G	G	C	0.09		
					C	T	G	G	T	0.08		

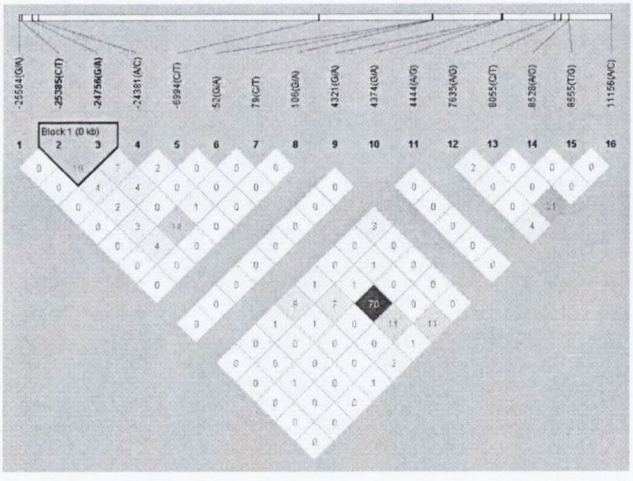
A. *NR1I2* (PXR) Cauc



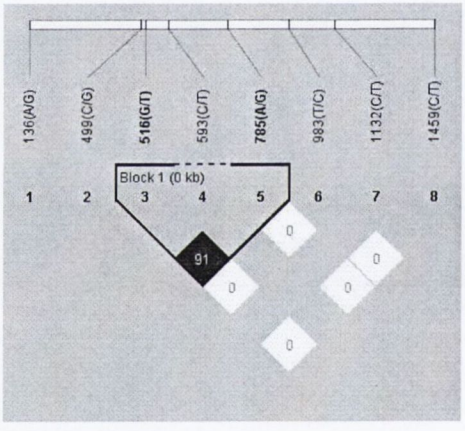
B. *CYP2B6* Cauc



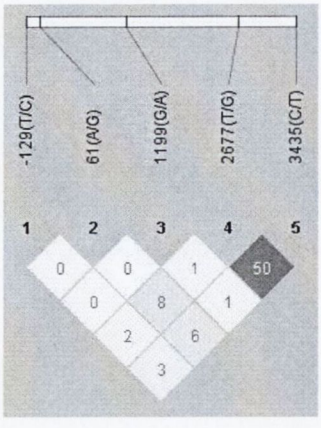
C. *NR1I2* (PXR) SSA



D. *CYP2B6* SSA



E. *ABCB1/MDR1* Cauc



F. *ABCB1/MDR1* SSA

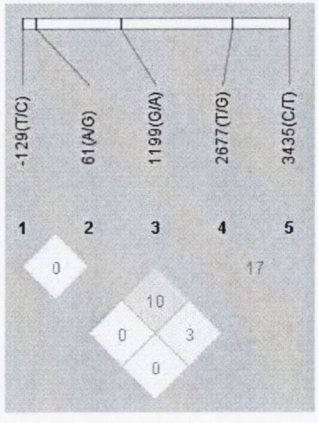


Figure 3.2 Linkage disequilibrium (LD) plots. A) LD plot for *NR1I2* (PXR) in the Caucasian (Cauc) population, B) LD plot for *CYP2B6* in the Caucasian population, C) LD plot for *NR1I2* (PXR) in the Sub-Saharan African (SSA) population, D) LD plot for *CYP2B6* in the Sub-Saharan African population, E) LD plot for *ABCB1/MDR1* in the Caucasian population, F) LD plot for *ABCB1/MDR1*) in the Sub-Saharan African population. White indicates $r^2 = 0$, black indicates $r^2 = 1$, shades of grey $0 < r^2 < 1$.

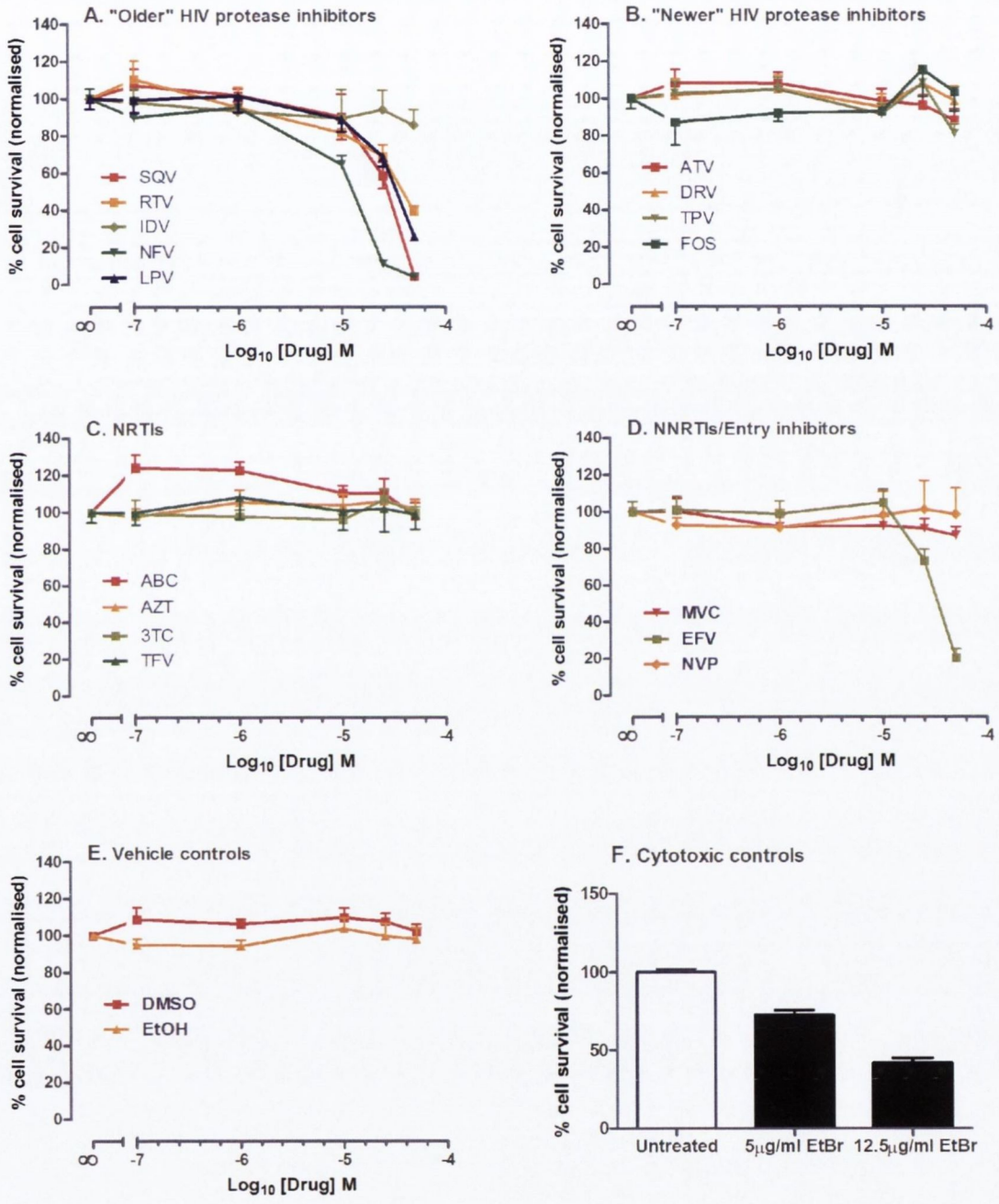


Figure 3.3 MTT cytotoxicity assays in HepG2 human hepatocarcinoma cells. Cells were treated for 48h with the indicated concentrations (n > 4). For every data point mean ± S.E.M. is shown. SQV = saquinavir, RTV = ritonavir, IDV = indinavir, NFV = nelfinavir, LPV = lopinavir, ATV = atazanavir, DRV = darunavir, TPV = tipranavir, FOS = fosamprenavir, ABC = abacavir, AZT = zidovudine, 3TC = lamivudine, TFV = tenofovir, MVC = maraviroc, EFV = efavirenz, NVP = nevirapine, DMSO = dimethylsulphoxide, EtOH = ethanol, EtBr = ethidium bromide.

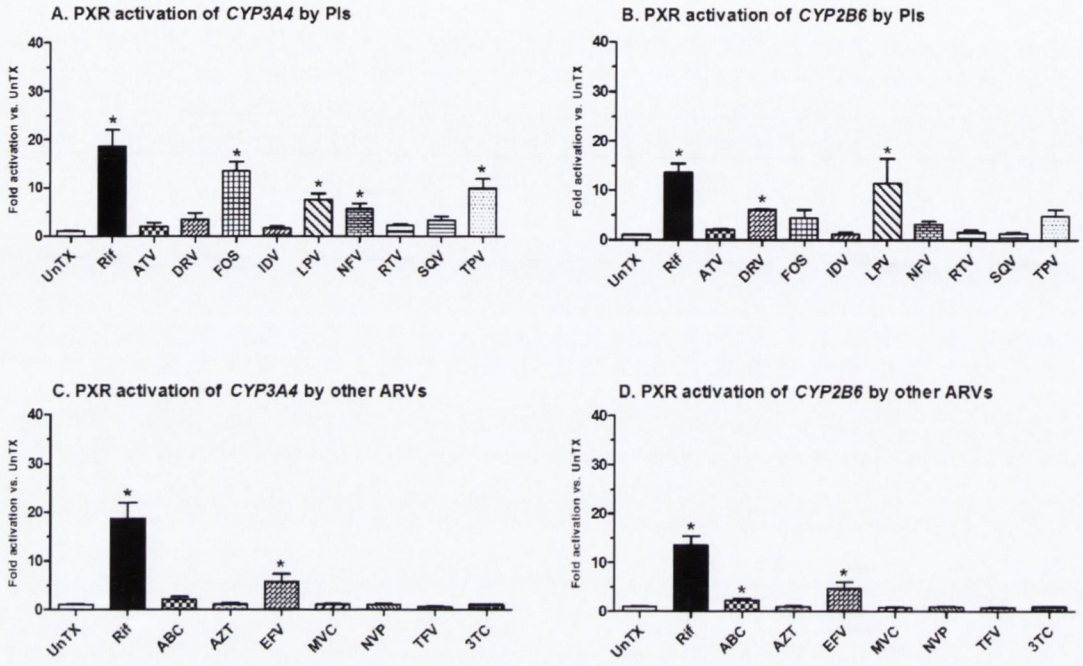


Figure 3.4 ARV-induced PXR-mediated *CYP3A4* and *CYP2B6* promoter activity. Results from Dual-Luciferase Reporter Assays performed in transfected HepG2 cells after 48h drug exposure. A) PI-induced PXR-mediated *CYP3A4* promoter activity, B) PI-induced PXR-mediated *CYP2B6* promoter activity, C) NRTI/NNRTI/entry inhibitor-induced PXR-mediated promoter activity of *CYP3A4*, D) NRTI/NNRTI/entry inhibitor-induced PXR-mediated promoter activity of *CYP2B6*. Data is presented as mean ($n \geq 3$) ± S.E.M. relative to average untreated, analysed by one-way ANOVA with Dunnett's *post hoc* analysis, *$P \leq 0.05$. ARV = antiretroviral, PI = protease inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NNRTI = non-nucleoside reverse transcriptase inhibitor, UnTX = untreated, Rif = rifampicin (positive control), ATV = atazanavir, DRV = darunavir, FOS = fosamprenavir, IDV = indinavir, LPV = lopinavir, NFV = nelfinavir, RTV = ritonavir, SQV = saquinavir, TPV = tipranavir, ABC = abacavir, AZT = zidovudine, EFV = efavirenz, MVC = maraviroc, NVP = nevirapine, TFV = tenofovir, 3TC = lamivudine.

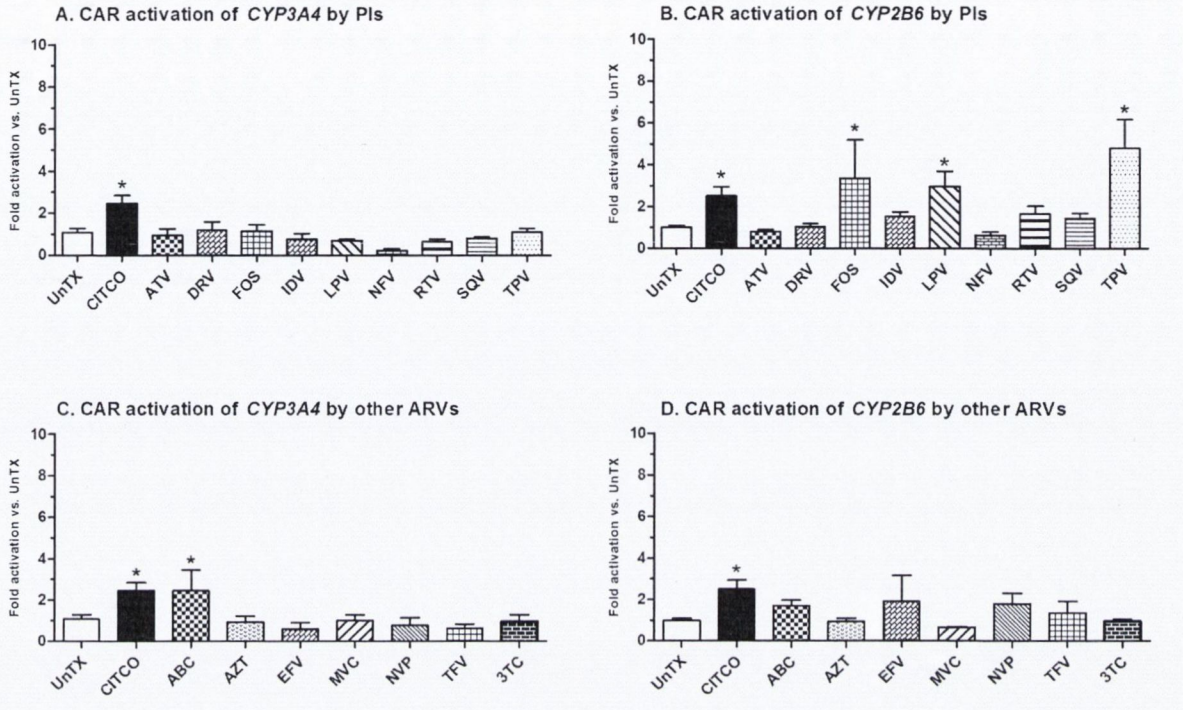


Figure 3.5 ARV-induced CAR-mediated *CYP3A4* and *CYP2B6* promoter activity. Results from Dual-Luciferase Reporter Assays performed in transfected HepG2 cells after 48h drug exposure. A) PI-induced CAR-mediated *CYP3A4* promoter activity, B) PI-induced CAR-mediated *CYP2B6* promoter activity, C) NRTI/NNRTI/entry inhibitor-induced CAR-mediated *CYP3A4* promoter activity, D) NRTI/NNRTI/entry inhibitor-induced CAR-mediated *CYP2B6* promoter activity. Data is presented as mean ($n \geq 3$) ± S.E.M. relative to average untreated, analysed by one-way ANOVA with Dunnett's *post hoc* analysis, *$P \leq 0.05$. ARV = antiretroviral, PI = protease inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NNRTI = non-nucleoside reverse transcriptase inhibitor, UnTX = untreated, CITCO = 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl)oxime (positive control), ATV = atazanavir, DRV = darunavir, FOS = fosamprenavir, IDV = indinavir, LPV = lopinavir, NFV = nelfinavir, RTV = ritonavir, SQV = saquinavir, TPV = tipranavir, ABC = abacavir, AZT = zidovudine, EFV = efavirenz, MVC = maraviroc, NVP = nevirapine, TFV = tenofovir, 3TC = lamivudine.

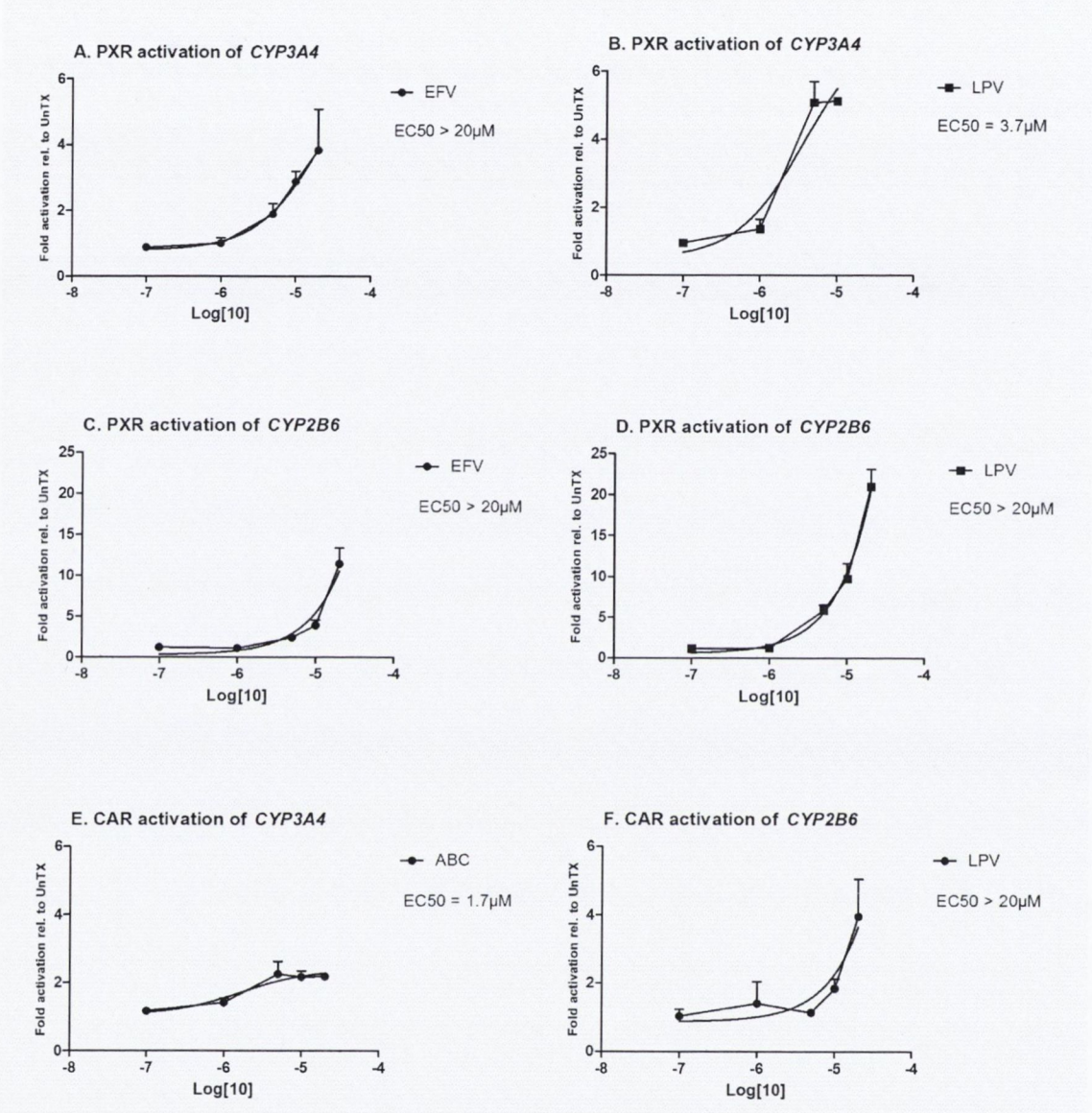


Figure 3.6 Dose-response curves for selected ARVs. One confirmed inducer of *CYP3A4* or *CYP2B6* promoter activity from each drug subclass: LPV (lopinavir, protease inhibitor), EFV (efavirenz, non-nucleoside reverse transcriptase inhibitor) and ABC (abacavir, nucleoside reverse transcriptase inhibitor). Dual-Luciferase Reporter Assays were performed with transfected HepG2 cells after 48h drug exposure. For every data point mean ± S.E.M. is shown (n = 3). A) EFV dose-response curve for PXR-mediated *CYP3A4* promoter activity, B) LPV dose-response curve for PXR-mediated *CYP3A4* promoter activity, C) EFV dose-response curve for PXR-mediated *CYP2B6* promoter activity, D) LPV dose-response curve for PXR-mediated *CYP2B6* promoter activity. E) ABC dose-response curve for CAR-mediated *CYP3A4* promoter activity, F) LPV dose-response curve for CAR-mediated *CYP2B6* promoter activity. EC_{50} values were calculated using GraphPad Prism version 5 (non-linear fit, sigmoidal dose-response curves). ARV = antiretroviral.

Table 3.7 Effect of low-dose ritonavir on *CYP3A4/CYP2B6* promoter activity by HIV protease inhibitors (PIs). Assessed by Dual-Luciferase Reporter Assays with PIs in combination with low-dose ritonavir (r): fold increases relative to single PIs (means ± S.D.). Results were analysed by unpaired Mann-Whitney t-tests, *P ≤ 0.05 regarded as indication of significant difference. ATV = atazanavir, DRV = darunavir, FOS = fosamprenavir, LPV = lopinavir, SQV = saquinavir, TPV = tipranavir.

	ATV/r	DRV/r	FOS/r	LPV/r	SQV/r	TPV/r
PXR/CYP3A4	1.66 ± 1.73	1.23 ± 0.74	1.05 ± 0.19	1.67 ± 0.50*	2.65 ± 0.98*	1.06 ± 0.52
PXR/CYP2B6	1.20 ± 0.13	1.21 ± 0.63	2.35 ± 1.92	1.98 ± 0.95	5.71 ± 1.40*	1.24 ± 0.67
CAR/CYP3A4	1.08 ± 0.89	1.25 ± 0.83	2.35 ± 0.50*	2.52 ± 0.92*	2.44 ± 1.06*	1.06 ± 0.47
CAR/CYP2B6	0.92 ± 0.20	1.28 ± 0.47	0.74 ± 0.34	1.75 ± 0.65	1.10 ± 0.30	1.04 ± 0.52

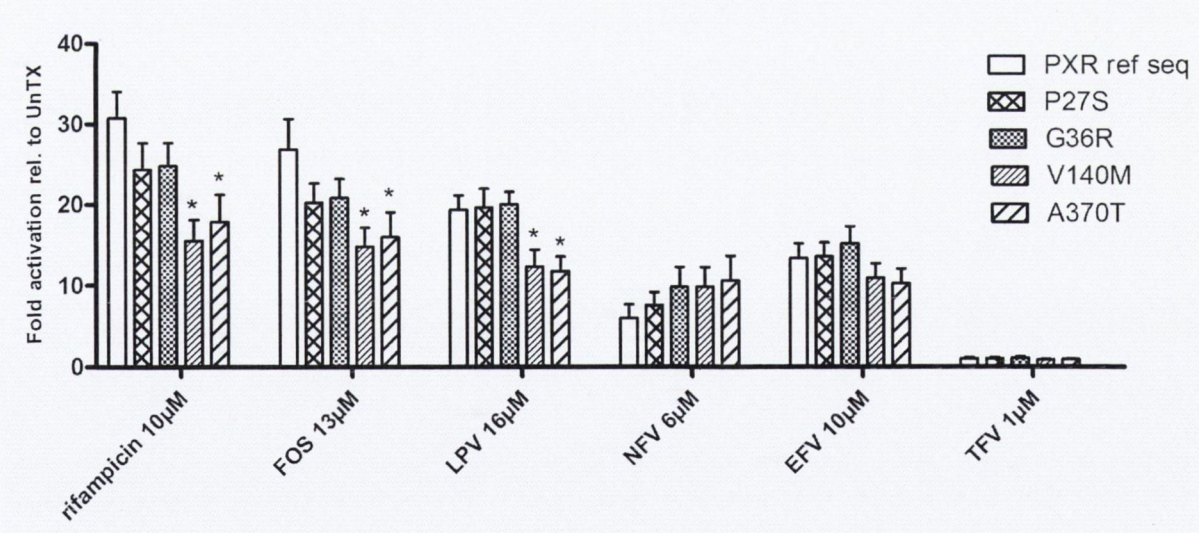


Figure 3.7 Impact of *NR1I2* (PXR) variants on ARV induction of *CYP3A4* promoter activity. Rifampicin and ARV-induced activation of *CYP3A4* promoter activity by PXR variants (relative to untreated promoter activity levels for each variant). Data is presented as mean (n ≥ 6) ± S.E.M., analysed by one-way ANOVA with Dunnett's *post hoc* analysis. *P ≤ 0.05. ARV = antiretroviral, PXR ref seq = "wildtype" PXR, UnTX = untreated, Rif = rifampicin (positive control), FOS = fosamprenavir, LPV = lopinavir, NFV = nelfinavir, EFV = efavirenz, TFV = tenofovir.

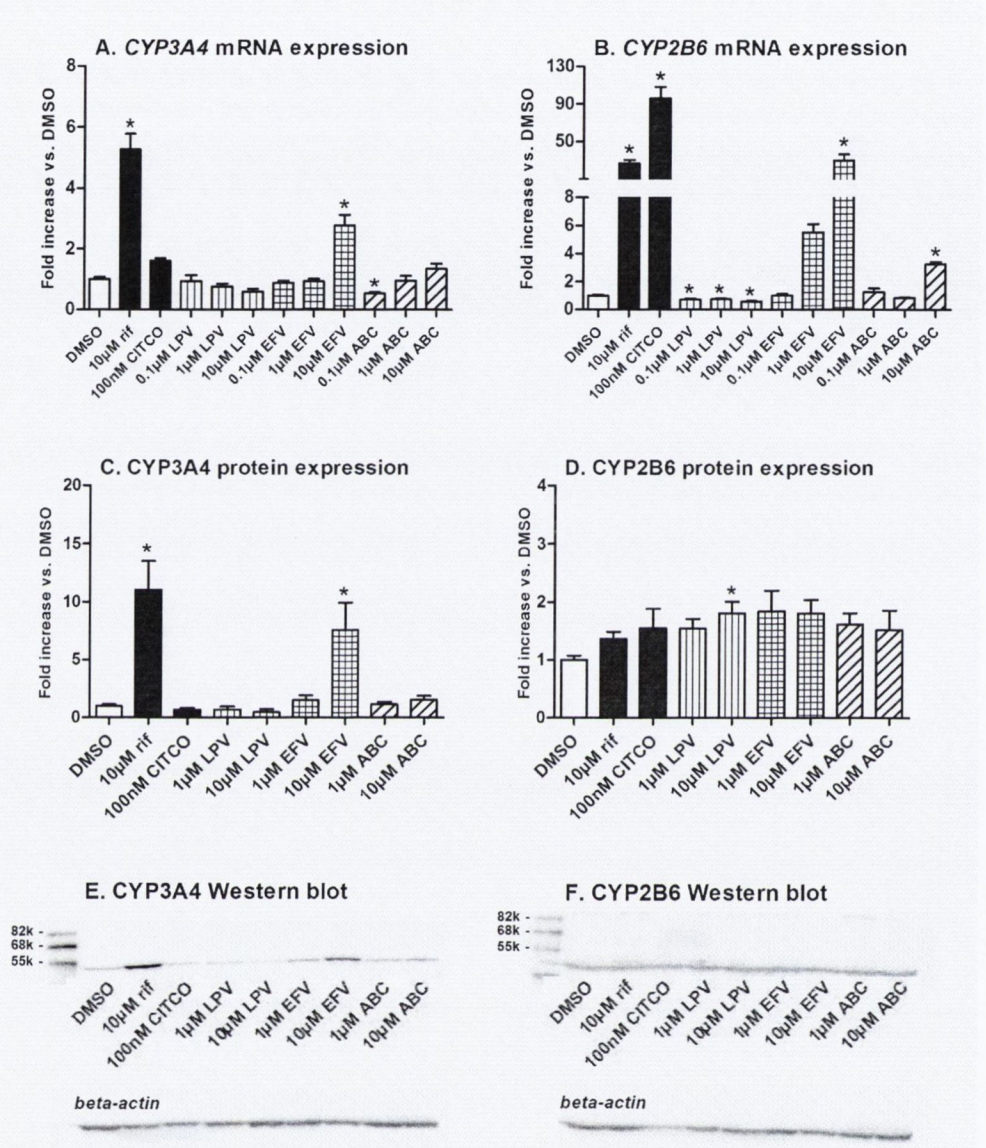


Figure 3.8 CYP3A4 and CYP2B6 mRNA and protein expression in primary human hepatocytes after 48h drug exposure (n = 4). A) *CYP3A4* mRNA expression as measured by real time PCR (relative to vehicle control), B) *CYP2B6* mRNA expression as measured by real time PCR (relative to vehicle control), C) CYP3A4 protein expression as measured by Western blot, D) CYP2B6 protein expression as measured by Western blot, E) representative blot for CYP3A4 (57kDa) with corresponding β-actin internal standard blot, F) representative blot for CYP2B6 (56kDa) with corresponding β-actin internal standard blot. Data was normalised to β-actin and presented as mean ± S.E.M. DMSO = dimethylsulphoxide (vehicle), rif = rifampicin (PXR inducer, positive control), CITCO = 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl)oxime (CAR inducer, positive control), LPV = lopinavir, EFV = efavirenz, ABC = abacavir.

3.5 Discussion

In this study, the frequency distribution of SNPs in genes selected for their established or likely impact on ARV drug metabolism was examined in a cohort of more than 1000 Caucasian and Sub-Saharan African HIV patients. The ability of 16 different ARVs at clinically relevant concentrations to induce promoter activity of *CYP3A4* or *CYP2B6* via nuclear receptors PXR and CAR was evaluated, as well as the effect of low-dose ritonavir in combination with a subset of the PIs. The impact on ARV-induced promoter activity of *CYP3A4* by four exonic, non-synonymous *NR1I2* (PXR) polymorphisms detected among the patients was subsequently assessed. Real-time PCR and Western blot analysis were also conducted with primary human hepatocytes to detect changes in CYP3A4 and CYP2B6 mRNA and protein expression following drug exposure.

The genotyping results of this study strengthen allele frequency data from smaller studies (ranging between 48-511 patients) of comparable groups (*NR1I2*[330, 333, 349, 377], *CYP3A4*[335, 337, 338, 380, 382, 384, 392, 393], *CYP2B6*[335, 393, 394] and *ABCB1/MDR1*[186, 194, 374, 395]. However, few studies have included a large number of subjects from Sub-Saharan Africa. Our study, containing 357 Sub-Saharan Africans, revealed a higher prevalence in this subgroup of the *CYP3A4* promoter polymorphism -392A>G as well as *CYP2B6* SNPs 516G>T, 983T>C and 785A>G, compared to the Caucasian patients. Another example is the *ABCB1* (*MDR1*) 3435C allele, associated with higher P-gp expression[395] and lower intracellular nelfinavir concentrations[396], which was twice as common among Sub-Saharan Africans. Also, for more than half of the *NR1I2* (PXR) SNPs screened for, the "minor alleles" linked to altered expression or activity were present at higher frequencies in the Sub-Saharan African population. Although no ancestry informative markers[397] were included in the screening, allele frequencies similar to data from previous studies (where available) are reassuring that the differences between ethnicities are authentic. The two deviations from HWE (*NR1I2* 7635A>G, *ABCB1* 2677G>T) among Sub-Saharan Africans can be explained by a degree of genetic diversity within this subcontinent: when the patients were divided into North-East, North-West and Southern regions these SNPs were in HWE in all three groups (however 8055C>T deviated from HWE in the Southern group). Increased plasma concentrations of NNRTIs[398] and predisposition to toxicity (typically cardiovascular, renal and psychiatric events[399]) seen among patients of African origin are mainly attributed to genetic variation in *CYP2B6*, although multiple polymorphisms in the *NR1I2* (PXR) gene may also be a contributing factor. The only SNP in *NR1I2* (PXR) clinically associated with alterations in ARV drug levels to date; -6994C>T which reduced atazanavir concentrations among homozygotes[334], was nearly twice as

common in the Caucasian population. Furthermore, some significant differences in allele frequency were noted between our Sub-Saharan African patients and mixed African American groups (*NR1I2* (PXR): Zhang *et al*[330]; *CYP3A4* and *ABCB1/MDR1*: Haas *et al*[335], see Table 3.5). This indicates that predictions of drug efficacy and toxicity in African HIV patients based on data from African American study populations should perhaps be interpreted with caution.

Results from Dual-Luciferase Reporter assays indicate that PXR has a more pronounced role than CAR in mediating ARV-induced promoter activity of *CYP3A4* and *CYP2B6* in a HepG2 cells. Its dominance over CAR as an induction pathway may be explained by a higher degree of ligand promiscuity due to a larger and more flexible ligand binding pocket[400], but as suggested by others[401] it is also possible that the constitutive activation of CAR in immortalised cell lines[402] renders the process of identifying activators of this nuclear receptor more difficult. Nevertheless, this study found fosamprenavir, lopinavir, tipranavir and abacavir to have CAR-activating abilities.

The majority of the drugs found to have inductive abilities were from the PI subclass; however, it does not appear to be a general characteristic as some PIs did not give rise to any significant increase of *CYP3A4/CYP2B6* promoter activity at the concentrations tested. This is consistent with earlier results from Dussault *et al*[324], who were also unable to detect PXR activation by indinavir and saquinavir at 10μM. However, this publication also presented negative results for nelfinavir which in our study increased PXR-mediated *CYP3A4* promoter activity significantly at 6μM. Conversely, Gupta *et al*[327] reported PXR activation by ritonavir, saquinavir, indinavir and atazanavir whose effects did not differ significantly from untreated controls in our study. These discrepancies may be due to the use of lower concentrations (with the exception of indinavir which was used at 15 μM) and a different cell line; HepG2 (human hepatocarcinoma, widely used for reporter assays) vs. CV-1 (African green monkey kidney cells) and LS180 (human colorectal adenocarcinoma) in the other studies. The degree of efavirenz induction of *CYP3A4* via the PXR pathway is comparable to luciferase reporter assays performed by Hariparsad *et al*[328] in HepG2, where a 3-4 fold increase was reached. A somewhat surprising result was the finding that abacavir increased both PXR-mediated *CYP2B6* promoter activity as well as CAR-mediated *CYP3A4* promoter activity, considering that as an NRTI it is subjected to very limited CYP450 metabolism and therefore an unlikely candidate for involvement in drug interactions through this pathway. Nevertheless, the inductive abilities of efavirenz and abacavir testify that these characteristics are not exclusive to PIs. The finding that low-dose ritonavir enhanced the response to most PIs, despite demonstrating no ability to activate PXR alone at the same concentration, is

interesting. This dualistic effect of enzyme inhibition and promoter activation is likely to contribute to the complexity of ritonavir-associated drug interactions.

When investigating the effect of *NR1I2* (PXR) polymorphism variants on rifampicin-activated *CYP3A4* promoter activity in HepG2 cells, we found a lower induction with V140M and A370T. Similar results were presented by Hustert *et al*[333] using LS174T cells, where the difference between the PXR reference sequence and variants however did not reach statistical significance. A lowered response was also seen in our experiments with the same PXR variants using fosamprenavir and lopinavir, but not with efavirenz. This could suggest an ARV drug class-specific effect. However, the same is not found with nelfinavir, and efavirenz failing to show significant changes with variant constructs is perhaps more likely to be due to a lower inductive power at this concentration. It is plausible that any CYP450-inductive effect imposed by fosamprenavir or lopinavir treatment could be diminished among patients in the cohort carrying one of these SNPs, potentially changing their drug metabolism in comparison with other patients.

Validation of the results in primary human hepatocytes exposed to lopinavir, efavirenz and abacavir, confirmed significant increases in both mRNA and protein expression for CYP3A4 as well as *CYP2B6* mRNA by efavirenz. CYP2B6 was also increased at an mRNA level by abacavir and at a protein level by lopinavir. Paradoxically, abacavir and lopinavir also decreased *CYP3A4* and *CYP2B6* mRNA expression, respectively.

A limitation to this study arises from the difficulty in determining accurate correlations between *in vivo* and *in vitro* drug concentrations; confounding factors include plasma protein binding. Our reporter assay experiments were performed in 10% serum and the concentrations used were plasma c_{max} values from clinical studies. Good correlations between plasma and intracellular concentrations have been demonstrated for some but not all ARVs[403]. It is possible that these estimates exceed the actual concentrations, although they are in keeping with the concentration range frequently used in *in vitro* reporter assays, which is a well evaluated tool for predicting *in vivo CYP3A4* induction[404]. Great variability has also been reported in ARV drug concentrations in patients[405]. Nevertheless, it would be of great value to validate this study clinically in order to establish the relationship between genotype and phenotype. In this large cohort however, where the patients are on complex drug regimens, it would be difficult to differentiate an effect of individual drugs.

In conclusion, we have shown that there are distinctive differences in prevalence of polymorphisms in genes of relevance for ARV drug interactions between Caucasian and Sub-Saharan African populations. This could affect the extent of PXR- and CAR-mediated *CYP3A4/CYP2B6* induction by ARVs, potentially influencing the bioavailability and/or toxicity of the inducing drug as well as co-administered drugs metabolised by these enzymes. Some SNPs in coding regions of the *NR1I2* (PXR) gene examined in this study are indeed functionally relevant and may have a considerable impact on ARV pharmacokinetics among carriers.

4 Off-target effects by antiretrovirals: a combined *in silico* and *in vitro* approach to assess nuclear receptor activation

4.1 Introduction

Nuclear receptors form a family of proteins which control the expression of a plethora of genes implicated in cell growth, inflammation, energy homeostasis, and drug metabolism. In humans, 48 different nuclear receptors have been identified. Structurally, they share several conserved functional domains including: an N-terminal ligand independent activation function domain (AF-1), a DNA binding domain (DBD) containing two zinc fingers, a C-terminal ligand binding domain (LBD) that accommodates small lipophilic molecules, and a ligand-dependent transcriptional activation function domain (AF-2). Some nuclear receptors, e.g. pregnane X receptor (PXR), constitutive androstane receptor (CAR) and liver X receptor (LXR), undergo heterodimerisation with retinoid X receptor (RXR) as a prerequisite for binding to specific response elements in the promoter region of target genes. Most RXR heterodimers reside in the nucleus bound to DNA, which in the absence of a ligand are thought to be complexed with co-repressor proteins, thus inhibiting gene transcription. Upon ligand binding, nuclear receptors undergo a conformational change which displaces the co-repressor and facilitates interaction with co-activator proteins, leading to gene transcription. Nuclear hormone receptors, on the other hand, reside in the cytoplasm bound to heat shock proteins (Hsp). Upon ligand binding, the receptors dissociate from Hsp and translocate to the nucleus where homodimerisation and recruitment of co-activators occurs to facilitate binding to response elements and subsequently gene transcription[243, 406].

We have previously demonstrated the ability of darunavir, fosamprenavir, lopinavir, nelfinavir, tipranavir, abacavir and efavirenz from the three major ARV drug classes (protease inhibitors/PIs, nucleoside reverse transcriptase inhibitors/NRTIs and non-nucleoside reverse transcriptase inhibitors/NNRTIs) to activate PXR, with consequences for drug interaction potential. Our study also showed that fosamprenavir, lopinavir, tipranavir and abacavir activate CAR[407]. The previous study focussed on PXR and CAR because of their pivotal role in xenobiotic metabolism. However, nuclear receptors are also involved in a variety of other critical endogenous processes such as reproduction, growth, metabolism, inflammation and central nervous system (CNS) function. Three important

subtypes of nuclear receptors involved in aforementioned processes are LXRs, estrogen receptors (ERs) and glucocorticoid receptor (GR).

4.1.1 Liver X receptors

Liver X receptors (LXRα/β) are activated by naturally occurring oxysterols which are intermediates in cholesterol metabolic pathways in the liver, adrenal glands and brain. These nuclear receptors modulate expression of hepatic cholesterol 7α-hydroxylase (CYP7A), the enzyme responsible for the rate-limiting step of conversion of cholesterol to bile acids; hence the activation by oxysterols serves as a feedback loop[408]. LXRs are also regulators of cholesterol metabolism and efflux through gene expression of ATP-binding cassette transporters ABCA1 and ABCG1 and apolipoproteins (e.g. ApoE). As such, they are of importance for atherosclerosis development in macrophages[408] and generate neuroprotective effects as cholesterol is essential for brain function[309]. In addition, both LXRα and LXRβ mediate repressive action on a set of inflammatory genes[409]. Another target gene is the sterol regulatory element binding protein 1c (*SREBP-1c*)[410]. Expression of this lipogenic transcription factor leads to increased fatty acid synthesis. In preadipocytes, adipogenesis is stimulated by LXR agonists through upregulation of peroxisome proliferator-activated receptor γ (PPARγ)[411].

4.1.2 Estrogen receptors

Estrogen receptors (ERα/β) are classical endocrine receptors which homodimerise upon binding to steroid hormones; a major endogenous ligand is 17β-estradiol. ERs are expressed in a broad range of tissues and are most commonly associated with reproductive function[412] and breast and prostate cancer development[308]. However, estrogens also have favourable effects on lipid levels, vascular tone and fibrinogen levels and are hence protective against cardiovascular disease[413]. ERα activation mediates anti-lipogenesis, improved insulin sensitivity and reduced adipose tissue mass, while ERβ activation disrupts glucose and lipid homeostasis[414]. Estrogen receptors have also been implicated in protective functions against neurotoxic stimuli and in models of CNS disease[308].

4.1.3 Glucocorticoid receptor

Glucocorticoid receptor (GR), also an endocrine receptor, is an important regulator of carbohydrate, protein and fat metabolism. During fasting, it stimulates hepatic gluconeogenesis and release of

amino acids in the periphery, protecting glucose-dependent tissues such as the brain and heart[415]. Glucocorticoids have a fundamental role in modulation of inflammation and immunity by suppression of cytokine and chemokine release, and by affecting differentiation or inducing apoptosis of immune cells[416]. Glucocorticoids are also critical for homeostasis of the hematopoietic, renal and reproductive systems[415]. Elevated cortisol levels and/or abnormalities in GR function have often been observed in psychotic major depression[310], while glucocorticoid receptor agonists can improve depressive symptoms[417]. Additionally, chronic glucocorticoid administration impairs cognition in humans[418].

Little is known regarding the effects of ARVs on nuclear receptors other than PXR and CAR. Few have investigated direct interactions between ARVs and nuclear receptor ligand binding domains (LBDs): Lenhard *et al*[419] found that saquinavir can compete with radiolabelled rosiglitazone in peroxisome proliferator-activated receptor γ (PPARγ)-LBD scintillation proximity assays while amprenavir, indinavir, nelfinavir and ritonavir had little effect on ligand binding to PPARγ. None of these PIs tested showed affinity to retinoid X receptor α (RXRα)-LBD. Dussault *et al*[141] found no significant binding to LXRα-LBD or ERα-LBD by 3μM ritonavir in reporter assays.

Others have used surrogate markers such as target gene mRNA or protein expression as indication of nuclear receptor activation, sometimes with conflicting results. Pou *et al*[420] demonstrated increased mRNA and protein levels of LXR targets ABCA1 and CD36 in THP-1 macrophages after exposure to 3.5μM ritonavir, with activation of LXRα as the authors' proposed mechanism of action. Nguyen *et al*[421] had previously shown increased SREBP-1, also a direct target gene of LXR, in differentiating adipocytes exposed to ritonavir. These findings were confirmed in rats by Riddle *et al*[422] who ascribed ritonavir-induced dyslipidemia to accumulation of SREBP-1 protein in the nucleus of liver and adipose tissue; however no change was detected in *SREBP-1* mRNA levels. Interestingly, other researchers have reported decreased protein levels of SREBP-1 in an adipocyte cell line after exposure to indinavir[423]. Dramatically lower levels of SREBP-1c mRNA were also detected in fat from HIV patients treated with indinavir or nelfinavir plus stavudine/lamivudine in comparison to healthy controls[424]. Due to intricate cross-regulation, it is however difficult to attribute changes in gene expression to specific nuclear receptors.

4.1.4 Nuclear receptor cross-regulation

It is well-known that a great degree of ligand overlap and cross-talk occurs between nuclear receptors. For example, the synthetic LXR agonist T0901317 has also been shown to activate PXR and induce expression of PXR target genes such as *CYP3A4* and *CYP2B6*[425, 426], and likewise the endogenous LXR ligand 24(S),25-epoxycholesterol can increase *Cyp3a* mRNA in Lxr-null mouse and rat hepatocytes[427]. The prototypical PXR inducer rifampicin decreases expression of LXR target genes *ABCA1* and scavenger receptor-BI (*SR-BI*, facilitates cholesterol efflux to lipoproteins) in human and rat hepatocytes[428]. Conversely, expression of LXR target gene *SREBP-1c* represses target genes of both PXR and CAR[429]. Different phytoestrogens have been reported as both agonists (equol[430]) and antagonists (coumestrol[431]) of PXR. One of the first publications describing mouse PXR[259] identified GR agonists (e.g. dexamethasone) and remarkably also GR antagonists (e.g. pregnenolone 16α-carbonitrile (PCN)) as activators. The latter turned out to be a less potent activator of human PXR[236, 263]. Nevertheless, GR can also directly promote transcription of *CYP3A4* as demonstrated by reporter assays using dexamethasone as an inducer and further supported by the presence of a glucocorticoid response element in the regulatory region of this gene[288].

4.1.5 Cross-regulation between LXRs, ERs and GR in overlapping metabolic effects

Both ERs and GR are involved in adipocyte development and bone remodelling; estrogen favours osteoblastogenesis over adipogenesis in bone marrow stromal cells[303] whereas glucocorticoids have the opposite effect, promoting osteogenic differentiation[302]. 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) plays an important role in mediating glucocorticoid action on preadipocyte differentiation, by governing the conversion of inactive cortisone to active cortisol[432]. It has also been demonstrated that activation of LXRs downregulates 11beta-HSD1 expression and activity *in vitro* and *in vivo*[433]. This was paralleled by reduced expression of glucocorticoid-responsive gene *PEPCK* (phosphoenolpyruvate carboxykinase), indicating LXR interference with peripheral cortisol activation.

Furthermore, it is well established that glucocorticoids can induce insulin resistance[305]. ER has also been implicated in development of insulin resistance; clinical trials have uncovered an association between estrogen hormone replacement therapy and reduced incidence of diabetes[306, 307]. LXR agonists have shown insulin-sensitising properties in mice by virtue of downregulation of gene

expression of gluconeogenetic enzymes in the liver and upregulation of glucose transporter 4 (*glut4*) in adipose tissue[304].

4.1.6 Adverse metabolic effects of highly active antiretroviral therapy (HAART)

Highly active antiretroviral therapy (HAART) has significantly reduced morbidity and mortality of HIV-infected patients. Successful HAART is dependent on adequate drug concentrations and tolerability of the drug. However, the achievement of these is often encumbered by drug interactions and adverse events. Long-term administration of HAART is associated with a multitude of off-target metabolic effects, such as lipid abnormalities, fat redistribution, glucose intolerance, atherosclerosis and osteoporosis[70]. Typical lipid abnormalities include increased levels of triglycerides, low density lipoprotein (LDL) cholesterol and total cholesterol, and are often accompanied by lipoatrophy of face and limbs and lipohypertrophy of the abdomen[434]. However, these effects are not observed to the same extent with all ARV drugs: Calza *et al*[72] demonstrated that ritonavir or lopinavir/ritonavir treatment gave rise to significantly higher incidence of increased serum triglycerides compared to other PIs (amprenavir, indinavir, nelfinavir, saquinavir). Greater changes in limb fat were also observed with stavudine/didanosine compared to zidovudine/lamivudine and with nelfinavir compared to efavirenz by Dubé *et al*[435]. Both insulin resistance[434] and reduced bone mineral density (BMD)[94] have been associated with PI use, however nucleoside analogues have also been implicated[74, 436].

Insulin resistance[437] and dyslipidemia are established risk factors for atherosclerosis in the general population. Mondy *et al*[438] found that insulin resistance is a predictor of endothelial dysfunction and cardiovascular risk in also in patients on HAART. Lorenz *et al*[76] established HAART as an independent risk factor for atherosclerosis: intima media thickness (IMT) in the carotid bifurcation was 19.7% higher in patients on HAART compared to treatment-naïve subjects. The D:A:D Study[77] showed significant associations between the development of myocardial infarction and recent use of abacavir and didanosine, but not with zidovudine, stavudine or lamivudine. The authors commented on the unexpectedness of this finding as the metabolic effects of abacavir in particular were thought to be minor in comparison to for example stavudine[439].

Some of these metabolic effects seen with HAART may be related to the actions of nuclear receptors. Oral administration of LXR agonists in mice results in hypertriglyceridemia[440], while development of atherosclerosis is suppressed[441]. HAART-associated lipodystrophy, characterised by a reduction of facial, extremity and buttock fat and sometimes combined with visceral and dorso-cervical fat accumulation[442], is remarkably similar to Cushing's syndrome. This could imply an increase in circulating cortisol, however an early case study of four PI-treated subjects revealed either normal urine levels or adequate suppression by standard dexamethasone treatment[443]. A more recent study reports a correlation between pseudo-Cushing's features in patients with HAART-associated lipodystrophy and increased regeneration of cortisol by 11beta-HSD1 in adipose tissue[444]. Alternatively, the symptoms could arise because of non-cortisol dependent direct activation of GR[445]. In addition, there is evidence of a protective effect by estrogen in the development of PI-induced atherosclerosis: it prevents the accumulation of cholesteryl esters after ritonavir exposure in macrophages *in vitro*[446]. LDL-R (low density lipoprotein receptor) null female mice treated with ritonavir or amprenavir developed fewer atherosclerotic lesions than males, an effect which was obliterated by genetic removal of ERα[447]. Neuropsychiatric symptoms have been described in patients treated with zidovudine, abacavir and nevirapine[85], although the single most common cause of HAART-induced CNS toxicity is efavirenz[87]. However, no previous studies have investigated LXR, ER and GR activation as a potential mechanism of efavirenz-mediated CNS toxicity despite the involvement of these nuclear receptors in neuropsychiatric function.

4.2 Aims

We have shown activation of nuclear receptors PXR and CAR by ARVs and how this might affect drug metabolising capacity. Little is known regarding ARV activation of other nuclear receptors such as LXRs, ERs, and GR. Given the potential metabolic effects of nuclear receptor activation, this represents an important deficit in the literature.

Importantly, studies to date have been limited by lack of a unifying methodological approach and confined to a small number of drugs, mainly PIs. The aim of this study was to explore the potential of ARVs of different drug classes to act as ligands for LXRα, LXRβ, ERα, ERβ and GR using a combined approach: *in silico* modelling, assessment of direct ligand binding by cell-free fluorescent co-activator assays and evaluation of nuclear receptor activation in cell-based luciferase reporter assays.

4.3 Materials and methods

4.3.1 Antiretroviral drugs and controls

Fosamprenavir, lopinavir and nelfinavir were kindly donated by GlaxoSmithKline (Hertfordshire, UK), Abbott (Abbott Park, IL, USA) and Pfizer (Groton, CT, USA), respectively. Gilead Sciences (Foster City, CA, USA) generously provided tenofovir, while Boehringer Ingelheim (Dublin, Ireland) supplied with nevirapine. Efavirenz was purchased from LGM Pharmaceuticals (Boca Raton, FL, USA) and indinavir, ritonavir and saquinavir from USP Reference Standards (Rockville, MD, USA). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Abacavir, atazanavir sulphate, bicyclam JM-2987, didanosine, emtricitabine, etravirine, flavopiridol, lamivudine, maraviroc, raltegravir, stavudine, TAK-779, tipranavir, zalcitabine, zidovudine and integrase inhibitor 118-D-24. Darunavir was provided by Tibotec, Inc. through the same program. Nuclear receptor agonists T0901317 (LXR), 17β-estradiol (ER) and dexamethasone (GR) as well as LXR antagonist geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma-Aldrich (Wicklow, Ireland). All drug stocks were dissolved in dimethylsulphoxide (DMSO) except TAK-779 which was dissolved in water as advised by the provider, 17β-estradiol and dexamethasone which were dissolved in ethanol as recommended by the manufacturer and GGPP which was supplied in methanol:NH_4OH (7:3).

4.3.2 *In silico* analysis: setup of nuclear receptor LBD docking models

In silico evaluation of a library of 26 antiretrovirals (Table 4.1, see Appendix for drug structures) as potential ligands of LXRα, LXRβ, ERα, ERβ and GR was performed by Dr Fernando Blanco at the Molecular Design Group (School of Biochemistry and Immunology, Trinity College Dublin). As the structures of these nuclear receptors are extensively documented in the Research Collaboration for Structural Collaboration Protein Data Bank (RCSB PDB www.rcsb.org; LXR > 44 structure hits / 33 citations, ER > 123 structure hits / 58 citations and GR > 73 structure hits / 32 citations), docking using the LBD structures was chosen as an appropriate methodology for carrying out the evaluation. In the selection of crystal structures for the analysis the following parameters were taken into account: resolution, R-value, R-free and EC_{50} of the associated ligand (Table 4.2). R-value and R-free are measures of the quality of the atomic model obtained from the crystallographic data. The R-value is calculated after refinement of the atomic model, using all available diffraction pattern data included

in the development of the model, and should ideally be close to 0 (typical values are around 0.2). A completely random set of atoms would give a value of ≈ 0.63. For calculation of R-free, 10% of the diffraction data omitted in the modelling and refinement process is used to validate the model, in order to avoid bias. R-free should be similar or just above the R-value for a model that is not over-interpreting the data (www.rcsb.org, Brünger 1992[448]). The structures of the receptors with co-crystallised ligands were pre-processed using Molecular Operating Environment software (MOE version 2010.10; Chemical Computing Group, Montreal, Canada). The positions of hydrogen atoms and partial charges were calculated and a molecular force field minimisation step was performed using AMBER99 (Assisted Model Building with Energy Refinement, force field specifically designed for proteins) implemented in MOE. Co-activators and secondary water molecules were removed and the shape and features of the LBDs explored using MOE applications. In order to be used in docking analysis with Fast Rigid Exhaustive Docking software (FRED version 2.2.5; OpenEye Scientific Software, New Mexico, USA), the five pre-processed receptors were prepared with the *fred_receptor* application. FRED performs exhaustive docking by enumerating rigid rotations and translations of each given conformer within the active site. Shape-based filters were used to rapidly eliminate compounds in the database that were not complementary to the binding site of interest. Ligand poses from the exhaustive docking were optimised by rigidly rotating and translating the molecule. The optimised poses were scored by the Chemgauss3 scoring function (FRED), representing an estimation of the binding affinity.

4.3.3 *In silico* analysis: validation of nuclear receptor LBD docking models

Validation tests were also carried out to evaluate the ability of the model to retrieve known active compounds from a database containing both active and inactive compounds (decoys). For ER and GR receptors the sets of actives/decoys were downloaded from www.dud.docking.org, a support website designed to help test docking algorithms by providing challenging decoys. For the LXR receptor, a set of actives/decoys was built using the standard parameters recommended on the same website, with 36 decoys for each active (Table 4.3). The decoys selected had similar physical and structural properties as actives but dissimilar topology, to challenge the model. The ranges of molecular descriptors found in the sets of active ligands are presented in Table 4.4. All the molecules were pre-processed with MOE to calculate positions of hydrogen atoms and partial charges and energy minimisation performed using MMFF94x (Merck Molecular Force Field). OMEGA software (version

2.4.3, OpenEye Scientific Software, New Mexico, USA) was utilised with default parameters for conformational searches in order to test 50 conformers for each active/decoy. Receiver Operating Characteristic (ROC) curves were constructed to assess the ability of each model to distinguish known actives from known inactives (Figure 4.2).

4.3.4 *In silico* analysis: evaluation of ARV compounds as nuclear receptor ligands

The developed and validated docking models of LXRα, LXRβ, ERα, ERβ and GR were subsequently used in docking studies of the 26 ARV compounds using FRED. For evaluation of each compound, molecules were pre-processed in MOE and energy minimisation was performed as described above. 250 conformers per compound were generated using OMEGA software and used for docking. Docking scores were calculated using the Chemgauss3 scoring function in FRED. Results from the docking analysis of ARVs were filtered using a set of molecular descriptors for known ligands of each of the nuclear receptors: number of hydrogen donors, hydrogen acceptors, nitrogen atoms, oxygen atoms, rotatable bonds, hydrophobic bonds, rings, logP and molecular weight (Table 4.4). Compounds falling outside of the range of these parameters, even those passing the docking test, were not considered as potential ligands in this study.

4.3.5 Assessment of direct receptor-LBD interactions: fluorescence co-activator assays

LanthaScreen Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) Coactivator Assay Kits (Invitrogen, Paisley, UK) were utilised to assess direct binding to nuclear receptors LXRα and ERα. Recruitment of co-activator is measured by monitoring FRET between a terbium-labelled antibody bound to the recombinant nuclear receptor LBD, and fluorescein on the co-activator peptide. This is brought about when co-activator binding affinity increases as a result of a conformational change in the LBD upon binding to a ligand. PGC1α (peroxisome-proliferator-activated receptor γ co-activator 1α) and TRAP220/DRIP-2 (thyroid hormone receptor-associated protein 220/vitamin D receptor-interacting protein 2) have been identified as co-activators of both LXRα[449, 450] and ERα[451, 452]. Assays were first validated using concentration ranges of known LXRα and ERα agonists; T0901317 and 17β-estradiol, respectively. Initial experiments were carried out to detect effects at 100μM with ARVs predicted as ligands of LXRα (darunavir, tipranavir, efavirenz, maraviroc, TAK-779 and flavopiridol) or ERα (efavirenz, flavopiridol) as well as a number of non-predicted ligands: these drugs were either confirmed PXR inducers (fosamprenavir, lopinavir, nelfinavir) or included as they are associated with

lipodystrophy (ritonavir), more favourable lipid profiles (atazanavir) or altered SREBP-1c levels (indinavir). Serial dilutions were made in DMSO and the assays performed according to the manufacturer's instructions. All test concentrations were run in triplicates in 384-well plates and every experiment included a vehicle control, a positive control (20μM T0901317 or 1μM 17β-estradiol) and a "no LBD" control. Dose-response curves were subsequently constructed for any positive hits. To test if compounds have LXRα antagonistic properties (as indicated by initial TR-FRET screening of single concentration drugs), the same protocol was carried out in the presence of 1.5μM T0901317 (EC_{80} of the agonist as measured by this assay). A known LXR antagonist, GGPP, was used as control. All assays were incubated for 2h at room temperature protected from light, followed by measurement of the 520/495 emission ratio using a BMG PheraStar instrument (BMG Labtech, Offenburg, Germany).

4.3.6 Reporter assays: plasmids

Human pCMX-LXRα and pCMX-LXRβ were generously provided by Professor David J Mangelsdorf, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, whereas 3xhLXRE-luc was donated by Professor Andrew J Brown, University of New South Wales, Sydney. Human pSG5-ERα and pSG5-ERβ were gifts from Professor Jan-Åke Gustafsson, Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm. Human 3xERE-TATA-luc was constructed by Professor Donald P McDonnell, Duke University Medical School, Durham, North Carolina, USA, and obtained through Addgene, Cambridge, MA, USA. Human pCMV6-GR and GR-luc were purchased from Origene (Rockville, MD, USA) and Panomics (Fremont, CA, USA), respectively. An internal standard, pRL-TK (expressing *Renilla* luciferase), was obtained from Promega (Madison, WI, USA).

4.3.7 Reporter assays: HepG2 cell culture and transfections

HepG2 cells (kindly donated by Dr Stephen Gray) were maintained in Minimum Essential Medium Eagle supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine and 100units + 0.1mg/ml penicillin-streptomycin (all from Sigma-Aldrich). For LXR and GR transfections, cells were seeded into 24-well plates (40,000 cells per well) the day before transient transfection using Lipofectamine LTX (Invitrogen) according to manufacturer's instructions with the following DNA quantities: 50ng pRL-TK + 5ng nuclear receptor expression plasmid + 400ng responsive element-luciferase construct. The transfections were allowed to proceed for 8-9h in serum- and antibiotic-free medium. For ER

transfections, the cells were seeded at the same density as above, but pre-treated 24h after seeding with phenol red-free Minimum Essential Medium (Gibco/Invitrogen) with 10% charcoal-stripped FBS, 2mM L-glutamine and 100units + 0.1mg/ml penicillin-streptomycin (Sigma-Aldrich) for 32-34h. Transfection was performed in phenol red-, serum- and antibiotic-free medium using the same reagent as above with 200ng pRL-TK + 500ng ER plasmid + 1µg 3xERE-TATA-luc over 15h.

4.3.8 Reporter assays: drug exposure and luciferase activity assessment

Transfected cells were washed with phosphate buffered saline (PBS, Sigma-Aldrich) and treatment initiated with 10µM of each antiretroviral drug diluted in phenol red-free Minimum Essential Medium (Gibco/Invitrogen) with 10% charcoal-stripped FBS, 2mM L-glutamine and 100units + 0.1mg/ml penicillin-streptomycin (Sigma-Aldrich). Exceptions from this uniform concentration were nelfinavir (1µM) and flavopiridol (100nM) which were used at lower concentrations for reasons of cytotoxicity. For experiments with LXR-transfected cells 5% foetal bovine lipoprotein-deficient serum (FB-LPDS, Intracel, Frederick, MD, USA) was used to reduce background activation of LXR. Similar to the TR-FRET assays, the drugs included were either predicted ligands of LXRs (darunavir, tipranavir, efavirenz, maraviroc, TAK-779, flavopiridol), ERs (efavirenz, flavopiridol), GR (efavirenz, flavopiridol), confirmed PXR inducers (efavirenz, fosamprenavir, lopinavir, nelfinavir) or included as they are associated with lipodystrophy (ritonavir), more favourable lipid profiles (atazanavir), or altered SREBP-1c levels (indinavir). Positive controls were: 10µM T0901317 for LXRs, 1µM dexamethasone for GR and 100nM 17β-estradiol (E2) for ERs. A vehicle control (0.1% DMSO) was also included. After completed drug exposure (24h for LXRs and GR, 30h for ERs), the cells were harvested and the Dual-Luciferase Reporter Assay System (Promega) utilised to measure transcription levels with the aid of a luminometer (Thermo Fisher Scientific, Dublin, Ireland). Reporter construct responses (firefly) were normalised to internal standard (*Renilla*) and fold increases calculated relative to vehicle controls.

4.3.9 Statistics

In TR-FRET co-activator assays, one-way ANOVA with Dunnett's *post hoc* analysis was utilised to identify compounds which caused significant increases in co-activator recruitment at 100µM (in comparison to DMSO control) in initial screening experiments. In dose-response experiments, curves were fitted using a sigmoidal dose-response equation and EC_{50}/IC_{50} values calculated using GraphPad Prism version 5.

Data from luciferase reporter assays were normalised to internal standards and also analysed by one-way ANOVA with Dunnett's *post hoc* analysis (GraphPad Prism version 5). Five independent experiments were performed in duplicates for each treatment and presented relative to DMSO controls as means ± S.E.M. P-values ≤ 0.05 were regarded as indication of significant difference. Student's t-tests were performed to clarify whether effects of certain ARVs were statistically significant in direct comparison to DMSO controls.

4.4 Results

4.4.1 *In silico* analysis: docking validation and ARV evaluation

A comparison of the nuclear receptor LBDs shows smaller sizes for ER (450Å^3)[453] and GR (599Å^3)[454], compared to LXR-LBD (700-800Å^3)[455] which is more extended. The three receptor types display predominantly hydrophobic LBDs, with a few specific hydrophilic areas potentially involved in hydrogen bond interactions (Figure 4.1). The validation of the docking models as measured by ROC area under curve coefficients deemed ERα (0.907) as excellent, LXRα (0.754) as fair, LXRβ (0.829) and ERβ (0.843) as good, while the ROC curve for GR (0.595) is close to a diagonal line (equivalent to random hits) and hence a poor quality model (Figure 4.2). Consequently, scoring results of the latter must be considered statistically less reliable. ARV docking scores and results from filtering by molecular descriptor parameters for each receptor are presented in Table 4.5 (LXRα), Table 4.6 (LXRβ), Table 4.7 (ERα), Table 4.8 (ERβ) and Table 4.9 (GR). For LXRα, the ARV compounds which passed the docking test and were compatible with molecular descriptors were (in order of best docking score): darunavir, maraviroc, flavopiridol, efavirenz, TAK-779 and tipranavir. Potential LXRβ ligands were TAK-779, maraviroc, flavopiridol, efavirenz, tipranavir and darunavir. Only efavirenz passed the ERα and ERβ *in silico* screening, while both flavopiridol and efavirenz were identified as potential GR ligands. The most common molecular descriptor leading to exclusion of compounds from the list of potential candidates, despite passing the docking test, was "number of hydrophobic atoms" (too few) for LXRα and LXRβ. The same molecular descriptor in addition to "number of nitrogens" (too many) were common reasons for exclusion In the ERα and ERβ analysis, while for the GR model the most common reasons for exclusion additionally were too many hydrogen acceptors or not fitting the molecular weight range (too small or too large). The molecules which did not pass the docking tests of all five receptors were almost always too large and had too few rotatable bonds.

4.4.2 Assessment of direct receptor-LBD interactions: fluorescence co-activator assays

To assess direct binding of ARV drugs (predicted as potential ligands *in silico*) to LXRα and ERα LBDs, TR-FRET co-activator assays were utilised. Initial screening experiments with high concentration (100μM) of drugs were carried out with both co-activators (PGC1α and TRAP220/DRIP-2) for LXRα and ERα. In LXRα experiments, recruitment of PGC1α yielded larger magnitudes of change than TRAP220/DRIP-2 and was subsequently used in the dose-response experiments with any positive hits. Positive control T0901317 was used to verify assay functionality (EC_{50} = 276nM, Figure 4.3A). PGC1α recruitment was increased by 60% with maraviroc, by 26% with darunavir and by 23% with tipranavir at the highest tested concentration (100μM) compared to DMSO controls. Dose-response curves are shown in Figure 4.3B; EC_{50} (maraviroc) was 16.8μM, EC_{50} (darunavir) was 21.7μM, and EC_{50} (tipranavir) was 30.0μM. Initial screening of ARV drugs in LXRα assays also indicated reduced TRAP220/DRIP-2 recruitment by efavirenz, TAK-779 and flavopiridol. Antagonist assays with T0901317 present (at EC_{80} = 1.5μM, calculated from dose-response curves with TRAP220/DRIP-2) confirmed attenuated co-activator recruitment by these drugs: efavirenz IC_{50} = 45.2μM (64.6% agonist effect remained at 100μM), TAK-779 IC_{50} = 206μM (60.8% agonist effect remained at 100μM) and flavopiridol IC_{50} = 26.4μM (64.3% agonist effect remained at 90μM) (Figure 4.4B). A known LXR antagonist, GGPP, was used as control (IC_{50} = 2.0 μM, Figure 4.4A).

In ERα TR-FRET assays, none of the ARVs tested affected recruitment of either of co-activator (PGC1α or TRAP220/DRIP-2). Positive control 17β-estradiol (E2, ER agonist) was used to ensure functional assays: IC_{50}(PGC1α) = 0.5nM, IC_{50}(TRAP220-DRIP2) = 0.7nM (Figure 4.5). Z'-factors[456] were calculated for all TR-FRET experiments to ascertain robustness and for the results presented values ranged between 0.64-0.84 indicating excellent assays (a value of 1 indicates a theoretically ideal assay with no variability).

4.4.3 Reporter assays

The ability of ARV drugs to activate nuclear receptors LXRα/β, ERα/β or GR in a complete cell system was assessed by *in vitro* luciferase reporter assays. Synthetic LXR agonist T0901317 (positive control) increased LXRα transcriptional activity with a fold change of 9.6 ± 1.3 (mean ± S.E.M.) compared to vehicle control, while LXRβ activity was increased by 2.8 ± 0.2. One-way ANOVA analysis of ARV effects on LXR transcriptional activity revealed agonistic effects by atazanavir, darunavir and ritonavir of both isoforms. For LXRα, fold changes of 2.8 ± 0.5 (atazanavir), 1.8 ± 0.2 (darunavir) and 3.5 ± 1.3

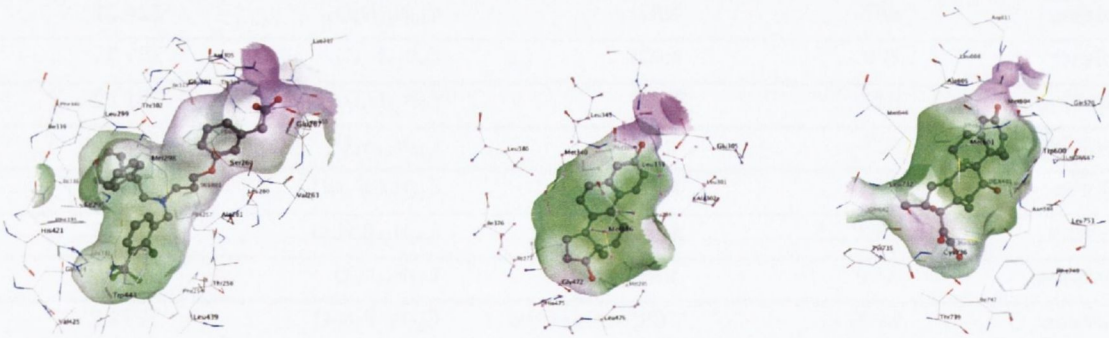
(ritonavir) were noted. For LXRβ, the corresponding values were 2.5 ± 0.9 (atazanavir), 2.0 ± 0.2 (darunavir) and 2.7 ± 1.4 (ritonavir). Efavirenz, on the other hand, reduced the activity of both receptor subtypes to 26 ± 7% (LXRα) and 7 ± 4% (LXRβ) of basal levels; although the reduction in LXRα activity was only significant effect when compared directly to DMSO control by Student's t-tests. Using the same statistical method, maraviroc-induced activity of LXRβ (1.6 ± 0.4) was classified as significantly different from DMSO, and similarly was the flavopiridol-induced reduction in activity of LXRα (52 ± 11% of basal levels) and LXRβ (32 ± 4%, Figure 4.6). Transcriptional activity of ERα was increased by efavirenz (13.6 ± 5.9) and tipranavir (5.5 ± 3.8) (Figure 4.7A). None of the ARV drugs tested had an effect on ERβ (Figure 4.7B) or GR promoter activation (Figure 4.8).

Table 4.1 ARV compounds included in the molecular modelling analysis. ARV = antiretroviral, PI = protease inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NNRTI = non-nucleoside reverse transcriptase inhibitor, Cdk = cyclin-dependent kinase.

ARV drug	Abbreviation	Class	Molecular formula	Mol. weight (g/mol)
Atazanavir	ATV	PI	$C_{38}H_{52}N_6O_7$	704.87
Darunavir	DRV	PI	$C_{27}H_{37}N_3O_7S$	546.66
Fosamprenavir	FOS	PI	$C_{25}H_{36}N_3O_9PS$	585.61
Indinavir	IDV	PI	$C_{36}H_{47}N_5O_4$	613.79
Lopinavir	LPV	PI	$C_{37}H_{48}N_4O_5$	628.81
Nelfinavir	NFV	PI	$C_{32}H_{45}N_3O_4S$	567.79
Ritonavir	RTV	PI	$C_{37}H_{48}N_6O_5S_2$	720.95
Saquinavir	SQV	PI	$C_{38}H_{50}N_6O_5$	670.85
Tipranavir	TPV	PI	$C_{31}H_{33}F_3N_2O_5S$	602.67
Abacavir	ABC	NRTI	$C_{14}H_{18}N_6O$	286.34
Didanosine	ddI	NRTI	$C_{10}H_{12}N_4O_3$	236.23
Emtricitabine	FTC	NRTI	$C_8H_{10}FN_3O_3S$	247.25
Lamivudine	3TC	NRTI	$C_8H_{11}N_3O_3S$	229.26
Stavudine	d4T	NRTI	$C_{10}H_{12}N_2O_4$	224.22
Tenofovir	TFV	NRTI	$C_9H_{14}N_5O_4P$	287.22
Zalcitabine	ddC	NRTI	$C_9H_{13}N_3O_3$	211.22
Zidovudine	AZT	NRTI	$C_{10}H_{13}N_5O_4$	267.25
Efavirenz	EFV	NNRTI	$C_{14}H_9ClF_3NO_2$	315.68
Etravirine	TMC125	NNRTI	$C_{20}H_{15}BrN_6O$	435.29
Nevirapine	NVP	NNRTI	$C_{15}H_{14}N_4O$	266.30
Maraviroc	MVC	CCR5 antagonist	$C_{29}H_{41}F_2N_5O$	513.68
TAK-779	-	CCR5 antagonist (investigational)	$C_{33}O_2N_2H_{38}$	495.69
bicyclam JM-2987	hydrobromide salt of AMD-3100	CXCR4 antagonist (investigational)	$C_{30}H_{70}Br_8N_8O_4$	506.83
Raltegravir	MK-0518	Integrase inhibitor	$C_{20}H_{20}FKN_6O_5$	444.42
118-D-24	-	Integrase inhibitor (investigational)	$C_{11}H_9N_3O_4$	247.2
Flavopiridol	-	Cdk inhibitor (investigational)	$C_{21}H_{20}O_5NCl$	402.85

Table 4.2 X-ray structures of nuclear receptor LBDs selected for *in silico* analysis. Presented are also parameters considered in the selection of these structures (relating to quality of models). PDB = Protein Data Bank (www.rscb.org). LBD = ligand binding domain, LXR = liver X receptor, ER = estrogen receptor, GR = glucocorticoid receptor, GW3965 = synthetic LXR ligand, AIT = (2S,3R)-3-(4-hydroxyphenyl)-2-[4-[(2R)-2-pyrrolidin-1-ylpropoxy]phenyl]-2,3-dihydro-1,4-benzoxathiin-6-ol (compound 19), E2 = 17β-estradiol, DEXA = dexamethasone. All structures correspond to human sequences. [a]Ligand affinity data from BindingDB (www.bindingdb.org).

Receptor	PDB Code	Resolution (Å)	R-Value	R-Free	Ligand	EC_{50} (nM)[a]
LXR α	3IPQ	2.00	0.201	0.234	GW3965	80.0 - 660.0
LXR β	1PQ6	2.40	0.209	0.262	GW3965	20.0 - 410.0
ER α	1XPC	1.60	0.184	0.251	AIT	0.04 - 1.3
ER β	3OLL	1.50	0.177	0.208	E2	0.1 - 30.0
GR	1M2Z	2.50	0.267	0.267	DEXA	0.2 – 7.2



LXR **ER** **GR**

Figure 4.1 Ligand binding pockets of LXR, ER and GR. Views of the three receptor families with prototypical ligands obtained with Molecular Operating Environment (MOE) software. Hydrophobic, neutral and hydrophilic regions are shown in green, white and violet respectively. LXR = liver X receptor, ER = estrogen receptor, GR = glucocorticoid receptor.

Table 4.3 Docking validation test data. N = number of input molecules, D = number of molecules passing docking validation, ROC AUC = Receiver Operating Characteristic area under curve (close to 1 indicates high true positive hit rate, close to 0 indicates high false positives rate).

Receptor	N Actives	N Decoys	D Actives	D Decoys	ROC AUC
LXRα	70	2564	70	504	0.754
LXRβ	70	2564	70	504	0.829
ERα	67	2570	67	2351	0.907
ERβ	67	2570	67	2351	0.843
GR	78	2947	78	2583	0.595

Table 4.4 Molecular descriptors of typical known ligands of nuclear receptors LXRα, LXRβ, ERα, ERβ and GR ("Actives" used in docking validation test). LXR = liver X receptor, ER = estrogen receptor, GR = glucocorticoid receptor.

Molecular descriptor	LXRα	LXRβ	ERα	ERβ	GR
Number of hydrogen donors	<3	<3	<4	<4	<3
Number of hydrogen acceptors	<6	<6	<6	<6	<5
Number of hydrophobic atoms	16-42	16-42	10-25	10-25	15-30
Number of nitrogens	<5	<5	<2	<2	<2
Number of oxygens	<7	<7	<6	<6	<5
Number of rotatable bonds	<18	<18	<6	<6	<6
Number of rings	<6	<6	<2	<2	<5
LogP	<12	<12	<6	<6	<8
Molecular weight (g/mol)	300-700	300-700	200-375	200-375	250-500

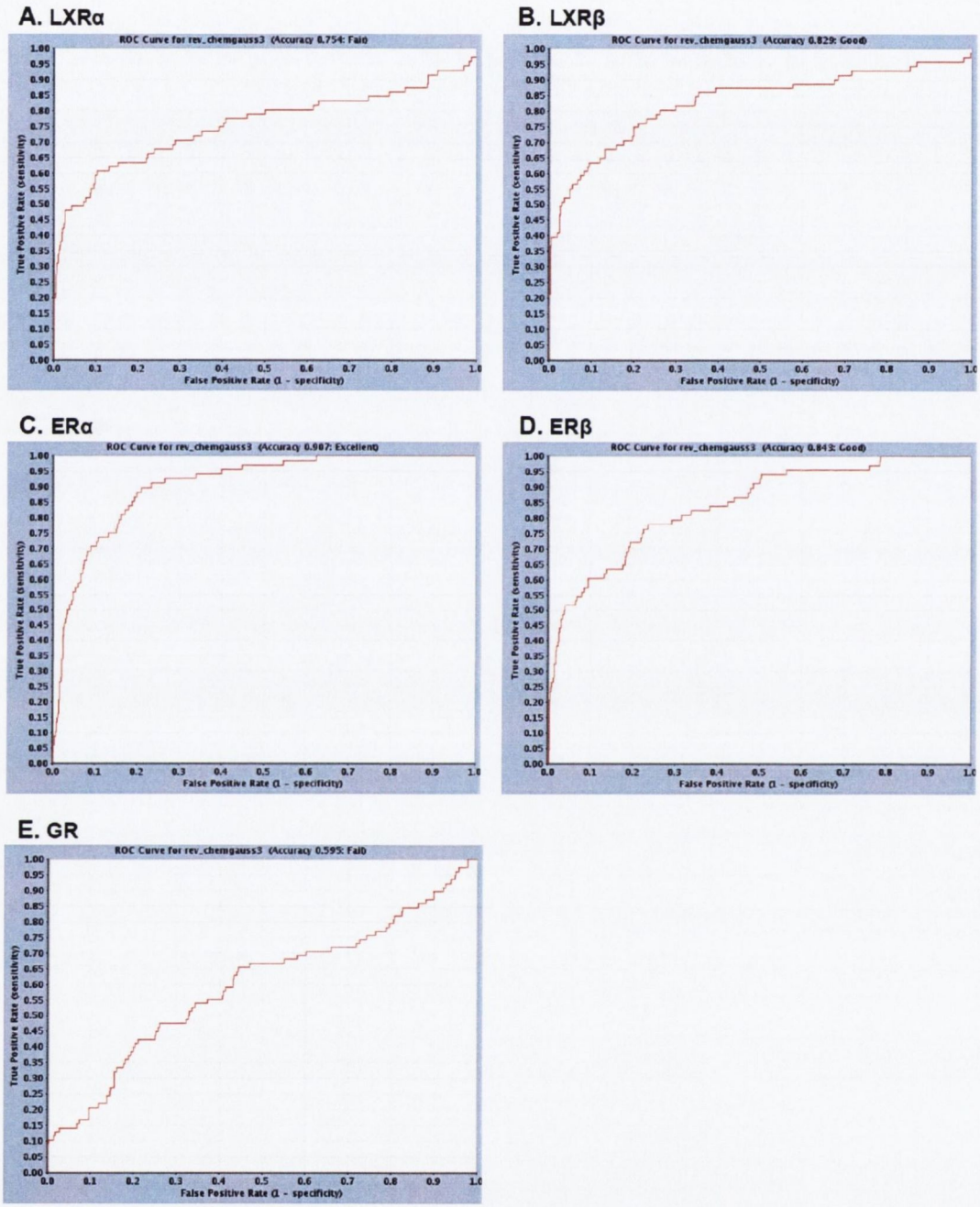


Figure 4.2 Receiver Operating Characteristic (ROC) curves for LXR, ER and GR models. Results from validation tests to evaluate the ability of the model to distinguish actives from inactives (decoys). Plotted are true positives rate (sensitivity, y-axis) vs. false positives rate (specificity, x-axis). A) LXRα ROC area under curve = 0.754, B) LXRβ ROC area under curve = 0.829, C) ERα ROC area under curve = 0.907, D) ERβ ROC area under curve = 0.843, E) GR ROC area under curve = 0.595. LXR = liver X receptor, ER = estrogen receptor, GR = glucocorticoid receptor.

Table 4.5 LXRα docking score results. S_{Dock} = docking score using Chemgauss3 scoring function (FRED software). Actives range for S_{Dock} indicates the limit above which 95% of the known actives scored. N_{Dock} = S_{Dock} value normalised to best scoring known active, a_don = number of hydrogen donors, a_acc = number of hydrogen acceptors, a_hyd = number of hydrophobic atoms, a_nN = number of nitrogens, a_nO = number of oxygens, b_rotN = number of rotatable bonds, rings = number of rings, MW = molecular weight (g/mol). F = compound failed docking test (listed in no specific order). Dark grey indicates compound outside actives range of molecular descriptors.

						LXRα					
Molecule	**S_{Dock}**	**N_{Dock}**	**a_don**	**a_acc**	**a_hyd**	**a_nN**	**a_nO**	**b_rotN**	**rings**	**log P**	**MW**
Actives range	***<-80.00 (95%)***		***< 3***	***< 6***	***16-42***	***< 5***	***< 7***	***< 18***	***< 6***	***< 12***	***300-700***
TMC125	-104.47	70.57	2	4	16	6	1	6	3	3.81	435.29
DRV	-101.01	68.23	3	6	24	3	7	13	4	2.15	546.66
MVC	-98.35	66.43	1	4	28	5	1	9	5	6.64	513.68
FLAV	-88.84	60.01	3	4	21	1	5	2	4	2.90	402.85
ABC	-88.58	59.84	3	4	10	6	1	4	4	0.41	286.34
RAL	-87.95	59.41	3	7	15	6	5	8	3	0.81	444.42
EFV	-86.71	58.57	1	1	17	1	2	3	3	4.10	315.68
118-D-24	-83.59	56.46	3	6	8	3	4	6	1	1.16	248.22
AZT	-78.76	53.20	2	6	7	5	4	4	2	-1.91	267.25
NVP	-78.45	52.99	1	3	12	4	1	1	4	1.90	266.30
IDV	-77.91	52.63	4	7	34	5	4	14	5	2.76	613.80
TFV	-75.36	50.90	4	7	5	5	4	5	2	-1.60	287.22
ddI	-73.74	49.81	2	5	5	4	3	2	3	0.13	236.23
ddC	-72.51	48.98	2	4	6	3	3	2	2	-0.50	211.22
FTC	-68.52	46.28	2	4	7	3	3	2	2	-0.52	247.25
NFV	-67.60	45.66	4	5	31	3	4	12	4	5.36	567.79
3TC	-67.16	45.37	2	4	6	3	3	2	2	-0.75	229.26
d4T	-63.50	42.89	2	4	7	2	4	2	2	-1.01	224.22
TAK-779	-62.81	42.43	1	2	32	2	2	7	5	6.45	495.69
TPV	-61.12	41.28	2	5	33	2	5	12	4	7.68	602.67
FOS	-59.82	40.41	5	8	24	3	9	15	3	1.50	585.61
JM2987	-40.54	27.38	3	3	27	8	0	4	3	0.92	506.83
LPV	-2.98	2.01	4	5	34	4	5	17	4	5.19	628.81
SQV	25.76	-17.40	5	7	34	6	5	16	5	3.31	670.85
ATV	F	-	5	7	34	6	7	22	3	4.74	704.87
RTV	F	-	4	6	36	6	5	22	4	5.00	720.96

Table 4.6 LXRβ docking score results. S_{Dock} = docking score using Chemgauss3 scoring function (FRED software). Actives range for S_{Dock} indicates the percentage of known actives above a given score. N_{Dock} = S_{Dock} value normalised to best scoring known active, a_don = number of hydrogen donors, a_acc = number of hydrogen acceptors, a_hyd = number of hydrophobic atoms, a_nN = number of nitrogens, a_nO = number of oxygens, b_rotN = number of rotatable bonds, rings = number of rings, MW = molecular weight (g/mol). F = compound failed docking test. Dark grey indicates compound outside actives range of molecular descriptors.

LXRβ											
Molecule	**S_{Dock}**	**N_{Dock}**	**a_don**	**a_acc**	**a_hyd**	**a_nN**	**a_nO**	**b_rotN**	**rings**	**log P**	**MW**
Actives range	***<-88.00 (95%)***		***< 3***	***< 6***	***16-42***	***< 5***	***< 7***	***< 18***	***< 6***	***< 12***	***300-700***
TAK-779	-107.86	72.09	1	2	32	2	2	7	5	6.45	495.69
TMC125	-103.74	69.33	2	4	16	6	1	6	3	3.81	435.29
RAL	-101.32	67.71	3	7	15	6	5	8	3	0.81	444.42
MVC	-99.84	66.72	1	4	28	5	1	9	5	6.64	513.68
ABC	-90.52	60.49	3	4	10	6	1	4	4	0.41	286.34
FLAV	-89.65	59.91	3	4	21	1	5	2	4	2.90	402.85
EFV	-84.91	56.74	1	1	17	1	2	3	3	4.10	315.68
TFV	-83.65	55.90	4	7	5	5	4	5	2	-1.60	287.22
TPV	-81.21	54.27	2	5	33	2	5	12	4	7.68	602.67
118-D-24	-79.15	52.90	3	6	8	3	4	6	1	1.16	248.22
NVP	-78.28	52.31	1	3	12	4	1	1	4	1.90	266.30
NFV	-78.20	52.26	4	5	31	3	4	12	4	5.36	567.79
AZT	-75.86	50.69	2	6	7	5	4	4	2	-1.91	267.25
LPV	-75.11	50.19	4	5	34	4	5	17	4	5.19	628.81
DRV	-74.39	49.71	3	6	24	3	7	13	4	2.15	546.66
FOS	-72.84	48.68	5	8	24	3	9	15	3	1.50	585.61
ddI	-71.42	47.73	2	5	5	4	3	2	3	0.13	236.23
FTC	-69.28	46.30	2	4	7	3	3	2	2	-0.52	247.25
3TC	-67.57	45.16	2	4	6	3	3	2	2	-0.75	229.26
ddC	-67.24	44.93	2	4	6	3	3	2	2	-0.50	211.22
IDV	-67.07	44.82	4	7	34	5	4	14	5	2.76	613.80
d4T	-66.77	44.62	2	4	7	2	4	2	2	-1.01	224.22
JM-2987	-37.83	25.28	3	3	27	8	0	4	3	0.92	506.83
ATV	-32.48	21.71	5	7	34	6	7	22	3	4.74	704.87
SQV	-27.33	18.27	5	7	34	6	5	16	5	3.31	670.85
RTV	F	-	4	6	36	6	5	22	4	5.00	720.96

Table 4.7 ERα docking score results. S_{Dock} = docking score using Chemgauss3 scoring function (FRED software). Actives range for S_{Dock} indicates the limit above which 95% of the known actives scored. N_{Dock} = S_{Dock} value normalised to best scoring known active, a_don = number of hydrogen donors, a_acc = number of hydrogen acceptors, a_hyd = number of hydrophobic atoms, a_nN = number of nitrogens, a_nO = number of oxygens, b_rotN = number of rotatable bonds, rings = number of rings, MW = molecular weight (g/mol). F = compound failed docking test (listed in no specific order). Dark grey indicates compound outside actives range of molecular descriptors.

ERα											
Molecule	**S_{Dock}**	**N_{Dock}**	**a_don**	**a_acc**	**a_hyd**	**a_nN**	**a_nO**	**b_rotN**	**rings**	**log P**	**MW**
Actives range	***<-75.00 (95%)***		***< 4***	***< 6***	***10–25***	***< 2***	***< 6***	***< 6***	***< 5***	***< 6***	***200-375***
AZT	-84.12	79.11	2	6	7	5	4	4	2	-1.91	267.25
FLAV	-80.55	75.76	3	4	21	1	5	2	4	2.90	402.85
TFV	-80.13	75.36	4	7	5	5	4	5	2	-1.60	287.22
118-D-24	-79.16	74.44	3	6	8	3	4	6	1	1.16	248.22
TMC125	-72.89	68.55	2	4	16	6	1	6	3	3.81	435.29
EFV	-72.73	68.40	1	1	17	1	2	3	3	4.10	315.68
d4T	-71.58	67.32	2	4	7	2	4	2	2	-1.01	224.22
MVC	-71.50	67.24	1	4	28	5	1	9	5	6.64	513.68
ddI	-70.11	65.94	2	5	5	4	3	2	3	0.13	236.23
NVP	-69.32	65.19	1	3	12	4	1	1	4	1.90	266.30
FTC	-64.09	60.28	2	4	7	3	3	2	2	-0.52	247.25
3TC	-63.11	59.35	2	4	6	3	3	2	2	-0.75	229.26
ddC	-62.27	58.56	2	4	6	3	3	2	2	-0.50	211.22
ABC	-61.25	57.60	3	4	10	6	1	4	4	0.41	286.34
ATV	F	-	5	7	34	6	7	22	3	4.74	704.87
DRV	F	-	3	6	24	3	7	13	4	2.15	546.66
FOS	F	-	5	8	24	3	9	15	3	1.50	585.61
IDV	F	-	4	7	34	5	4	14	5	2.76	613.80
LPV	F	-	4	5	34	4	5	17	4	5.19	628.81
NFV	F	-	4	5	31	3	4	12	4	5.36	567.79
RTV	F	-	4	6	36	6	5	22	4	5.00	720.96
SQV	F	-	5	7	34	6	5	16	5	3.31	670.85
TPV	F	-	2	5	33	2	5	12	4	7.68	602.67
TAK-779	F	-	1	2	32	2	2	7	5	6.45	495.69
JM-2987	F	-	3	3	27	8	0	4	3	0.92	506.83
RAL	F	-	3	7	15	6	5	8	3	0.81	444.42

Table 4.8 ERβ docking score results. S_{Dock} = docking score using Chemgauss3 scoring function (FRED software). Actives range for S_{Dock} indicates the limit above which 95% of the known actives scored. N_{Dock} = S_{Dock} value normalised to best scoring known active, a_don = number of hydrogen donors, a_acc = number of hydrogen acceptors, a_hyd = number of hydrophobic atoms, a_nN = number of nitrogens, a_nO = number of oxygens, b_rotN = number of rotatable bonds, rings = number of rings, MW = molecular weight (g/mol). F = compound failed docking test (listed in no specific order). Dark grey indicates compound outside actives range of molecular descriptors.

			ERβ								
Molecule	**S_{Dock}**	**N_{Dock}**	**a_acc**	**a_don**	**a_hyd**	**a_nN**	**a_nO**	**b_rotN**	**rings**	**logP**	**MW**
Actives range	***<-70.00 (95%)***		***< 4***	***< 6***	***10-25***	***< 2***	***< 6***	***< 6***	***< 5***	***< 6***	***200-375***
AZT	-77.63	80.09	6	2	7	5	4	4	2	-1.91	267.25
118-D-24	-77.21	79.66	6	3	8	3	4	6	1	1.16	248.22
TFV	-73.19	75.52	7	4	5	5	4	5	2	-1.60	287.22
FLAV	-72.44	74.74	4	3	21	1	5	2	4	2.90	402.85
d4T	-69.59	71.80	4	2	7	2	4	2	2	-1.01	224.22
NVP	-69.28	71.48	3	1	12	4	1	1	4	1.90	266.30
TMC125	-64.62	66.67	4	2	16	6	1	6	3	3.81	435.29
EFV	-64.20	66.23	1	1	17	1	2	3	3	4.10	315.68
FTC	-62.50	64.49	4	2	7	3	3	2	2	-0.52	247.25
ddI	-60.06	61.97	5	2	5	4	3	2	3	0.13	236.23
ddC	-57.74	59.57	4	2	6	3	3	2	2	-0.50	211.22
ABC	-57.56	59.39	4	3	10	6	1	4	4	0.41	286.34
3TC	-57.14	58.96	4	2	6	3	3	2	2	-0.75	229.26
RAL	2.41	-2.49	7	3	15	6	5	8	3	0.81	444.42
JM2987	9.28	-9.57	3	3	27	8	0	4	3	0.92	506.83
ATV	F	-	5	7	34	6	7	22	3	4.74	704.87
DRV	F	-	3	6	24	3	7	13	4	2.15	546.66
FOS	F	-	5	8	24	3	9	15	3	1.50	585.61
IDV	F	-	4	7	34	5	4	14	5	2.76	613.80
LPV	F	-	4	5	34	4	5	17	4	5.19	628.81
NFV	F	-	4	5	31	3	4	12	4	5.36	567.79
RTV	F	-	4	6	36	6	5	22	4	5.00	720.96
SQV	F	-	5	7	34	6	5	16	5	3.31	670.85
TPV	F	-	2	5	33	2	5	12	4	7.68	602.67
MVC	F	-	1	4	28	5	1	9	5	6.64	513.68
TAK-779	F	-	1	2	32	2	2	7	5	6.45	495.69

Table 4.9 GR docking score results. S_{Dock} = docking score using Chemgauss3 scoring function (FRED software). Actives range for S_{Dock} indicates the limit above which 95% of the known actives scored. N_{Dock} = S_{Dock} value normalised to best scoring known active, a_don = number of hydrogen donors, a_acc = number of hydrogen acceptors, a_hyd = number of hydrophobic atoms, a_nN = number of nitrogens, a_nO = number of oxygens, b_rotN = number of rotatable bonds, rings = number of rings, mw = molecular weight (g/mol). F = compound failed docking test (listed in no specific order). Dark grey indicates compound outside actives range of molecular descriptors.

GR											
Molecule	**S_{Dock}**	**N_{Dock}**	**a_don**	**a_acc**	**a_hyd**	**a_nN**	**a_nO**	**b_rotN**	**rings**	**log P**	**weight**
Actives range	***<-64.00 (95%)***		***< 3***	***< 5***	***15-30***	***< 2***	***< 5***	***< 6***	***< 5***	***< 8***	***250-500***
FLAV	-90.85	66.14	3	4	21	1	5	2	4	2.90	402.85
EFV	-89.75	64.74	1	1	17	1	2	3	3	4.10	315.68
TFV	-86.07	62.67	4	7	5	5	4	5	2	-1.60	287.22
AZT	-85.04	62.43	2	6	7	5	4	4	2	-1.91	267.25
118-D-24	-84.72	62.40	3	6	8	3	4	6	1	1.16	248.22
ABC	-82.78	59.46	3	4	10	6	1	4	4	0.41	286.34
d4T	-76.27	55.97	2	4	7	2	4	2	2	-1.01	224.22
ddI	-73.84	53.26	2	5	5	4	3	2	3	0.13	236.23
NVP	-72.28	52.51	1	3	12	4	1	1	4	1.90	266.30
TMC125	-71.26	52.09	2	4	16	6	1	6	3	3.81	435.29
FTC	-67.92	48.74	2	4	7	3	3	2	2	-0.52	247.25
3TC	-66.14	47.90	2	4	6	3	3	2	2	-0.75	229.26
ddC	-65.00	47.06	2	4	6	3	3	2	2	-0.50	211.22
RAL	-63.86	33.87	3	7	15	6	5	8	3	0.81	444.42
JM2987	-45.95	32.20	3	3	27	8	0	4	3	0.92	506.83
DRV	-43.69	25.56	3	6	24	3	7	13	4	2.15	546.66
MVC	-34.69	20.66	1	4	28	5	1	9	5	6.64	513.68
FOS	-28.04	13.81	5	8	24	3	9	15	3	1.50	585.61
TPV	-18.74	9.10	2	5	33	2	5	12	4	7.68	602.67
NFV	-12.35	0.00	4	5	31	3	4	12	4	5.36	567.79
ATV	F	-	5	7	34	6	7	22	3	4.74	704.87
IDV	F	-	4	7	34	5	4	14	5	2.76	613.80
LPV	F	-	4	5	34	4	5	17	4	5.19	628.81
RTV	F	-	4	6	36	6	5	22	4	5.00	720.96
SQV	F	-	5	7	34	6	5	16	5	3.31	670.85
TAK-779	F	-	1	2	32	2	2	7	5	6.45	495.69

Table 4.10 Summary of docking scores for all nuclear receptor models. Docking scores (N_{Dock}) for ARV compounds (normalised to Chemgauss3 score of best active = 100). Missing values indicates compounds did not pass the docking test, dark grey indicates compounds were outside the "actives range" for one or more molecular descriptor.

ARV	LXRα	LXRβ	ERα	ERβ	GR
Atazanavir (ATV)	-	21.71	-	-	-
Darunavir (DRV)	68.23	49.71	-	-	25.56
Fosamprenavir (FOS)	40.41	48.68	-	-	13.81
Indinavir (IDV)	52.63	44.82	-	-	-
Lopinavir (LPV)	2.01	50.19	-	-	-
Nelfinavir (NFV)	45.66	52.26	-	-	0.00
Ritonavir (RTV)	-	-	-	-	-
Saquinavir (SQV)	-17.40	18.71	-	-	-
Tipranavir (TPV)	41.28	54.27	-	-	9.10
Abacavir (ABC)	59.84	60.79	57.60	59.39	59.46
Didanosine (ddI)	49.81	47.73	65.94	61.97	53.26
Emtricitabine (FTC)	46.28	46.30	60.38	64.49	48.74
Lamivudine (3TC)	45.37	45.16	59.35	58.96	47.90
Stavudine (d4T)	42.89	44.62	67.32	71.80	55.97
Tenofovir (TFV)	50.90	55.90	75.36	75.52	62.67
Zalcitabine (ddC)	48.98	44.93	58.56	59.57	47.06
Zidovudine (AZT)	53.20	50.69	79.11	80.09	62.43
Efavirenz (EFV)	58.57	56.74	68.40	66.23	64.74
Etravirine (TMC125)	70.57	69.33	68.55	66.67	52.09
Nevirapine (NVP)	52.99	52.31	65.19	71.48	52.51
Maraviroc (MVC)	66.43	66.72	67.24	-	20.66
TAK-779	42.43	72.09	-	-	-
bicyclam JM-2987	-	-	-	-	-
Raltegravir (RAL)	59.41	67.71	-	-2.19	33.87
118-D-24	56.46	52.90	74.44	79.66	62.40
Flavopiridol (FLAV)	60.01	59.91	75.76	74.74	66.14

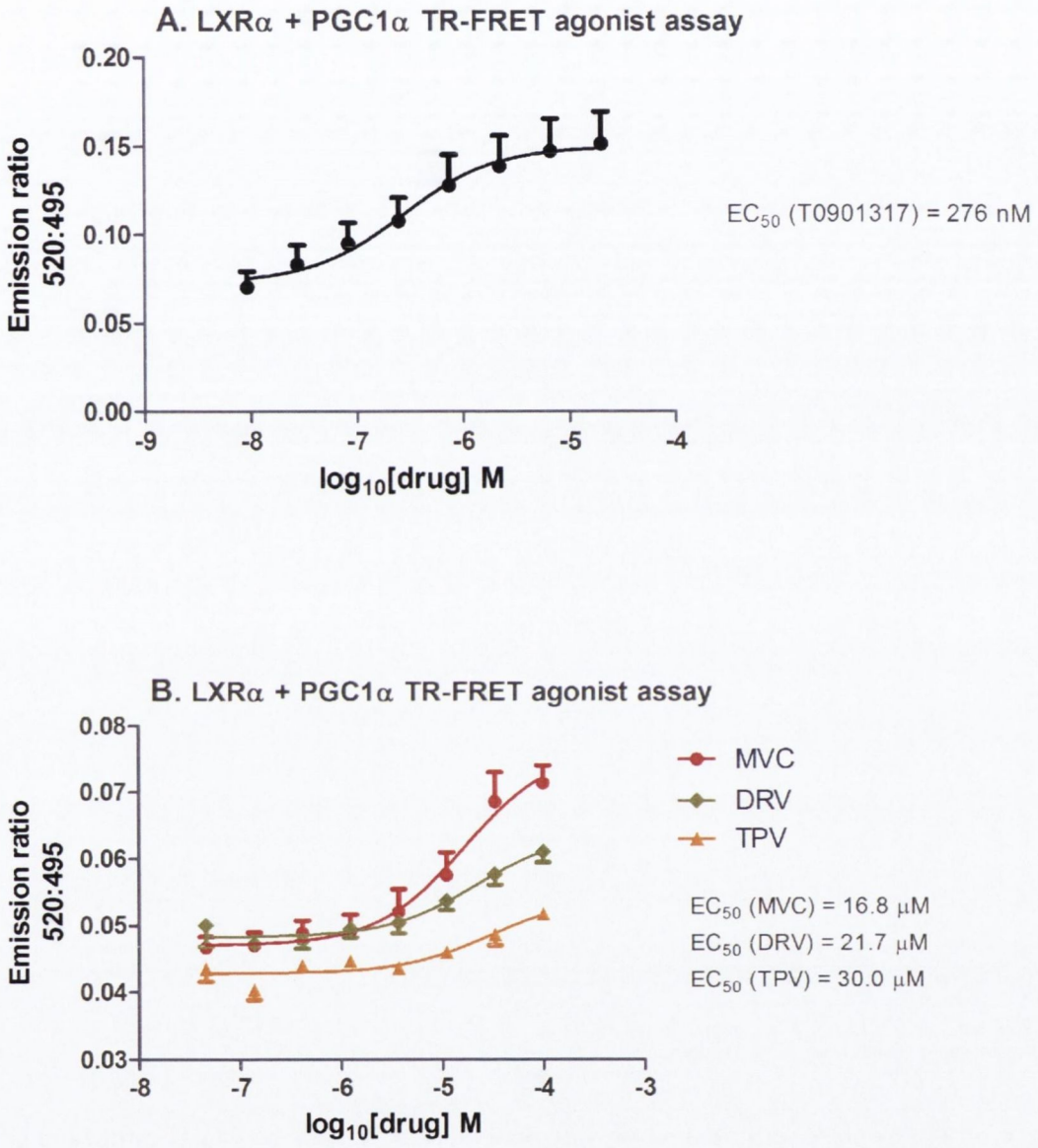


Figure 4.3 TR-FRET LXRα co-activator assays. A) Validation of PGC1α co-activator assay using T0901317, synthetic LXR agonist. B) Agonistic effects by maraviroc (MVC), darunavir (DRV) and tipranavir (TPV) on LXRα PGC1α recruitment. Two independent experiments were performed with triplicate wells (n = 6). TR-FRET emission ratio measured after 2h incubation at room temperature in the dark. Results are presented as means ± S.E.M. EC_{50} values were determined using a sigmoidal dose-response equation in GraphPad Prism version 5.

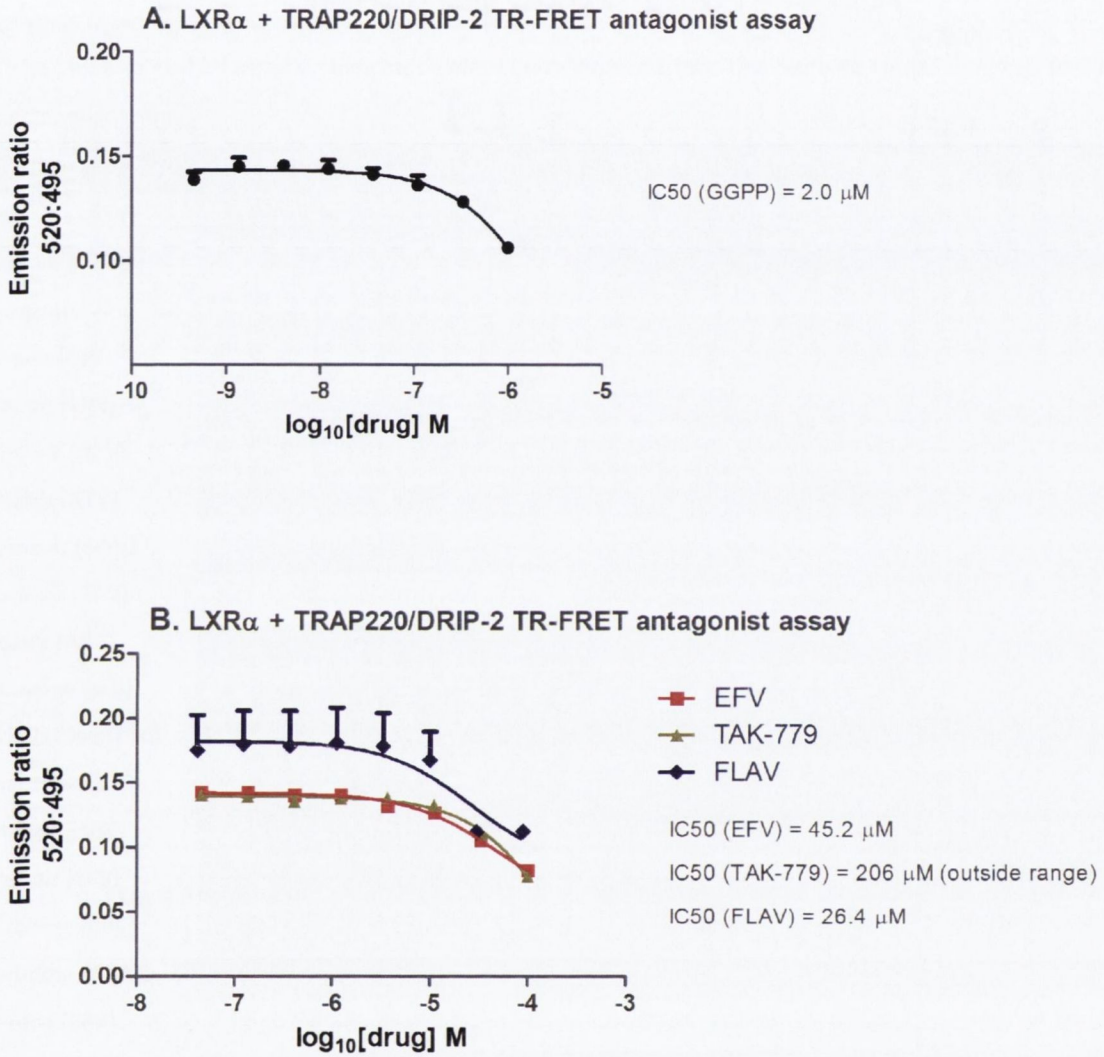


Figure 4.4 TR-FRET LXRα co-activator assays, antagonist mode in presence of 1.5μM T0901317 (LXR agonist, EC_{80}). A) Validation of TRAP220/DRIP-2 co-activator assay (antagonist mode) using geranylgeranyl pyrophosphate (GGPP), known LXR antagonist. B) Antagonistic effects by efavirenz (EFV), TAK-779 and flavopiridol (FLAV) on LXRα TRAP220/DRIP-2 recruitment. Two independent experiments were performed with triplicate wells (n = 6). TR-FRET emission ratio measured after 2h incubation at room temperature in the dark. Results are presented as means ± S.E.M. IC_{50} values were determined using a sigmoidal dose-response equation in GraphPad Prism version 5.

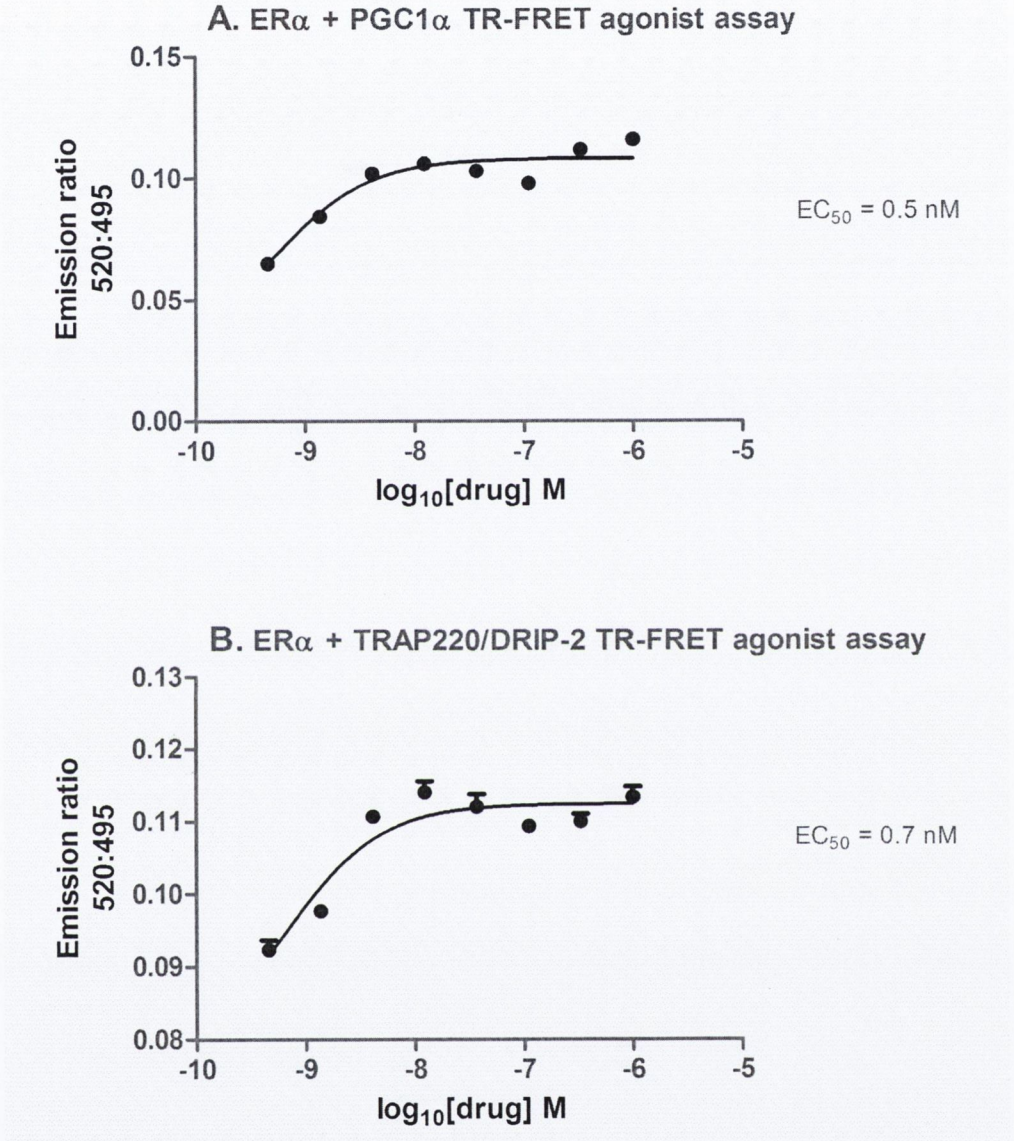


Figure 4.5 TR-FRET ERα co-activator assays. A) Validation of PGC1α co-activator assay using 17β-estradiol (E2, a known ER agonist). B) Validation of TRAP220/DRIP-2 co-activator assay using 17β-estradiol (E2). Experiments were performed with triplicate wells (n = 3). TR-FRET emission ratio measured after 2h incubation at room temperature in the dark. Results are presented as means ± S.E.M. EC_{50} values were determined using a sigmoidal dose-response equation in GraphPad Prism version 5.

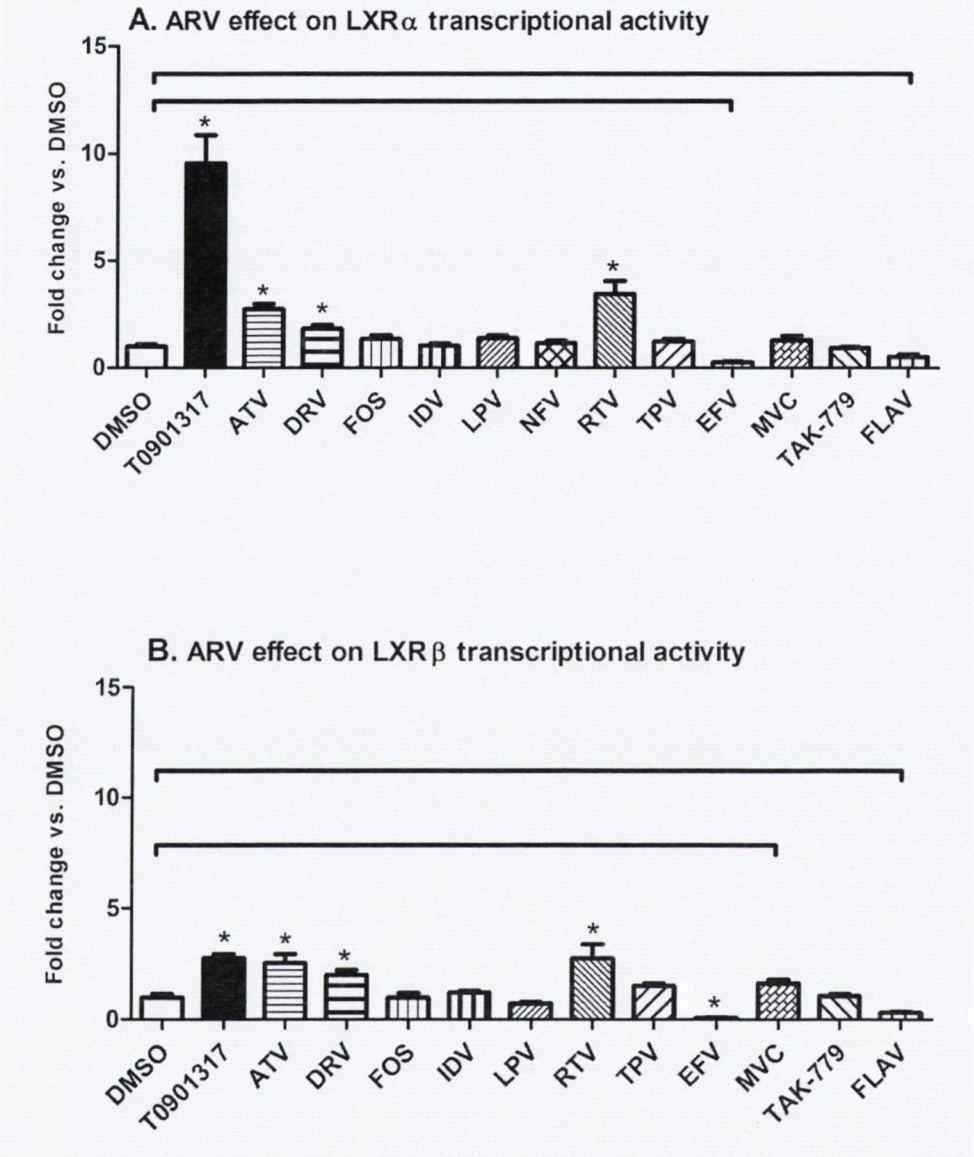


Figure 4.6 Effect of ARVs on LXR transcriptional activity as measured by Dual-Luciferase Reporter Assays: A) LXRα, B) LXRβ. HepG2 cells were transfected with nuclear receptor expression plasmid and the corresponding responsive element-luciferase construct. Five independent experiments were performed in duplicates for each treatment. All ARV drugs were used at 10μM except nelfinavir (NFV, 1μM) and flavopiridol (FLAV, 100nM), due to cytotoxicity. LXR positive control T0901317 (10μM) and a vehicle control (0.1% DMSO) were included. *P < 0.05 by one-way ANOVA analysis, bars denote significant difference compared to DMSO as analysed by Mann-Whitney t-tests. (DMSO = dimethylsulphoxide, T0901317 = LXR positive control, ATV = atazanavir, DRV = darunavir, FOS = fosamprenavir, IDV = indinavir, LPV = lopinavir, NFV = nelfinavir, RTV = ritonavir, TPV = tipranavir, EFV = efavirenz, MVC = maraviroc, FLAV = flavopiridol).

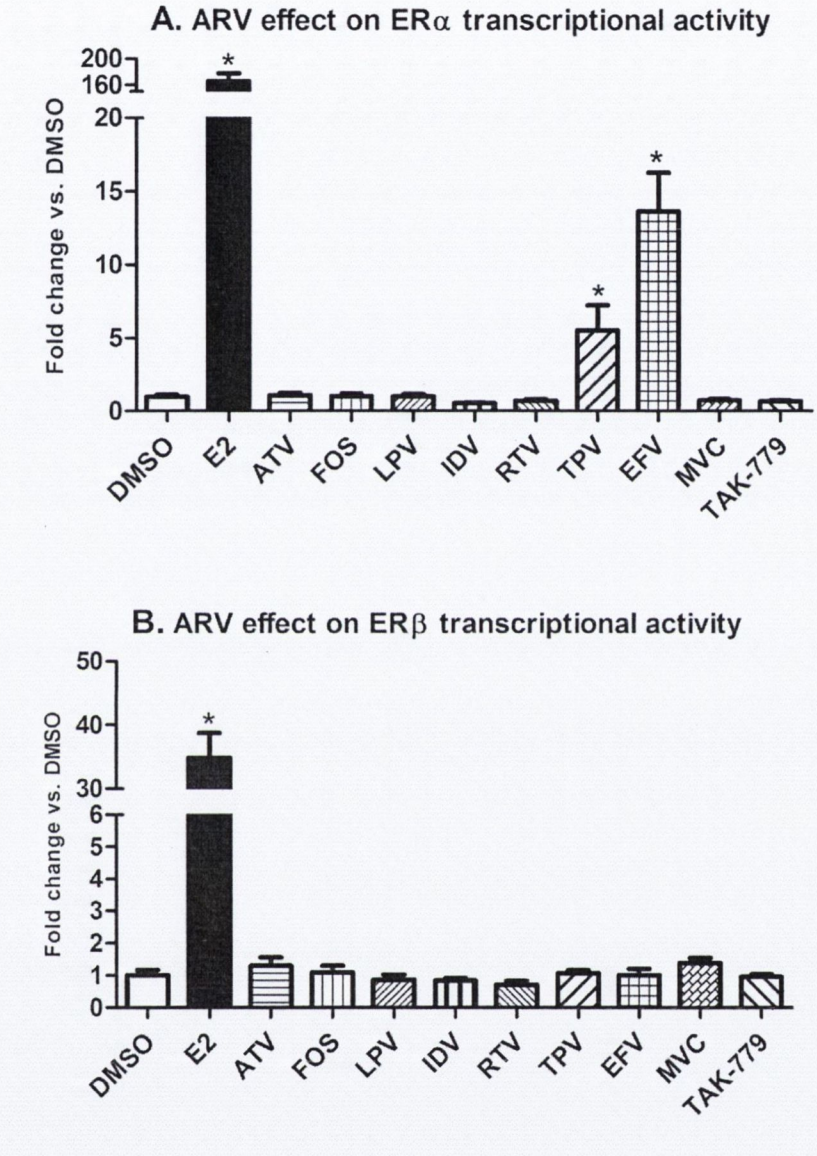


Figure 4.7 Effect of ARVs on ER transcriptional activity as measured by Dual-Luciferase Reporter Assays: A) ERα, B) ERβ. HepG2 cells were transfected with nuclear receptor expression plasmid and the corresponding responsive element-luciferase construct. Five independent experiments were performed in duplicates for each treatment. All ARV drugs were used at 10μM. ER positive control E2 (17β-estradiol, 100nM) and a vehicle control (0.1% DMSO) were included. *P < 0.05 by one-way ANOVA analysis. (DMSO = dimethylsulphoxide, E2 = 17β-estradiol (ER positive control), ATV = atazanavir, FOS = fosamprenavir, LPV = lopinavir, IDV = indinavir, RTV = ritonavir, TPV = tipranavir, EFV = efavirenz, MVC = maraviroc).

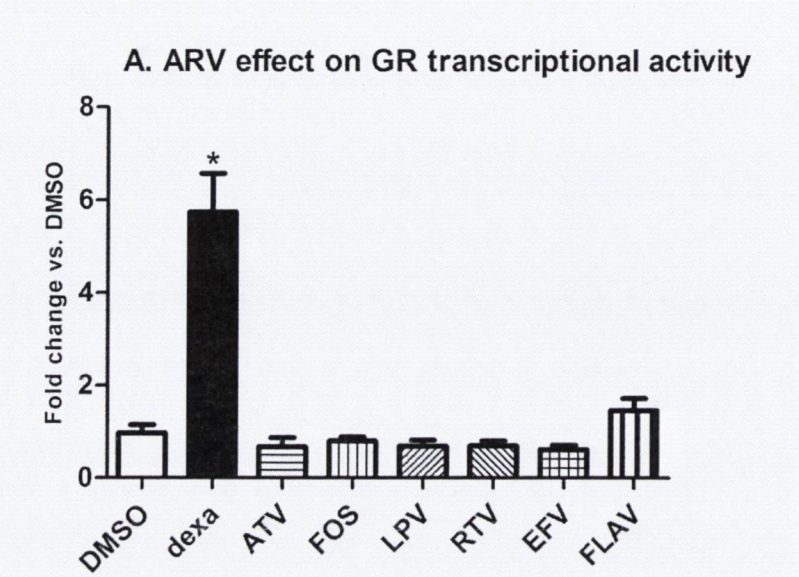


Figure 4.8 Effect of ARVs on GR transcriptional activity as measured by Dual-Luciferase Reporter Assays. HepG2 cells were transfected with nuclear receptor expression plasmid and the corresponding responsive element-luciferase construct. Five independent experiments were performed in duplicates for each treatment. All ARV drugs were used at 10μM except flavopiridol (FLAV, 100nM). GR positive control dexamethasone (1μM) and a vehicle control (0.1% DMSO) were included. *P < 0.05 by one-way ANOVA analysis. (DMSO = dimethylsulphoxide, dexa = dexamethasone (GR positive control), ATV = atazanavir, FOS = fosamprenavir, LPV = lopinavir, RTV = ritonavir, EFV = efavirenz, FLAV = flavopiridol).

4.5 Discussion

In this study, we have combined structure-based *in silico* analysis with cell-free and cell-based *in vitro* validation, to assess nuclear receptor activation by ARV compounds. Based on LBD docking and structural analysis, darunavir and tipranavir were the only PIs predicted as nuclear receptor ligands for LXRα and LXRβ. In addition, maraviroc, flavopiridol, efavirenz and TAK-779 were deemed as possible LXRα and LXRβ ligands. In the ERα and ERβ modelling studies only efavirenz emerged as a potential ligand, while both efavirenz and flavopiridol were considered as good candidates in assessment of the GR-LBD. In TR-FRET LXRα co-activator assays, maraviroc, darunavir and tipranavir were confirmed as agonists. Efavirenz, TAK-779 and flavopiridol had antagonistic effects and attenuated co-activator recruitment by LXR agonist T0901317. No evidence of direct LBD-binding and co-activator recruitment was observed in TR-FRET ERα co-activator assays with any of the compounds tested (except for positive control 17β-estradiol).

PIs atazanavir, darunavir and ritonavir significantly increased both LXRα and LXRβ transcriptional activity in luciferase reporter assays. Efavirenz and flavopiridol attenuated basal LXR activity, consistent with the antagonistic effect observed in TR-FRET assays. The CCR5 antagonist maraviroc increased LXRβ activity. In ER-transfected cells, tipranavir and efavirenz increased ERα transcriptional activity, while none of the ARVs tested had an impact on ERβ activity.

Most PIs are structurally large moieties that possess hydrophilic features such as hydroxyl groups, amines, trifluorides and phosphate groups; as such they are unlikely ligands of nuclear receptors whose ligand-binding pockets are restricted in size and in general are mostly hydrophobic. Notably, the LXR LBD is larger and more flexible than those of ER and GR (see Figure 4.1), therefore may accommodate more bulky structures and also demonstrates a greater number of predicted ligands for LXRα and LXRβ in our study. Several ARV compounds from the NRTI and NNRTI drug classes (in general smaller molecules than PIs) scored well in the docking studies. However, with the exception of efavirenz they were excluded as ligand candidates due to low hydrophobicity and for ERs and GR they also exceeded the limit of nitrogen atoms. It is possible that compounds which were outside the range of only one molecular descriptor could still bind to the nuclear receptor LBD, however for the purpose of this study these compounds were strictly filtered from the list of potential ligands.

Although the *in silico* predictions and *in vitro* results corresponded well in general, there were some discrepancies. Atazanavir and ritonavir activated LXR in the reporter assay despite very poor results in the docking study and structural analysis. In TR-FRET assays, an initial assessment of PGC1α co-activator recruitment resulted in a 24% increase by atazanavir and a 19% increase by ritonavir at 100μM which were significant (data not shown). However, subsequent dose-response experiments failed to yield converging curves and confirm these findings. This could indicate involvement of other co-factors present in the cell-based reporter assay but not in the cell-free TR-FRET experiments. For example, steroid receptor co-activator 1 (SRC-1) has been linked to stimulation of LXR transactivity[457, 458]; the recruitment of this co-activator was not investigated in this study. Furthermore, increased nuclear receptor transcriptional activity could also be due to effects on co-repressor dissociation as opposed to co-activator recruitment[459] – in fact partial agonists can induce a state where interaction with both co-activators and co-repressors are favoured to the unliganded state[460]. A strong interaction between LXRα and nuclear receptor co-repressor (N-CoR) in particular has been demonstrated[461]. Alternatively, the effects on LXRs by atazanavir and ritonavir seen in the reporter assays may be due to allosteric effects. Examples of allosteric modulators of nuclear receptor activity have been previously described: in experiments studying progesterone receptor (PR), non-dose-dependent attenuation of progesterone induction and incomplete displacement of progesterone by antagonists indicate allosteric binding sites[462]. This is a more plausible explanation as atazanavir and ritonavir are unlikely to fit in the LXR ligand-binding pocket due to their size and hydrophilicity; poor results in the *in silico* docking assay also support this hypothesis. An allosteric mechanism may also be the explanation for the ERα-inductive effect by tipranavir in reporter assays, as interaction with the ERα-LBD was neither predicted *in silico* nor observed in TR-FRET assays. Although efavirenz was regarded as a suitable ERα ligand based on docking score and drug structure, and increased ERα activity more than 10-fold in reporter assays, direct binding was not supported by TR-FRET results. It is possible that the effect seen in transfected HepG2 cells is specific to other co-activators than those present in these TR-FRET experiments (PGC1α or TRAP220/DRIP-2). Despite being a predicted ligand of ERβ and GR, efavirenz had no effect on these in reporter assays. Moreover, although an antagonistic effect by TAK-779 on the isolated LBD was observed in TR-FRET assays, no significant effect was detected in the cell model, potentially due to low potency. Another predicted LXR ligand, tipranavir, had agonistic effects in LXRα TR-FRET experiments however the effect of tipranavir did not reach statistical significance in reporter assays (LXRα: 1.2-fold increase, LXRβ: 1.5-fold increase). This highlights the fact that docking and structural analysis by molecular descriptors are only theoretical

approaches to estimate the affinity of a compound to the nuclear receptor LBD, and should be validated in a biological system. In our study, the poor validation results of the GR model will also have to be taken into account. Nevertheless, one of the two predicted GR ligands; flavopiridol, increased GR activity slightly (1.5-fold, albeit not significantly) in reporter assays.

The LBDs of LXRα and LXRβ are almost completely conserved and consequently most LXR agonists identified to date activate both subtypes[253]. However, a few exceptions have been identified[457, 463]. Although LXRα and LXRβ appear to share target genes, selective agonists could elicit differential responses due to differences in tissue distribution. In contrast, amino acid sequence identity between ERα and ERβ LBDs is lower (56%), thus many natural and pharmacological ligands exhibit isoform selectivity[308]. This is consistent with our findings; ARV drug effects on LXRα and LXRβ activity in reporter assay experiments were highly correlated whereas important differences were noted between ERα and ERβ activation.

Due to the role of nuclear receptors in a wide range of physiological responses, identifying therapeutic agents and other chemical entities that modulate their activity is important because of the potential implications of disturbed homeostasis. It may be possible to link adverse drug effects of HAART such as metabolic abnormalities to the activation of nuclear receptors.

In silico computational methods are often used in drug discovery as a means of screening large libraries of compounds and identifying possible receptor ligands which would not be possible or cost-effective by *in vitro* or biochemical assays. Cell-based transactivation assays have been used by pharmaceutical companies to evaluate PXR activation and CYP3A4 induction potential for many years[464]. However, critics may call attention to differences in gene expression profiles between the immortalised cell lines commonly used in this type of assay and "healthy" cells *in vivo*[465]. It has however been demonstrated that HepG2 cells express both DRIP-2[466] and PGC1α[467]. The output from reporter gene assays using full-length receptor expression plasmids does not however give information about the nature of the interaction, i.e. whether it is due to direct binding to the ligand-binding pocket or indirect effects. Cell-free TR-FRET co-activator assays offer highly sensitive and robust assessment of direct interactions with LBDs and identify necessary components of transcriptional activation. It also allows differentiation between agonists and antagonists. A notable drawback is the limitation to specific co-activators.

The LXR activation by atazanavir, darunavir and ritonavir observed in reporter assays is interesting. Published *in vitro* experiments exposing mouse skeletal muscle cells to atazanavir, darunavir, and lopinavir in combination with ritonavir resulted in increased expression of LXR target gene *SREBP-1*[468], a transcription factor involved in the regulation of lipid homeostasis[469]. Furthermore, treatment with ritonavir has a recognised association with hypertriglyceridemia[470, 471]; a typical effect of LXR inducing compounds[440]. From a clinical point of view, the finding that atazanavir and darunavir act as LXR agonists is more surprising as these more recently developed PIs are generally associated with more favourable lipid profiles[472, 473]. However, a recent study reported increased triglyceride levels in healthy volunteers treated with ritonavir-boosted atazanavir or darunavir[474]. Similarly, lipohypertrophy of the dorso-cervical region of the neck has been reported in a female HIV-patient treated with unboosted atazanavir plus raltegravir[475]. Moreover, hypertriglyceridemia can also be generated through a PXR-mediated and SREBP-independent pathway, as demonstrated by experiments with rifampicin-treated humanised mice[281]. This effect may be more dominant than LXR activation, and indeed ritonavir appears to be a more potent inducer of PXR than atazanavir[146] (although neither of these PIs significantly induced PXR in our experiments in chapter 3, ritonavir was only used at 1μM and unpublished results showed significant PXR activation at 10μM).

Efavirenz had multiple effects: it reduced LXRα and LXRβ basal activation and additionally increased ERα activation. The suppression of LXR activity is supported by a report of reduced expression of LXR target gene *SREBP-1c* after efavirenz exposure[476]. The LXR antagonistic and ERα agonistic effects by efavirenz are of interest given that efavirenz treatment is associated with depression, anxiety and impaired neurocognition[320, 477, 478]. Activation of LXR has neuroprotective effects[309], while researchers have demonstrated altered monoamine levels in female rat brains after ER agonist exposure[479]. Moreover, ERα-selective activation results in anxiogenic responses in female rats in contrast to ERβ-selective activation which is anxiolytic[480].

Tipranavir was also identified as an ERα agonist in this study. Multiple cases of intracranial haemorrhage have been reported in patients treated with this PI, causing the FDA to issue a warning in 2006. *In vivo* and *in vitro* investigations into this matter revealed decreased platelet aggregation as well as thromboxane B2 formation following tipranavir treatment[481]. Coincidently, estradiol (an ER agonist) also reduces production of thromboxane B2[482] and inhibits platelet aggregation[483].

ERα, but not ERβ, also has an important role in maintaining bone homeostasis[484]: a suggested mechanism is by estrogen-mediated down-regulation of the osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) ratio[485], a cytokine system that is essential for osteoclast biology. An investigation into the effects of PIs on an osteoblast-like cell line revealed reduced OPG/RANKL by tipranavir[486]. Interestingly, while a common PI-associated adverse effect is osteopenia, there appears to be no published reports of reduced bone mineral density (BMD) with tipranavir. In patients switching from a PI-based to an efavirenz-based regimen, a reduction in OPG and RANKL was seen by Mora *et al*[487].

In addition, the activation of ERα by efavirenz could potentially provide a contributing mechanism for development of gynecomastia which is strongly associated with the use of this NNRTI[488]. In a case study from 2002[489], a patient with gynecomastia was successfully treated with ER-antagonist tamoxifen. A recent publication[490] was able to show direct binding of efavirenz to ERα by competitive binding FRET and induced proliferation of breast cancer cell line MCF-7, further supporting our findings.

Flavopiridol, a cyclin-dependent kinase (Cdk) inhibitor in clinical trials as a potential anti-cancer drug due to antiproliferative effects, has also been reported to inhibit HIV-1 replication *in vitro*[491]. The suggested mechanism is through inhibition of a Cdk which in complex with viral transactivator Tat is necessary for RNA transcription. A high potency of this compound (IC_{50} = 8nM) could potentially overcome issues of cytotoxicity. Flavopiridol scored high in LXR and GR docking assays, and as it also fits the range of molecular descriptors for ligands of these receptors it was included in the *in vitro* assessment. TR-FRET co-activator assays showed antagonistic effects of flavopiridol, which were subsequently confirmed in LXR reporter assays. In GR reporter assays a small (1.5-fold) but non-significant increase in transcriptional activity was noted.

There was no significant effect on GR transcriptional activity by any of the ARV drugs tested in this study. However, pseudo-Cushing's syndrome seen in patients on HAART could also be caused by activation of PXR; cases of misdiagnosis have been described in patients receiving rifampicin-treatment for tuberculosis[492] and a study using transgenic mice confirms the ability of PXR agonists to disrupt glucocorticoid homeostasis and bring about adenocorticotropic hormone (ACTH)-independent hypercortisolism[287]. Indeed, many ARV drugs are inducers of PXR (see chapter 3).

Furthermore, as HIV disease and its treatment is highly complex, adverse effects of HAART are likely to be multifactorial and it may not be possible to ascribe effects to the isolated activation of one nuclear receptor. In addition, there is a great degree of cross-talk between nuclear receptors and the activation of one may often have an indirect impact on others. However, identifying direct effects of single ARV drugs on individual nuclear receptors can help explain at least in part the underlying mechanisms of HAART-associated adverse events. It would be of interest to extend this investigation to include other nuclear receptors: PPARs (in particular PPARγ) are major players in adipocyte[493] and osteoblast[494] differentiation, insulin resistance and inflammation[495]. Although Lenhard *et al*[419] examined competitive binding of PPARγ and RXRα by a number of PIs and found only significant binding to the former by saquinavir, many new drugs are now on the market which were not included in this study. In fact, another group reported overactivation of the renin-angiotensin system by lopinavir and atazanavir, in part through a PPARγ-dependent signalling pathway[496]. Vitamin D receptor (VDR) also plays an important role in insulin secretion, lipid metabolism, autoimmune disorders, cell proliferation, and cardiovascular diseases[497, 498]. Efavirenz, which in our studies has been proven to interact with several nuclear receptors, has also been associated with severe vitamin D deficiency[499]. Similar to LXR, farnesoid X receptor (FXR) is involved in the regulation of CYP7A, the rate-limiting step of cholesterol breakdown[500], and activation of FXR has anti-atherosclerotic effects[501]. These are some examples of other nuclear receptors involved in biological processes related to well-documented adverse effects of HAART. Assessment of the ability of new ARV therapeutics under development to interact with a panel of nuclear receptors may aid in predicting off-target interactions and reducing the risk of metabolic abnormalities.

In summary, we have utilised a combined approach incorporating *in silico* analysis, cell-free LBD-binding experiments and cell-based reporter assays to identify ligands of LXRα, LXRβ, ERα, ERβ and GR from a library of ARV drugs. We have demonstrated that several ARV drugs have the ability to act as ligands of LXRα, LXRβ and/or ERα. These results may provide additional information regarding potential contributing mechanisms for some of the overlapping adverse effects experienced by patients on various HAART regimens. Further investigations to elucidate the downstream effects and clinical relevance of LXRα/β and ERα activation by ARVs, as well as assessment of ARV binding to other nuclear receptors not investigated in this study, are warranted.

5 Effect of efavirenz on *ABCA1* and *ApoE* expression in human neuroblastoma cells and rat primary cortical cultures

5.1 Introduction

Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) widely used as first-line antiretroviral (ARV) therapy in the treatment of HIV disease in Europe and USA[30, 45, 502]. Efavirenz has been associated with central nervous system (CNS) side effects in more than 50% of patients following initiation of therapy[86, 319]. These side effects include: sleep disorders and dizziness, that occur predominantly in the first few weeks, and anxiety, depression and cognitive disturbances such as memory disorders and impaired concentration that may persist beyond three months[320] and occasionally in long-term treatment (>1 year)[319]. In many cases these adverse events result in discontinuation or switch of therapy (25.1% of patients in the TRT-5 Group[88]and 16.4% of patients in the EuroSIDA Study[89]). The underlying mechanisms of the CNS effects of efavirenz, which has intermediate (grade 3) CNS penetration in a revised effectiveness scale[84] and is thus effective at reducing viral loads in cerebrospinal fluid[503], are unclear. However, a significant correlation has been found between risk of toxicity and plasma drug levels: in a study by Gutiérrez *et al*[319] patients with efavirenz concentrations above 2.74μg/ml were 5.68 times more likely to experience neuropsychiatric adverse events.

5.1.1 Liver X receptors and cholesterol homeostasis in the brain

In chapter 4, we described an inhibitory effect by efavirenz on liver X receptors (LXRs) α and β. These nuclear receptors (in particular LXRβ[504]) are expressed in the brain, and similar to their role in the liver have important functions as regulators of cholesterol homeostasis[505]. Genes under transcriptional control of LXRs include sterol regulatory element binding proteins (SREBPs, transcription factors which stimulate expression of genes involved in synthesis and uptake of fatty acids and cholesterol[254]), ATP binding cassette transporters A1 and G2 (ABCA1 and ABCG2, mediators of cholesterol efflux[506]) and apolipoprotein E (ApoE, a facilitator of ABC-transporter-mediated cholesterol efflux[312]). Cholesterol is concentrated in the brain, more than in other body tissues[309], and as a major structural component of cell membranes it is essential for CNS function. A high cholesterol turnover is necessary for neuron repair and remodelling[507]. Human LXRα and LXRβ display 77%

sequence identity in both DNA-binding domains (DBDs) and ligand-binding domains (LBDs). In addition, they are highly conserved between humans and rodents[508].

5.1.2 LXR and neurodegeneration

The importance of LXR for CNS function is illustrated by LXR double knockout ($LXR\alpha^{-/-}\beta^{-/-}$) mice which exhibit neurodegeneration and dysmyelination[311]. Conversely, activation of LXRs invokes neuroprotective mechanisms: in experiments using wildtype mice[316], transgenic mice[313, 314], CHO-APP cells and primary human neurons[312], LXR agonists (T0901317, GW3965, 27-hydroxycholesterol) have been shown to reduce production of soluble β-amyloid (Aβ), a suggested marker for neurodegeneration and Alzheimer's disease (AD) progression[315]. Aβ, which in excess leads to the formation of extracellular neuritic plaques, is characteristic of AD in combination with intracellular neurofibrillary tangles consisting of Tau protein[509]. Kim *et al*[312] also detected increased levels of amyloid precursor protein (APP) in addition to reduced levels of Aβ after exposing CHO-APP cells to 27-hydroxycholesterol, an endogenous LXR ligand, supporting the hypothesis of LXR-mediated inhibition of Aβ processing. Aβ is derived from sequential proteolytic cleavage of APP[510], a transmembrane protein whose function is not yet fully understood. Cleavage of APP can be achieved through two different pathways; α-secretase and β-secretase, which have distinct cleavage sites, producing large soluble ectodomains of APP (sAPPα or sAPPβ). Consecutive cleavage by γ-secretase within the transmembrane domain yields either a rapidly degraded P83 fragment (following α-secretase cleavage) or Aβ (following β-secretase cleavage) (Figure 5.1). The exact site of intramembrane γ-secretase cleavage can vary, resulting in Aβ fragments of different length: Aβ40 is the most common species followed by Aβ42[510].

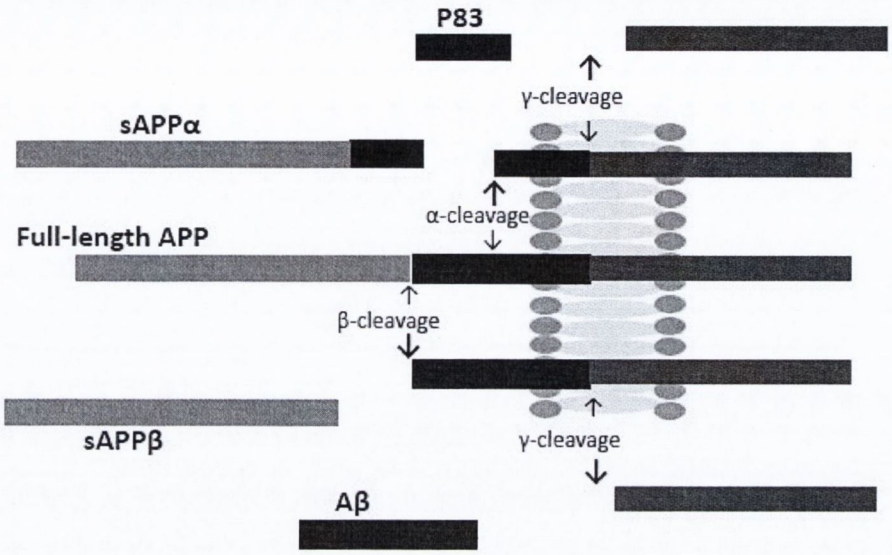


Figure 5.1 Schematic diagram of APP processing (simplified from Zhang *et al* 2011[510], not drawn in proportion). APP = β-amyloid precursor protein, sAPPα = soluble APPα, sAPPβ = soluble APPβ, Aβ = β-amyloid.

Some controversy remains with respect to the Aβ-lowering effect of LXR agonists, as one research group reported increased Aβ42 by T0901317[511]. However, this could be attributed to the finding that T0901317 modulates the site of APP cleavage by γ-secretase *in vitro*, selectively raising Aβ42 levels through this cholesterol-independent pathway[512].

5.1.3 The role of ABCA1 in neuroprotective effects exerted by LXR

Further evidence of LXR neuroprotective effects comes from rodent experiments: LXR agonist GW3965 improves object recognition memory in mice[513] and T0901317 promotes recovery after brain injury by reducing β-amyloid levels[514]. The researchers attributed these effects to increased expression of ABCA1, an LXR target gene which has an important role in cholesterol efflux[506]. Since APP, β-secretase and Aβ are all present in cholesterol-rich lipid rafts in cell membranes[515, 516] where also γ-secretase activity has been confirmed[517], this constitutes a probable site for APP β-cleavage[518]. LXR-mediated depletion of cholesterol affecting association of APP with lipid rafts may represent a link between increased ABCA1 efflux activity and reduction in Aβ production[519]. In contrast, the non-amyloidogenic α-secretase cleavage pathway appears to take place outside lipid rafts[520].

Interestingly, challenging human neuroblastoma cells with Aβ peptide results in elevated levels of *ABCA1* gene expression and the same has also been observed in hippocampal neurons of AD cases

compared to controls[521]. This could indicate a protective mechanism in response to the challenge. Moreover, retrospective epidemiological studies indicate a decreased risk of developing AD among individuals treated with lipid-lowering agents (statins)[522-524]. Confirmation that this is cholesterol-dependent and not related to the inhibition of mevalonate production induced by statins comes from a study using a specific inhibitor of the final step of the cholesterol biosynthetic pathway, where reduction of Aβ was achieved in a mouse model of AD[525].

5.1.4 The role of ApoE in neurodegeneration

Apolipoprotein E (ApoE) is one of the main cholesterol carriers in the CNS[526] and another target gene of LXR[312]. It has been linked in numerous studies with AD: the ε4 allele increases the risk of cognitive decline and development of dementia[527] and is the only confirmed genetic factor associated with predisposition to late onset AD[317, 318]. Several underlying mechanisms have been proposed, for example impaired cholesterol transport from astrocytes to neurons[528] as well as effects on synaptic plasticity[529] and neuronal survival[530]. ApoE also influences APP trafficking[531] and promotes proteolytic degradation of Aβ, hence facilitating its clearance and preventing the deposition of plaques[532]. Interestingly, a Chinese study demonstrated 3-fold increased odds of developing neurocognitive impairment among HIV-infected individuals with at least one *APOE* ε4 allele, however when comparing HAART-treated patients only the difference was no longer significant (P = 0.053)[533].
The LXR-ABCA1-ApoE regulatory axis is regarded as a promising new target for AD therapeutics[534], although development of brain-specific LXR agonists are required as systemic effects include hypertriglyceridemia due to induction of hepatic lipogenesis[535].

5.1.5 β-amyloid and HAART

Interestingly, Green *et al*[536] described autopsy findings of increased depositions of Aβ in the brain of HAART-treated compared to HAART-naïve AIDS cases, however the publication offers no information regarding details of the drug regimens. A similar study by Anthony *et al*[537] found no evidence of increased premature Aβ-depositions in HAART patients compared to non-HIV-infected age-matched controls, although the number of HIV patients on treatment in this study was relatively small (n = 9). The researchers did however observe elevated levels of hyperphosphorylated Tau in the hippocampus of ARV-treated subjects. No published studies to date have investigated the effect of efavirenz in particular on the Aβ processing pathway.

5.2 Aims

The inhibitory effect of efavirenz on LXR transcriptional activity was demonstrated in chapter 4 by luciferase reporter assays and co-activator assays confirmed direct antagonism of T0901317 recruitment of the TRAP220/DRIP-2 complex to LXRα-LBD. We hypothesise that the CNS effects of efavirenz, manifesting in patients as cognitive disorders and impaired memory, could be partly attributed to suppression of LXR activity and hence decreased expression of LXR target genes which have been associated with Aβ levels. Therefore the aims of this study were to 1) investigate the effects of efavirenz on genes encoding ABCA1 and ApoE, and 2) quantify APP and Aβ40 protein levels following exposure to efavirenz, in human neuroblastoma cells and primary rat cortical cultures.

5.3 Materials and methods

5.3.1 Cell culture of SH-SY5Y human neuroblastoma cells, cytotoxicity assays and drug treatments

The human neuroblastoma cell line SH-SY5Y was obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham with 2mM L-glutamine, 100units + 0.1mg/ml penicillin-streptomycin, 1% non-essential amino acids and 10% foetal bovine serum (FBS, all from Sigma-Aldrich, Wicklow, Ireland). For MTT cytotoxicity experiments 15,000 cells per well were seeded into 96-well plates the day before exposure to a dilution series (0.1μM, 1μM, 10μM, 25μM and 50μM) of efavirenz (LGM Pharmaceuticals, Boca Raton, FL, USA), T0901317 (synthetic LXR agonist, Sigma-Aldrich) or vehicle dimethylsulphoxide (DMSO, Sigma-Aldrich) in complete medium with 5% FBS. 2h prior to the end of the experiments, 10μl MTT (Sigma-Aldrich, 3mg/ml in phosphate buffered saline, PBS) was added to each well. The culture medium was removed after 24h drug exposure and the purple formazan complexes produced by mitochondrial reductase were dissolved in DMSO. Absorbance was measured at 540 nm in a Bio-Tek ELx808 Absorbance Microplate Reader (Vermont, US) and values from drug-treated wells normalised to vehicle controls. Experiments were repeated four times, in duplicates. For quantitative real time PCR experiments and protein detection 300,000 cells per well were seeded into 12-well plates. The following day cells were washed in PBS (Sigma-Aldrich) and 24h drug exposure initiated in complete phenol red-free DMEM/F-12 Ham with 5% foetal bovine lipoprotein-deficient serum (FB-LPDS, Intracel, Frederick, MD, USA) to reduce background activation of LXR. At least four replicates were collected per data point.

5.3.2 Isolation of primary rat cortical cells, cytotoxicity assays and drug treatments

Primary cortical cells were isolated from neonate 1-day old Wistar rats. Dissected cortices were dissociated with 0.3% trypsin (Sigma-Aldrich, in PBS) for 25 min at 37°C, after which the trypsin was inactivated with a PBS solution containing soy bean trypsin inhibitor, $MgSO_4$ and DNase (Sigma-Aldrich) and cells passed through a cell strainer. After centrifugation (2000 x g, 3 min, 20°C) the pellet was resuspended in neurobasal medium (NBM) with 2mM Glutamax, 100units + 0.1mg/ml penicillin-streptomycin, 10% heat-inactivated horse serum and 1% B-27 (penicillin-streptomycin from Sigma-Aldrich, all other media components from Gibco/Invitrogen, Paisley, UK). 125,000 cells per well were plated onto poly-L-lysine-coated coverslips in 24-well plates and incubated for 3-4 days. The cortical cultures were then pre-treated with phenol red- and serum-free NBM (to reduce background activation of LXR) with 2mM Glutamax, 100units + 0.1mg/ml penicillin-streptomycin and 1% B-27 for 24h, prior to exposure to efavirenz, T0901317 and vehicle control (DMSO) for 24h in the same phenol red- and serum-free medium.

To assess cytotoxicity of efavirenz in the primary rat cortical cultures, an MTT assay was performed: 2h prior to the end of the 24h exposure to varying concentrations of efavirenz (0.1μM, 1μM, 10μM and 20μM) as well as vehicle control (0.1% DMSO) or a cytotoxic control (10μM doxorubicin), 25μl MTT (Sigma-Aldrich, 5mg/ml in PBS) was added to each well. The MTT cytotoxicity assays were henceforth carried out as described above (n = 4).

5.3.3 Real time PCR: SH-SY5Y and primary rat cortical cultures

After 24h exposure the medium was removed and cells were washed once with PBS. RNA isolation was achieved using TRIsure (Bioline, London, UK) according to the manufacturer's instructions. RNA samples (800ng for SH-SY5Y and 400ng for rat primary cultures) were treated with DNaseI (Sigma-Aldrich) to remove genomic DNA and cDNA synthesis performed using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT, Sigma-Aldrich) with random hexamers (Bioline) according to the manufacturer's protocols. Real-time PCR was carried out in an Applied Biosystems 7900HT instrument using QuantiTect SYBR Green Mastermix and Primer Assays (Qiagen, West Sussex, UK): human *ABCA1*, *ApoE* and *ACTB* (β-actin, housekeeping gene) for SH-SY5Y and rat *Abca1*, *Apoe* and *Actb* for rat primary cortical cultures. β-actin is commonly employed as an internal standard and has been classified as one of the most stable housekeeping genes for rat cortex[538]. To assess amplification efficiency, standard curves were constructed for each primer set prior to analysis of experiments.

5.3.4 APP and Aβ-40 quantification by Western blots

Following drug exposure, SH-SY5Y cells and primary rat cortical cultures were lysed in modified RIPA buffer (50mM Trizma base, 150mM NaCl, 2mM EDTA, 0.5% NP40) with protease inhibitor cocktail (Sigma-Aldrich) and frozen at -70°C until further analysis of intracellular protein. Medium was collected, centrifuged at 1,500 x *g* for 5 min at 4°C to remove cell debris and protease inhibitor cocktail added to the supernatant which was then frozen at -70°C until further analysis. A trichloroacetic acid (TCA)/deoxycholate (DOC) method was used for protein precipitation from the culture medium: samples were mixed with 1/100 of its volume of 2% DOC (in H_2O) and incubated on ice for 30 min. TCA was added to give a final concentration of 15% followed by vortexing to prevent large conglomerates from forming, then samples were left at 4°C overnight. The following day, the precipitates were centrifuged at 15,000 x *g* for 10 min whereby TCA and contaminants were aspirated. Pellets were washed with ice cold ethanol, vortexed and left at room temperature for 5 min. Pellets were once again centrifuged at 15,000 x *g* for 10 min and the ethanol removed. This wash step was repeated once, followed by drying of the pellets under a slow stream of nitrogen.

Prior to loading onto 10% SDS polyacrylamide gels, 5x sample buffer (12% SDS, 50% glycerol, 250mM pH 6.8 Tris-HCl, 0.5% bromophenol blue) was added to RIPA and culture medium samples, followed by boiling for 5 min. A molecular weight marker (Chemiblot, Millipore, Cork, Ireland) was also loaded onto the gel. Gels were run at 100V and proteins subsequently transferred onto Hybond-P PVDF membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) by semi-dry technique (100mA, 1 hour), and then blocked with 5% milk powder (Marvel, PremierFoods, Ireland) in TBST (pH 7.5 10mM Trizma base, 100mM NaCl, 1M HCl, 0.1% Tween-20) for 1 hour at room temperature or alternatively at 4°C overnight. Probing with primary antibodies rabbit polyclonal anti-APP (Millipore, MA, USA, cat no 07-667) or mouse monoclonal anti-amyloid β40 (clone G2-10 cat no MABN11, also Millipore) was performed at 4°C overnight. Following washing with TBST, probing with horseradish peroxidise (HRP)-conjugated secondary antibodies (polyclonal swine anti-rabbit-HRP P0217 for anti-APP or polyclonal goat anti-mouse-HRP P0447 for anti-amyloid β40, both from Dako Denmark A/S, Glostrup, Denmark) was performed for 1h at room temperature, followed by enhanced chemiluminescence (ECL) detection as described by Haan & Behrmann[539] using a Fuji LAS 4000 chemiluminescent imager. Stripping of the membranes was achieved with a solution of 62.5mM Tris (pH 6.8), 2% SDS and 12.5µl β-mercaptoethanol/100ml, incubated in a 50°C water bath for 45 min. The membranes were subsequently blocked once again with 5% milk powder in TBST, followed by re-probing with internal

standard β-actin (HRP-conjugated, Santa Cruz Biotechnology Inc., CA, USA) and ECL detection. Band intensities were measured by densitometry using GeneTools software (Syngene, Cambridge, UK).

5.3.5 Statistics

Absorbance values from drug-treated cells in MTT cytotoxicity assays were normalised to vehicle-treated controls and presented as percentages. Real time PCR experiments were analysed using the Pfaffl method[540] where amplification efficiency (E_p) is defined by the formula: $10^{(-1/slope)}$. Slopes were determined from standard curves by linear regression. Results from drug exposure experiments were normalised relative to vehicle controls and presented as means ± S.E.M. In single-drug experiments, comparisons were made between vehicle controls and different treatments by one-way ANOVA with Dunnett's *post-hoc* analysis. In T0901317/efavirenz combination experiments in SH-SY5Y, *ABCA1* gene expression after T0901317 exposure alone at different concentrations was compared to the same concentration in combination with 10μM efavirenz by Mann-Whitney t-tests. The same analysis was performed with T0901317/efavirenz combination experiments in rats. Protein expression measured by Western blot was normalised to β-actin, presented as means ± S.E.M and analysed by one-way ANOVA with Dunnett's *post-hoc* analysis.

Significant difference was defined as a P-value ≤ 0.05 in all experiments. All statistical analyses were performed using GraphPad Prism version 5.

5.4 Results

5.4.1 Assessing cytotoxicity of efavirenz by MTT assays

Efavirenz and T0901317 showed cytotoxic effects (defined as cell survival < 80%) in SH-SY5Y only at the highest tested concentration (50μM), while in primary rat cortical cultures no cytotoxic effects were observed by efavirenz (highest tested concentration 20μM) (Figure 5.2). In experiments for mRNA and protein quantification, concentrations of efavirenz and T0901317 did not exceed 10μM.

5.4.2 Validation of real time PCR primers

In order to assess amplification efficiency of the QuantiTect Primer Assays used, standard curves were constructed using serial dilutions of cDNA from vehicle-treated cells ($r^2 > 0.95$, Figure 5.3). Efficiency (E_p) was calculated from standard curve slopes using the formula defined by Pfaffl[540]. For the different

primer pairs, amplification efficiencies were as follows: E_p(*ABCA1*) = 2.18, E_p(*APOE*) = 1.94, E_p(*ACTB*) = 2.60, E_p(*Abca1*) = 2.10, E_p(*Apoe*) = 1.98, E_p(*Actb*) = 1.97.

5.4.3 Expression of LXR target genes following efavirenz exposure

Real-time PCR was performed to assess changes in mRNA expression of *ABCA1* and *APOE* in SH-SY5Y and *Abca1* and *Apoe* in primary rat cortical cultures after treatment with efavirenz.

In SH-SY5Y, the positive control T0901317 indicated a functional LXR transcriptional activation pathway with a 16.4-fold increase in *ABCA1* expression (Figure 5.4A). 10μM efavirenz significantly lowered *ABCA1* expression in SH-SY5Y to 30 ± 6% (mean ± S.E.M) compared to basal levels. Combination experiments with varying concentrations of T0901317 with or without 10μM efavirenz showed significant ($P < 0.05$) attenuation of the T0901317-induced *ABCA1* increase at 0.1μM T0901317 (Figure 5.4B). No change in *APOE* mRNA expression was detected after efavirenz exposure; however T0901317 did not significantly induce expression of this gene either (Figure 5.4C).

In primary rat cortical cultures, there was a trend towards decreased expression of *Abca1* and *Apoe* as a result of efavirenz treatment although these changes were not significant (Figure 5.5). *Abca1* mRNA was increased 2.5-fold in the presence of 0.1μM T0901317, however this was not significant. No change was seen in *Apoe* mRNA expression following T0901317 exposure.

5.4.4 Effect of efavirenz on β-amyloid processing

Intracellular APP expression after T0901317 or efavirenz treatment of SH-SY5Y or primary rat cortical cultures was assessed by Western blots. Neither the LXR agonist T0901317 nor the confirmed LXR antagonist efavirenz caused any change in APP expression in either of the *in vitro* models (Figure 5.6). As no Aβ40 was detected in any of the Western blots from either culture medium with or without protein precipitation or RIPA cell lysates (data not shown), and the antibody was not validated using a positive control, no conclusions could be drawn from these experiments regarding the effect of efavirenz on Aβ40 levels.

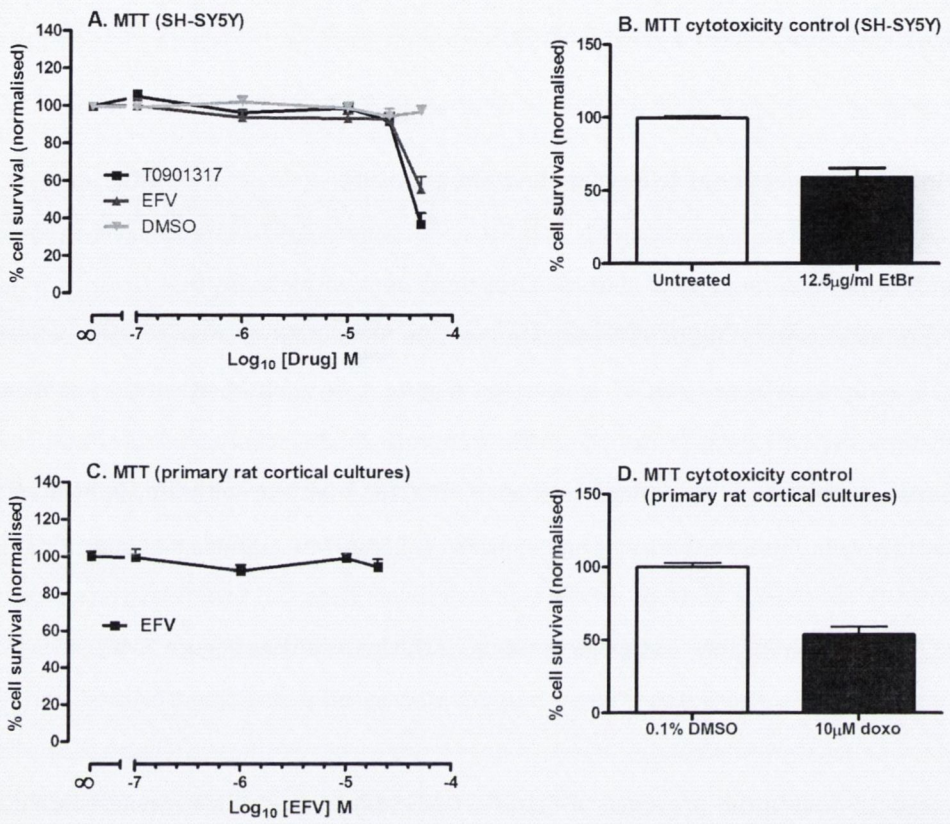


Figure 5.2 MTT cytotoxicity assays in SH-SY5Y human neuroblastoma cells and primary rat cortical cultures. Cells were treated for 24h with the indicated concentrations (n = 4). T0901317 = synthetic LXR agonist, EFV = efavirenz, DMSO = vehicle, EtBr = ethidium bromide (cytotoxic control), doxo = doxorubicin (cytotoxic control).

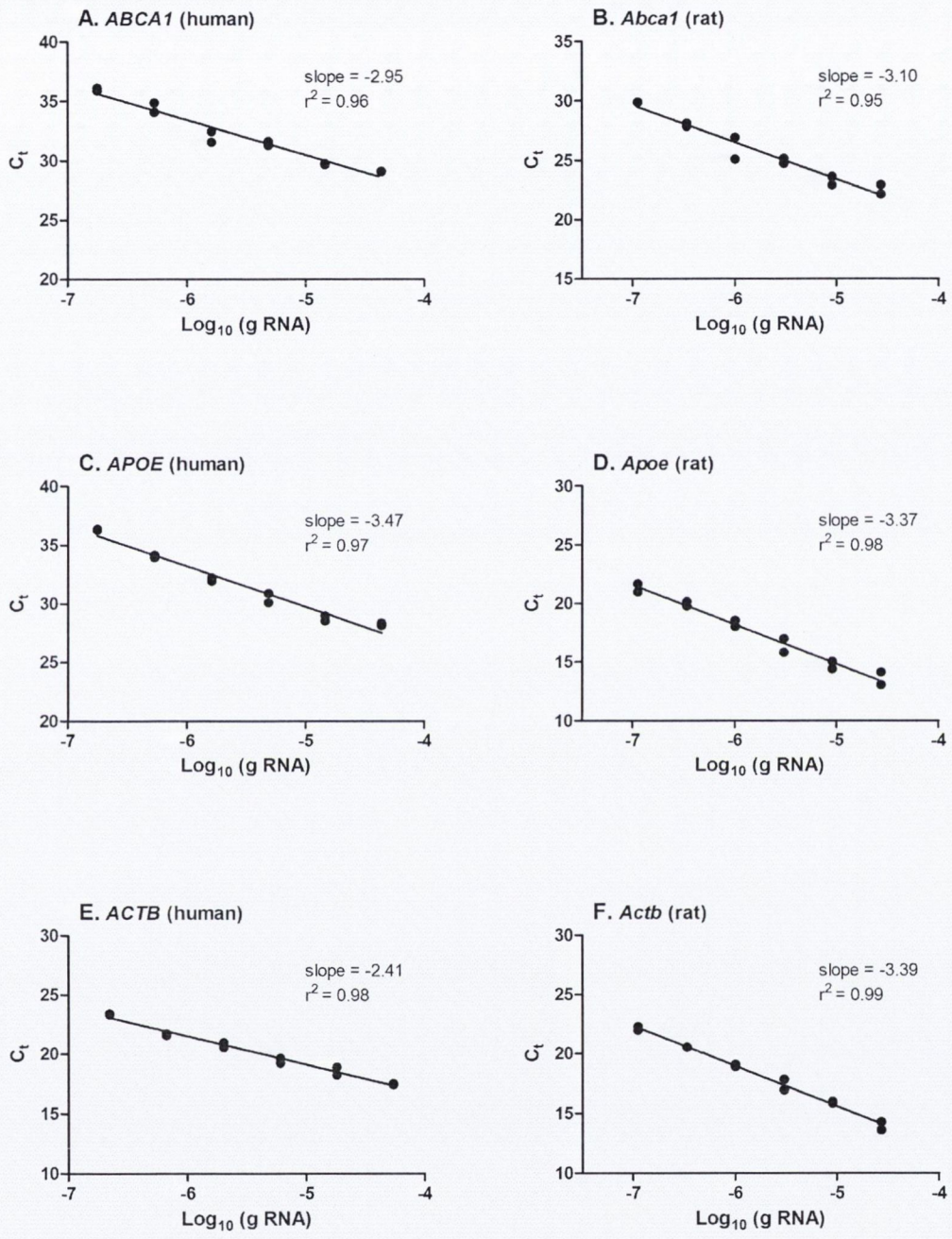


Figure 5.3 Standard curves of primers used for real time PCR. Serial dilutions were made of cDNA reverse transcribed from human and rat RNA in order to assess amplification efficiency of QuantiTect Primer Assays and apply the Pfaffl method to analyse real time PCR results. A) human *ABCA1*, B) rat *Abca1*, C) human *APOE*, D) rat *Apoe*, E) human *ACTB*, F) rat *Actb*. *ABCA1/Abca1* = ATP-binding cassette transporter A1, *APOE/Apoe* = Apolipoprotein E, *ACTB/Actb* = β-actin.

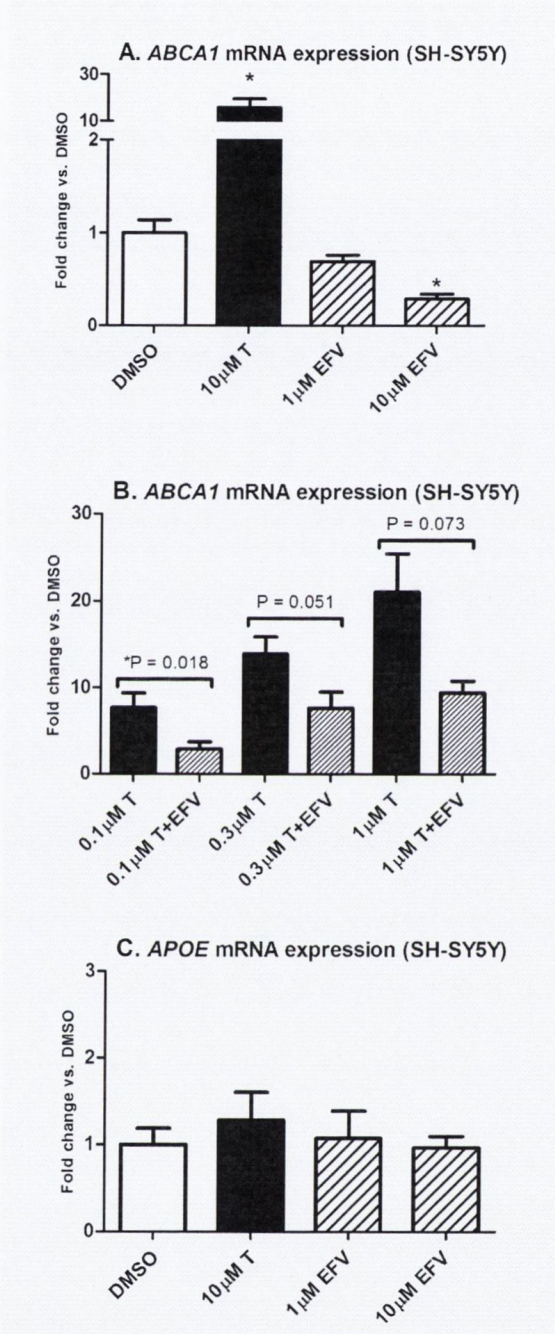


Figure 5.4 mRNA expression of *ABCA1* and *APOE* in SH-SY5Y. The human neuroblastoma cell line was exposed to indicated concentrations of T0901317 (T, LXR agonist), efavirenz (EFV) or vehicle (DMSO) for 24h in phenol red-free medium with 5% FB-LPDS (see Materials and methods). RNA was isolated and reverse transcribed, followed by real-time PCR analysis (n = 4-7). A) *ABCA1* mRNA expression following T0901317 or efavirenz exposure, B) *ABCA1* mRNA expression following exposure to varying concentrations of T0901317 with or without 10μM efavirenz, C) *APOE* mRNA expression following T0901317 or efavirenz exposure.

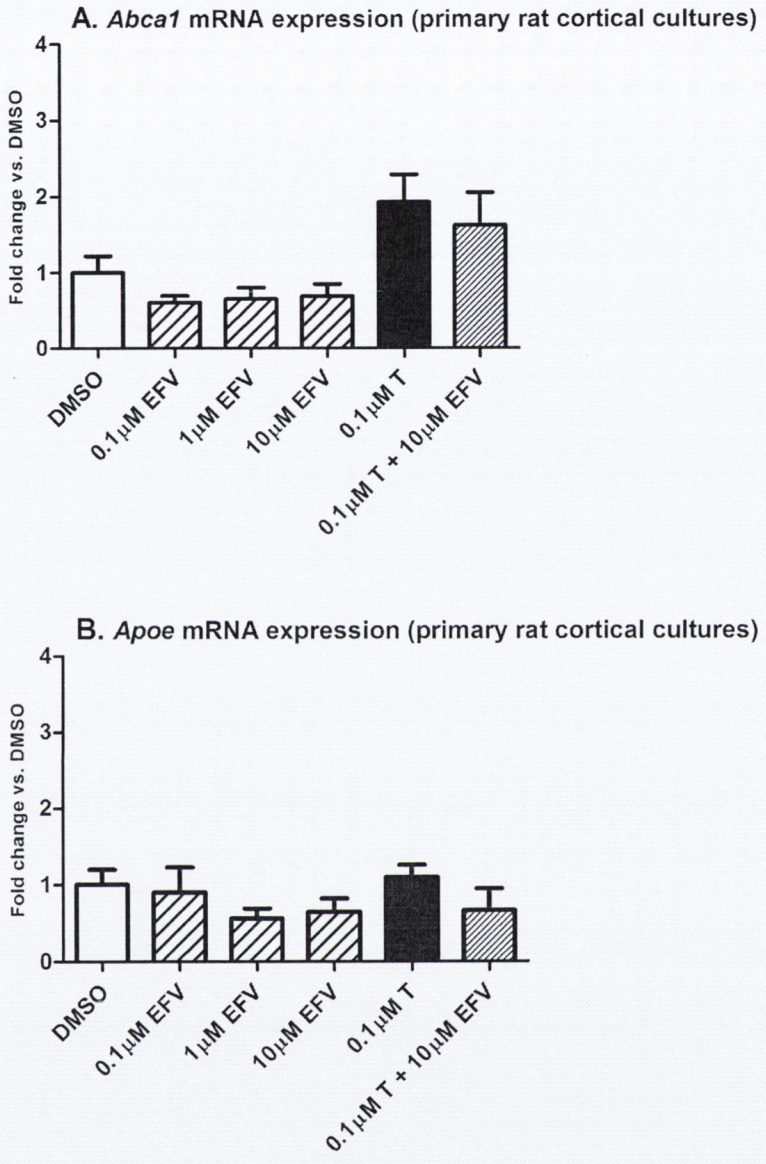


Figure 5.5 mRNA expression of *Abca1* and *Apoe* in primary rat cortical cultures. Cells were exposed to indicated concentrations of T0901317 (T, LXR agonist), efavirenz (EFV) or vehicle (DMSO) for 24h in phenol red- and serum-free medium (see Materials and methods). RNA was isolated and reverse transcribed, followed by real time PCR analysis (n = 4-6). A) *Abca1* mRNA expression following T0901317, efavirenz or T0901317 + efavirenz exposure, B) *Apoe* mRNA expression following T0901317, efavirenz or T0901317 + efavirenz exposure.

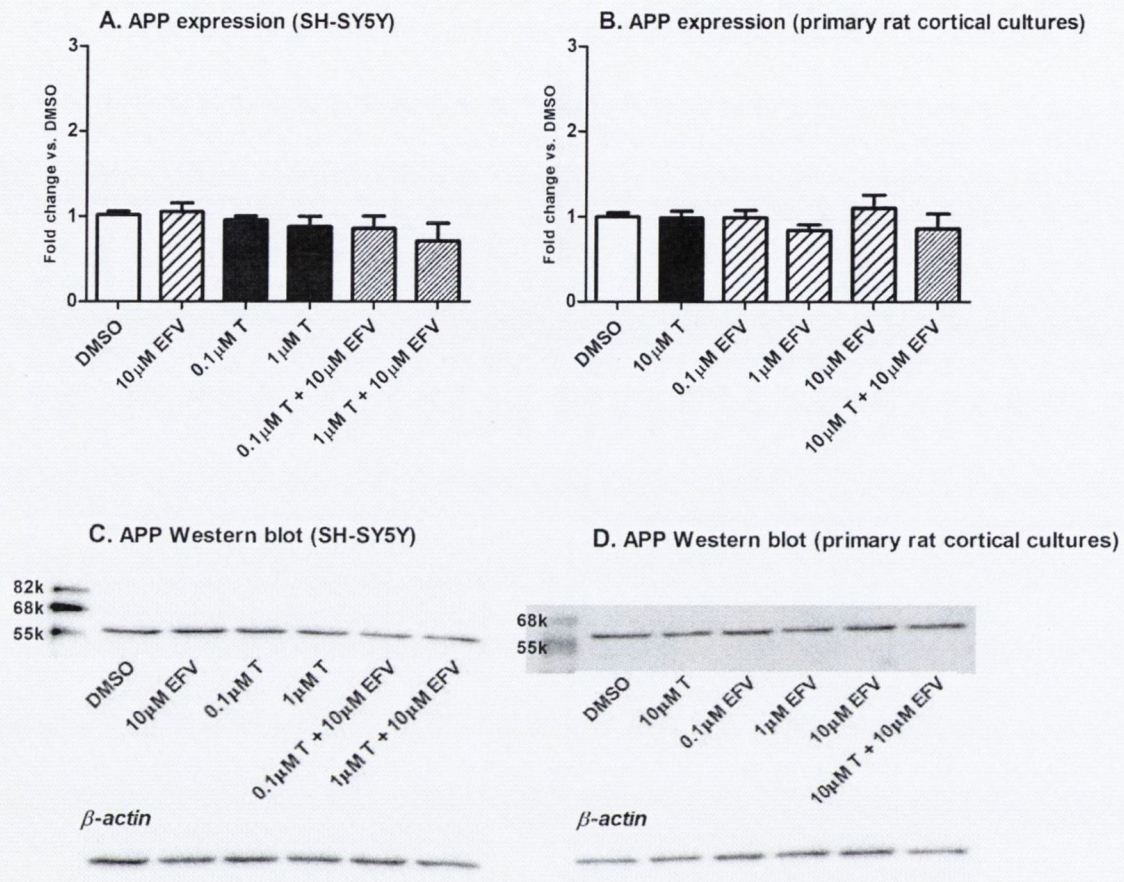


Figure 5.6 APP protein expression in SH-SY5Y and primary rat cortical cultures. Cells were exposed to indicated concentrations of T0901317 (T, LXR agonist), efavirenz (EFV) or vehicle (DMSO) for 24h in phenol red-free medium supplemented with 5% FB-LPDS (SH-SY5Y, n = 4) or phenol red- and serum-free medium (primary rat cortical cultures, n = 5, see Materials and methods). Western blots were performed with RIPA lysates. APP expression was normalised to β-actin and presented relative to vehicle controls as means ± S.E.M. A) APP expression in SH-SY5Y following efavirenz, T0901317 or T0901317+efavirenz exposure, B) APP expression in primary rat cortical cultures following efavirenz, T0901317 or T0901317+efavirenz exposure, C) representative APP (55kDa) blot with corresponding β-actin blot for SH-SY5Y, D) representative APP (55kDa) blot with corresponding β-actin blot for primary rat cortical cultures. APP = amyloid precursor protein.

5.5 Discussion

Efavirenz was identified as an antagonist of LXR activity in chapter 4, showing direct competition of binding to LXRα-LBD with agonist T0901317 in TR-FRET assays. In this chapter, the effects of efavirenz on LXR target genes with links to levels of Aβ – implicated in development of neurodegenerative disorders - were investigated *in vitro* using SH-SY5Y human neuroblastoma cells and primary rat cortical cultures. Expression of *ABCA1* was decreased in efavirenz-treated SH-SY5Y human neuroblastoma cells and attenuation of T0901317-induced expression of the same gene was also confirmed. It should be mentioned that activators of pregnane X receptor (PXR) have previously been reported to downregulate the expression of LXR target genes in a human hepatocarcinoma cell line[428], and we confirmed PXR activating abilities of efavirenz in chapter 3. It is possible that the reduction of *ABCA1* expression provoked by efavirenz is a dual effect of direct LXR and PXR interactions. However, other researchers observed upregulation of the same gene in mice *in vivo*[541] and in other human cell lines[542] after exposure to PXR agonists. No significant effects on *APOE* expression were detected in SH-SY5Y, although T0901317 did also not induce *APOE*. Increased levels of both ApoE mRNA and protein have been demonstrated in primary human neurons after exposure to T0901317[312], although consistent with our results no upregulation of ApoE was seen subsequent to oxysterol-treatment in SH-SY5Y[543], indicating negligent LXR-regulation of ApoE in this cell line. Another nuclear receptor involved in ApoE regulation is estrogen receptor (ER): activation of the α isoform increases mRNA and protein levels of ApoE in the hippocampus both *in vitro* and *in vivo*, whereas activation of the β isoform has the opposite effect[544]. As activation of ERα by efavirenz was also demonstrated in chapter 4, it is possible that any efavirenz-induced LXR inhibition of ApoE expression may be counteracted by ERα activation. Both ERα and ERβ are expressed in SH-SY5Y cells[545].

A similar but non-significant decrease in both *Abca1* and *Apoe* was seen in primary rat cortical cultures after exposure to efavirenz. Similar to SH-SY5Y experiments, T0901317 did not induce gene expression of *Apoe*. This is consistent with T0901317 effects presented by Koldamova *et al*[313] in mice: increased Abca1 protein expression (accompanied with decreased Aβ40) but no effect on ApoE. T0901317 induced a 2.5-fold increase in *Abca1* mRNA (however not significant); changes of the same magnitude in primary rat neuronal cultures[511] and mouse cerebral cortex sections *in vivo*[546] have also been described by other researchers. The dissimilarities in response between human and rat cells in this study may be explained by species differences in LXR gene regulation, of which some examples have been reported[547, 548]. Additionally, other investigations into the expression patterns of LXR and

related target genes in the rat brain revealed very low or barely detectable levels of *Lxra*, *Abca1* and *Apoe* in neurons while expression in glial cells including astrocytes is considerably higher[549, 550]. *Lxrb* on the other hand was detected in all three cell types[549], although levels are low in general in the postnatal rat brain according to Kainu *et al*[504]. Lefterov *et al*[551] performed gene expression profiling in APP transgenic mice in response to LXR stimulus and found upregulation of *Apoe* only in astrocyte, glial or mixed cultures, supporting this cell type-specific response. Whitney *et al*[505] on the other hand did not detect any influence of LXR activation on *Apoe* expression in wildtype mice. Serum-free growth conditions for several days may restrict glial proliferation and result in mainly neuronal cultures[505]. Since the primary neonatal rat cortical cultures in this study were pre-treated for 24 hours with serum-free medium to avoid background activation of LXR, it is possible that the proportion of neurons in these cultures was augmented.

No change in APP protein levels was detected after T0901317- or efavirenz-stimulation in either SH-SY5Y or primary rat cortical cultures. Different strategies may be adopted to better assess the impact of LXR modulators on amyloid processing: some investigators successfully enhanced this effect by using 9-cis-retinoic acid in combination with LXR agonists to stimulate the activity of retinoid X receptor (RXR), the heterodimer partner of LXR[313]. Another approach is to include a cholesterol acceptor such as apolipoprotein AI (ApoAI, also present in the CNS[526]) in the culture medium to facilitate apolipoprotein-mediated cholesterol efflux[314] as one of the main hypotheses for LXR effects on Aβ processing involves the activity of ABCA1. Nevertheless, other researchers have also reported no changes in cellular full-length APP despite significant decreases in Aβ levels after exposure to LXR agonists[313, 552]. This could indicate an effect on clearance and degradation of Aβ rather than on processing steps. Hoe *et al*[553] also published evidence of an alternative mechanism via tissue inhibitor of matrix metalloproteinase-3 (TIMP-3): Overexpression of TIMP-3 inhibits the activity of α-secretase (which is in fact a zinc metalloproteinase[554]), thus promoting the β-secretase pathway for APP processing and consequently increasing production of Aβ. Higher-than-normal levels of TIMP-3 were also detected in the frontal cortex of AD patients. LXR activation downregulates expression of TIMP-3 mRNA *in vitro*, presumably leading to removed inhibition of α-secretase and re-routing the APP processing to this pathway with decreased levels of Aβ as a result. However, when these investigators examined the effect of T0901317 exposure *in vivo* using mouse models they did not find any significant effects on APP processing, despite confirming increased TIMP-3 protein expression.
Unfortunately, attempts to quantify Aβ40 in this study were unsuccessful. As this soluble amyloid species correlates particularly well with the neurodegeneration of AD disease and distinguishes it

from non-dementia related pathology[315], and additionally greater LXR effects have been observed on Aβ40 than Aβ42 levels in above mentioned studies[313, 314], assessing the impact of efavirenz on Aβ40 would be highly relevant. It may be that endogenous levels of Aβ in SH-SY5Y and primary rat cortical cultures are too low for detection; indeed many researchers use APP transgenic animals or overexpressing cell lines. Nonetheless, there are examples of publications where the investigators quantified endogenous Aβ from embryonic wildtype mouse cortices[555] (by ELISA), from SH-SY5Y[556] (by immunoprecipitation followed by Western blot), and from primary human neurons as well as CHO-APP cells[312] (loading culture medium straight onto SDS polyacrylamide gels for Western blots).

Although primary human neuronal cultures would be the preferable *in vitro* model to examine the effects of pharmaceuticals on gene expression in the human brain, for ethical and practical reasons these are often difficult to obtain. While rodent primary cultures in general are more readily available, it is often useful to also include human-derived cell lines to account for species differences in response. SH-SY5Y human neuroblastoma cells have been extensively used for neurotoxicity studies[557] and as a model of neurodegenerative disease[558, 559]. However, as most transformed cell lines, SH-SY5Y displays some phenotypic differences compared to non-transformed cells (such as overexpression of anti-apoptotic genes[560]), which may be a limitation of this model. Moreover, according to the supplier loss of neuronal characteristics have been described for SH-SY5Y with increasing passage numbers, for this reason experiments were only performed up to passage ten.

Neurocognitive disorders in HIV patients are likely to be the result of many factors, for example HIV infection itself is associated with neurodegeneration[561] and many cytokines observed in HIV infection[562] promote amyloidogenesis[563]. In this study we demonstrated downregulation of *ABCA1* by efavirenz in a human neuroblastoma cell line, which may provide some mechanistic insights into the CNS effects associated with efavirenz use. Further investigations into downstream effects of LXR inhibition and implications of *ABCA1* downregulation by efavirenz are warranted.

6 Discussion and conclusions

The universal theme of this thesis is activation of nuclear receptors by antiretroviral (ARV) drugs. This superfamily of receptors regulates the expression of genes involved in a wide variety of important physiological processes such as cell growth and differentiation, energy homeostasis, inflammation and endobiotic as well as xenobiotic metabolism. On account of their ligand-dependent activity, nuclear receptors serve as an interface between cellular or organism environment and gene expression[243]. Activation of nuclear receptors by foreign substances may cause a disruption of metabolic equilibrium and lead to abnormalities.

Although highly active antiretroviral therapy (HAART) has improved HIV care considerably, many challenges remain: Adverse effects are common, sometimes leading to therapy switch or discontinuation[56]. Many ARV drugs also give rise to drug interactions, an effect partly explained by inhibition of metabolising enzymes and drug transporters[564]. However, an alternative mechanism is by activation of nuclear receptors. Previous studies[141, 146, 300] have established some HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz as activators of xenobiotic sensor pregnane X receptor (PXR). PXR, together with the closely related constitutive androstane receptor (CAR), regulate gene expression of several members of the cytochrome P450 (CYP450) metabolising enzyme family as well as drug efflux transporter P-glycoprotein (P-gp). Consequently, altered PXR and CAR activity may have a substantial impact on drug bioavailability.

The overall aim of this thesis was to use a combined methodological approach to investigate interactions between ARVs and nuclear receptors with both xenobiotic and endobiotic functions. Relevant downstream effects such as expression of drug metabolising enzymes and drug or cholesterol transporters were also examined. In addition, the influence of genetic variability on nuclear receptor activation by ARVs was assessed.

The first study (chapter 3) utilised luciferase reporter assays to assess the ability of a wide range of ARV compounds to activate PXR or CAR and induce transcription of *CYP3A4* and *CYP2B6*, which are important ARV drug metabolisers. Several ARV drugs from different subclasses were found to have this ability, demonstrating that it is not a class-specific effect. Addition of low-dose ritonavir, which is often used as a pharmacoenhancer to increase plasma levels of a partner PI in treatment of HIV, further enhanced CYP450 induction for several PIs despite exerting no significant effect alone.

Primary human hepatocytes were exposed to varying concentrations of a subset of PXR/CAR agonists, confirming inducing effects on mRNA and/or protein expression levels. In our studies the impact of PXR/CAR induction was more pronounced for CYP3A4 than CYP2B6 at the protein level, whereas pharmacogenetics appears to be the main determinant for CYP2B6 activity reported in the literature[191, 192, 565]. Nevertheless, these results highlight the usefulness of *in vitro* reporter assays for screening drugs in development for PXR/CAR inducing abilities to minimise the risk of potential drug interactions – a necessity in drug development. A double transgenic mouse strain expressing human PXR and CYP3A4 has been developed and successfully used to assess the metabolic stability of certain PIs following PXR induction[566]. It is important to keep in mind that as many PIs are also inhibitors of CYP450 enzymes and P-gp, the potential net result may be a reduction in activity of the enzyme/transporter. This has been illustrated by Fellay *et al*[567], who reported strong inhibition of CYP3A4 activity *in vivo* by several PIs, of which ritonavir had the highest potency. Treatment with efavirenz on the other hand resulted in increased CYP3A4 activity, an effect which was completely abrogated in combination with ritonavir. An interesting computational docking study by Mannu *et al*[568] revealed that efavirenz may also induce increased CYP3A4 metabolic activity by binding to a unique position in its active site.

Many genetic factors of importance for ARV drug bioavailability have been identified, primarily polymorphisms in metabolising enzyme and drug transporter genes[569]. Typically, pharmacogenetics studies of ARV effects are predominantly carried out with Caucasian subjects and native Africans are underrepresented despite the world's highest HIV prevalence on the African continent. In the first study of this thesis, we examined the prevalence of 37 single nucleotide polymorphisms (SNPs) and one 3bp insertion in *NR1I2* (PXR), *CYP3A4*, *CYP2B6* and *ABCB1/MDR1* with proven or predicted effect on drug levels in a cohort of 1013 HIV-infected Caucasians and Sub-Saharan Africans. We showed several significant differences in 22 out of 29 polymorphisms detected between these populations, with higher frequencies of the minor alleles among Sub-Saharan Africans for the majority of these SNPs. This may bring additional understanding to the higher prevalence of certain ARV adverse effects (mainly cardiovascular, renal and psychiatric) among black HIV patients[299], relevant as treatment switch or discontinuation due to toxicity is more common among non-whites[56]. Following a comparison of our results from the genetic screening of Sub-Saharan Africans with available data from studies including African Americans, we also noted significant differences in some allele frequencies in *NR1I2* (PXR), *CYP3A4* and *ABCB1/MDR1* SNPs, suggesting a degree of genetic diversity

between these groups. This indicates that prudence should perhaps be adopted in extrapolating results of pharmacokinetic studies from African Americans to Sub-Saharan Africans.

In the final part of our first study, we assessed the impact of polymorphisms in *NR1I2* (PXR) detected in the cohort and resulting in amino acid changes, on ARV induction of *CYP3A4* transcription. This was achieved by using luciferase reporter assays with PXR variants. Two of these SNPs (V140M and A370T) displayed significantly lower inducibility by rifampicin, fosamprenavir and lopinavir, illustrating the potential relevance of *NR1I2* (PXR) polymorphism for ARV pharmacokinetics.

In conclusion, we have demonstrated the ability of a variety of ARV drugs to activate PXR and CAR with implications for drug interactions. Nuclear receptor polymorphism can affect ARV induction; however the prevalence of SNPs in *NRI12* (PXR) and other genes of relevance for drug interactions differs between Caucasians and Sub-Saharan Africans. It would be of great interest to further investigate the influence of nuclear receptor polymorphism on activation by ARV therapeutics *in vivo*. Limitations of this type of study include the difficulty in identifying a measurable phenotype unaffected by confounding factors.

While the potential implications of PXR activation for drug interactions are well recognised, activation of other nuclear receptors by ARVs is less well studied. HAART treated patients often experience adverse effects, for example fat redistribution, lipid abnormalities, insulin resistance, increased cardiovascular risk, osteopenia[70] and neuropsychiatric complications[85]. Based on the fact that liver X receptors (LXRs), estrogen receptors (ERs) and glucocorticoid receptor (GR) are involved in gene regulation related to all these processes[308-310, 408-418], in addition to well-documented ligand overlap between PXR/CAR and other nuclear receptors[264], our hypothesis that ARV drugs may act as ligands of LXRs, ERs and GR was explored in the second study (chapter 4). A library of available ARV compounds was subjected to screening by *in silico* evaluation using molecular docking models of LXRα/β, ERα/β and GR. Results were filtered using molecular descriptors of known ligands. This was followed by assessment of direct binding by ARVs to ligand binding domains (LBDs) using cell-free TR-FRET co-activator assays. Nuclear receptor activation in a cellular environment was evaluated by *in vitro* luciferase reporter assays in HepG2 cells transfected with nuclear receptor expression plasmids and response element-luciferase constructs for LXRα, LXRβ, ERα, ERβ and GR.

All predicted ligands of LXRα, LXRβ and ERα showed activity in either TR-FRET or reporter assays or both. In reporter assays, we identified four agonists of LXRα and/or LXRβ: atazanavir, ritonavir, darunavir and maraviroc, although direct receptor binding had been detected in TR-FRET experiments

with the latter two only. This is supported by the nuclear receptor modelling which did not predict atazanavir and ritonavir as LXR ligands due to their size and polarity which would prevent these compounds fitting into the ligand-binding pocket. The LXR activation by atazanavir and ritonavir detected in the cell-based assays is therefore more likely due to allosteric effects. Efavirenz and flavopiridol, which were both predicted LXR ligands, showed antagonistic effects in reporter assays and direct competition of binding to the LXRα-LBD with LXR agonist T0901317 was confirmed in TR-FRET co-activator recruitment assays. Efavirenz and tipranavir both activated ERα in reporter assay experiments, for which efavirenz was a predicted ligand. However, neither of these two drugs caused significant recruitment of co-activators PGC1α or TRAP220/DRIP-2 to ERα-LBD. Nevertheless, these results do not exclude the involvement of other co-activators or co-repressors, in addition to the possibility of allosteric mechanisms which is perhaps the most likely explanation for tipranavir activation as its structure failed the ERα docking test. No significant activation was detected in ERβ and GR reporter assays with the drugs tested. Our study shows that ARVs can have both agonistic and antagonistic actions on LXRs and ERs, either by direct binding to LBDs or by other in our study uncategorised interactions, possibly allosteric. By relying on *in silico* ligand-binding domain modelling alone to inform the choice of drugs for *in vitro* validation of nuclear receptor activation, these effects would have been missed. This highlights the usefulness of multiple methodologies. Further investigations into these interactions and their consequences are warranted.

The NNRTI efavirenz forms part of first-line HAART in both Europe and USA[30, 45] and is usually administered in combination with two nucleoside reverse transcriptase inhibitors (NRTIs). It has the benefit of potent and durable viral suppression[570] and a long half-life[571]. However, neuropsychiatric adverse effects such as insomnia, anxiety, memory deficits and impaired cognition are common[320]. In some cases these effects are severe and result in treatment switch or discontinuation (25.1% of patients in the TRT-5 Group[88]and 16.4% of patients in the EuroSIDA Study[89]). The mechanisms of efavirenz central nervous system (CNS) adverse events are unclear, although it is known that it penetrates the blood-brain barrier and toxicity is associated with drug plasma levels[319].

Previous studies have linked LXR regulation of ABCA1 to β-amyloid (Aβ) processing[313, 314, 316], an important marker of neurodegenerative disease progression. Another target gene of LXR implicated in cognitive impairment is apolipoprotein E (apoE)[527]. Both ABCA1 and ApoE are involved in the homeostasis of cholesterol, necessary for neuronal function. Disruption of neuronal cellular

membranes and lipid rafts where Aβ processing occurs has been suggested as a potential mechanism for these effects. Given that LXR agonists reduce neurodegeneration[572] and improve neurocognition[513], we hypothesised that the well-documented CNS adverse effects by efavirenz may be partly attributed to the LXR antagonism we demonstrated in chapter 4. Therefore, the aim of the third study (chapter 5) was to investigate the downstream effects of reduced LXR activity by efavirenz, with focus on target genes *ABCA1* and *APOE*, and additionally on amyloid precursor protein (APP) and Aβ40. Efavirenz exposure resulted in downregulation of *ABCA1* mRNA expression in human SH-SY5Y neuroblastoma cells and attenuation of T0901317-induced transcription, consistent with expected effects of LXR antagonism. However, no effect on *APOE* was noted either by efavirenz or LXR agonist T0901317 in SH-SY5Y, indicating low LXR-dependence in this cell line. In primary rat cortical cultures no significant changes in *Abca1* or *Apoe* were observed after efavirenz exposure, although both of these showed a trend towards lower expression. We did not detect any impact of efavirenz on amyloid precursor protein (APP) in either of the two *in vitro* systems. Nevertheless, other researchers have reported altered levels of soluble Aβ without changes in intracellular APP[313]. A shift from β-secretase cleavage to α-secretase cleavage would for example reduce Aβ secretion but have no impact on APP levels. Alternatively, decreased Aβ could also be the result of increased degradation. A limitation of this study was that attempts to quantify Aβ40 were unsuccessful. Hence, no conclusions can be reached regarding the influence of efavirenz on Aβ levels. The confirmed inhibition of *ABCA1* expression does however suggest that it may have an impact. Further investigation may shed some light on the neurocognitive impairment experienced by many HIV patients on an efavirenz-containing drug regimen.

Despite most ARVs (an exception being CCR5 antagonists) having been designed to interact with viral proteins, the adverse events seen in clinical practice suggest off-target host effects. Activation of PXR is a well-known phenomenon of many unrelated pharmaceuticals leading to induced expression of metabolising enzymes and drug transporters, increasing the risk of drug interactions. Pan *et al* (2011)[573] has already demonstrated the usefulness of combining virtual ligand-based screening with luciferase reporter assays as a means of identifying new PXR ligands. PXR is widely regarded as a "promiscuous" nuclear receptor with a very flexible ligand binding pocket; hence it is not surprising that several ARV drugs can activate PXR. In this thesis we have shown in addition that ARVs from several different drug classes can act as ligands of nuclear receptors LXRα/β and ERα, potentially resulting in divergent metabolic effects. It should also be noted, however, that apart from their role

as xenobiotic sensors, PXR and CAR are likewise involved in several endogenous processes: PXR overstimulation has been associated with hypercholesterolemia[574] and hyperlipidemia[575] while CAR activation can cause triglyceridemia[576] and exacerbate liver steatosis[577]. ARV activation of various nuclear receptors may contribute to the off-target effects of HAART. Further research into drug-specific interactions with different nuclear receptors and downstream effects in terms of target genes as well as non-transcriptional responses is required. Additionally, studies into the clinical implications of nuclear receptor polymorphism in the context of ARV-receptor interactions are required. In the future it may be of interest to screen drugs in development for activation of an array of nuclear receptors in order to better predict the risk of HAART metabolic and adverse effects. Where interactions between already established ARV therapeutics and specific nuclear receptors are identified as clinically relevant, a strategy of using selective agonists/antagonists to modulate these off-target effects could also be considered, although potential effects on ARV plasma drug levels would have to be taken into account. However, ideally these properties should perhaps be avoided among new drug candidates to minimise the risk of drug interactions and undesirable side effects. It is also likely that metabolic effects seen in clinical practice such as lipodystrophy are multifactorial in origin, as opposed to the result of activation of a single nuclear receptor.

In summary, we have demonstrated that ARV drugs of different structural classes can interact with nuclear receptors PXR, CAR, LXRα, LXRβ and ERα, with implications for drug interactions and off-target effects. Genetic differences between Caucasians and Sub-Saharan Africans in *NR1I2* (PXR) and other genes of relevance for drug metabolism were described, and we confirmed a functional impact of coding *NR1I2* (PXR) SNPs on ARV-induced *CYP3A4* promoter activity. It is possible that polymorphism in other nuclear receptor genes may also influence their interaction with ARV compounds. This thesis highlights the need for further research into downstream effects as well as clinical consequences of nuclear receptor agonism/antagonism by ARV drugs.

7 Future directions

This thesis investigated interactions between antiretroviral (ARV) drugs, used in the treatment of HIV infection, and nuclear receptors, important transcription factors governing the expression of genes involved in both xenobiotic and endobiotic processes. Agonistic and/or antagonistic effects were demonstrated for several ARV drugs from different classes. These results inspire to further investigation, for example:

- The clinical relevance of coding and non-coding *NR1I2* (PXR) SNPs: effects on inducibility of CYP450 expression by ARVs. A clinical study including 50 HIV-infected subjects has been carried out at the GUIDE clinic, St James's Hospital, Dublin, for this purpose and sample analysis is ongoing.

- Downstream effects of LXRα/β and ERα activation by ARVs, such as expression of target genes involved in cholesterol and lipid homeostasis, adipocyte differentiation, inflammation, and balance of monoamine levels.

- Effect of efavirenz exposure on levels of β-amyloid (Aβ) in CNS models, as this was not clarified in this thesis.

- Impact of genetic polymorphism in *NR1H3* (LXRα), *NR1H2* (LXRβ) and *NR3A1* (ERα) and clinical relevance of ARV interactions with these nuclear receptor.

- ARV binding to nuclear receptors other than those examined in this thesis, for example farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPARs) and vitamin D receptor (VDR).

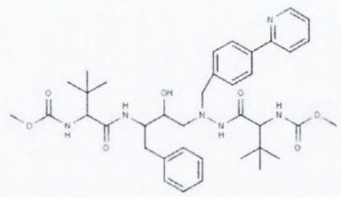
Appendix: ARV drug structures for in silico evaluation of nuclear receptor interactions

Drug (abbreviation)

a. **IUPAC name**
b. **chemical formula**
c. **2D structure**
d. **drug subclass** (PI = HIV protease inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NNRTI = non-nucleoside reverse transcriptase inhibitor)

1. Atazanavir (ATV)

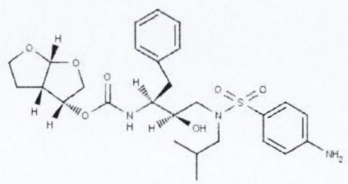
a. methyl N-[(2S)-1-[[(2S,3S)-3-hydroxy-4-[[[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-[(4-pyridin-2-ylphenyl)methyl]amino]-1-phenylbutan-2-yl]amino]-3,3-dimethyl-1-oxobutan-2-yl]carbamate
b. $C_{38}H_{52}N_6O_7$



c.
d. PI

2. Darunavir (DRV)

a. [(3R,3aS,6aR)-2,3,3a,4,5,6a-hexahydrofuro[5,4-b]furan-3-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2-yl]carbamate
b. $C_{27}H_{37}N_3O_7S$

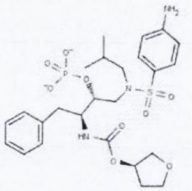


c.
d. PI

3. Fosamprenavir (FOS)

a. [(3S)-oxolan-3-yl] N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-1-phenyl-3-phosphonooxybutan-2-yl]carbamate

b. $C_{25}H_{36}N_3O_9PS$

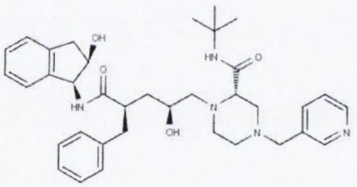
c. 

d. PI

4. Indinavir (IDV)

a. (2S)-N-tert-butyl-1-[(2S,4R)-2-hydroxy-5-[[(1S,2R)-2-hydroxy-2,3-dihydro-1H-inden-1-yl]amino]-5-oxo-4-(phenylmethyl)pentyl]-4-(pyridin-3-ylmethyl)piperazine-2-carboxamide

b. $C_{36}H_{47}N_5O_4$

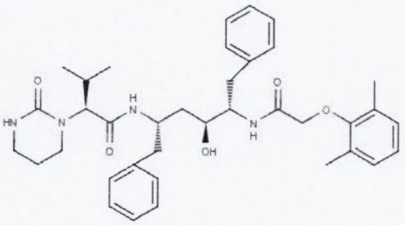
c. 

d. PI

5. Lopinavir (LPV)

a. (2S)-N-[(2S,4S,5S)-5-[[2-(2,6-dimethylphenoxy)acetyl]amino]-4-hydroxy-1,6-di(phenyl)hexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide

b. $C_{37}H_{48}N_4O_5$

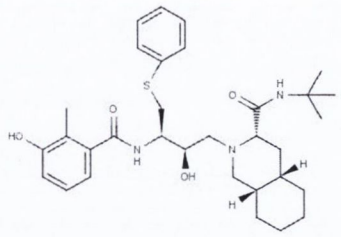
c. 

d. PI

6. Nelfinavir (NFV)

a. [3S-[2(2S*, 3S*),3α,4aβ,8aβ]]-N-(1,1-dimethylethyl)decahydro-2-[2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-(phenylthio)butyl]-3-isoquinoline carboxamide

b. $C_{32}H_{45}N_3O_4S$



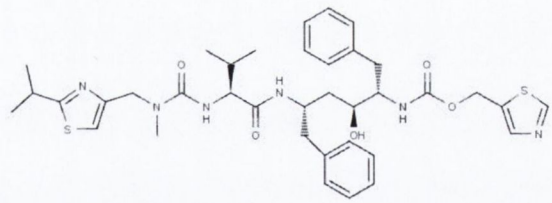
c.

d. PI

7. Ritonavir (RTV)

a. 2,4,7,12-Tetraazatridecan-13-oic acid, 10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11- bis(phenylmethyl)-5-thiazolylmethyl ester [5S-(5R*,8R*,10R*,11R*)]-. 5-Thiazolylmethyl [(aS)-a-[(1S,3S)-1-hydroxy-3-[(2S)-2-[3-[(2-isopropyl-4-thiazolyl)methyl]-3-methylureido]-3-methylbutyramido]-4-phenylbutyl]phenethyl]carbamate

b. $C_{37}H_{48}N_6O_5S_2$



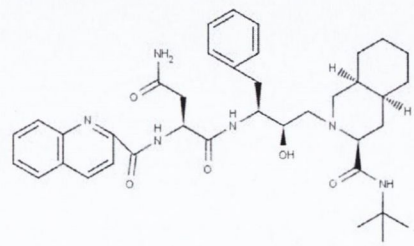
c.

d. PI

8. Saquinavir (SQV)

a. N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[[N-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4aS,8aS)-isoquinoline-3(S)-carboxamide methanesulfonate

b. $C_{38}H_{50}N_6O_5$



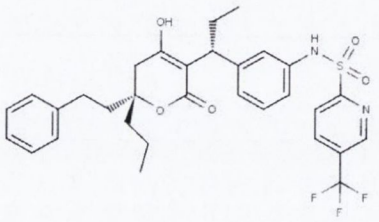
c.

d. PI

9. Tipranavir (TPV)

a. 2-Pyridinesulfonamide, N-[3-[(1R)-1-[(6R)-5,6-dihydro-4-hydroxy-2-oxo-6-(2-phenylethyl)-6-propyl-2H-pyran-3yl]propyl]phenyl]-5-(trifluoromethyl)

b. $C_{31}H_{33}F_3N_2O_5S$



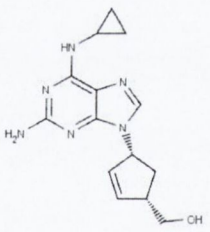
c.

d. PI

10. Abacavir (ABC)

a. (1S,cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt) (2:1)

b. $C_{14}H_{18}N_6O$



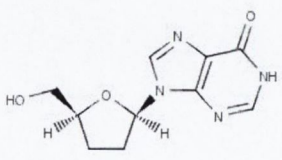
c.

d. NRTI

11. Didanosine (2,3'-Dideoxyinosine, ddI)

a. 2',3'-dideoxyinosine

b. $C_{10}H_{12}N_4O_3$



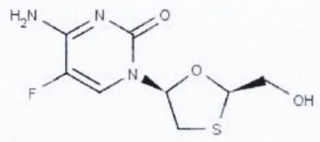
c.

d. NRTI

12. Emtricitabine (FTC)

a. 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine

b. $C_8H_{10}FN_3O_3S$



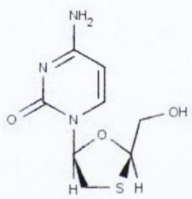
c.

d. NRTI

13. Lamivudine (3TC)

a. (2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one

b. $C_8H_{11}N_3O_3S$



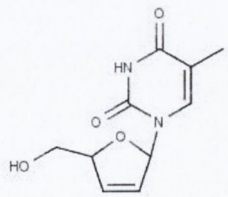
c.

d. NRTI

14. Stavudine (d4T)

a. 2',3'-didehydro-3'-deoxythymidine

b. $C_{10}H_{12}N_2O_4$



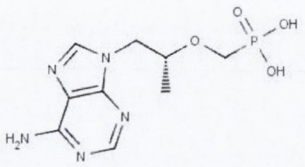
c.

d. NRTI

15. Tenofovir (TFV)

a. 9-[(R)-2-(phosphonomethoxy)propyl]adenine

b. $C_9H_{14}N_5O_4P \cdot H_2O$



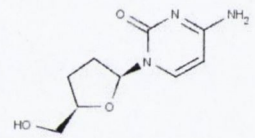
c.

d. NRTI

16. Zalcitabine (2',3'-dideoxycytidine, ddC)

a. 4-amino-1-[(2R,5S)-5-(hydroxymethyl)oxolan-2-yl]pyrimidin-2-one

b. $C_9H_{13}N_3O_3$



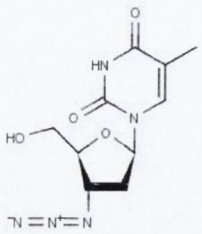
c.

d. NRTI

17. Zidovudine (AZT)

a. 3'-azido-3'-deoxythymidine

b. $C_{10}H_{13}N_5O_4$



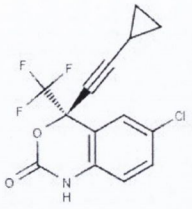
c.

d. NRTI

18. Efavirenz (EFV)

a. (S)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one

b. $C_{14}H_9ClF_3NO_2$



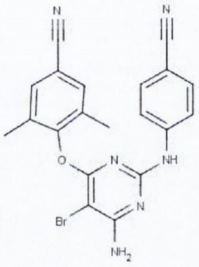
c.

d. NNRTI

19. Etravirine (TMC125)

a. 4-[[6-amino-5-bromo-2-[(4-cyanophenyl) amino]-4-pyrimidinyl]oxy]-3,5-dimethylbenzonitrile

b. $C_{20}H_{15}BrN_6O$



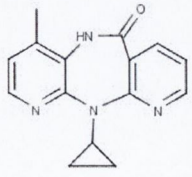
c.

d. NNRTI

20. Nevirapine (NVP)

a. 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2',3'-e][1,4] diazepin-6-one

b. $C_{15}H_{14}N_4O$



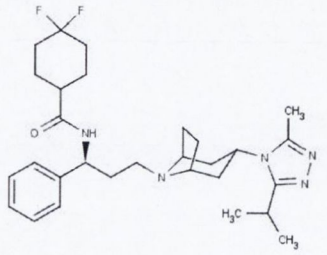
c.

d. NNRTI

21. Maraviroc (MVC)

a. 4,4-difluoro-N-{(1S)-3-[exo-3-(3-isopropyl-5-methyl-4H-1,2,4-triazol-4-yl)-8-azabicyclo[3.2.1]oct-8-yl]-1-phenylpropyl}cyclohexanecarboxamide

b. $C_{29}H_{41}F_2N_5O$



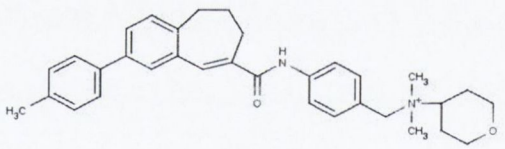
c.

d. CCR5 antagonist

22. TAK-779

a. *N,N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride

b. $C_{33}O_2N_2H_{38}$



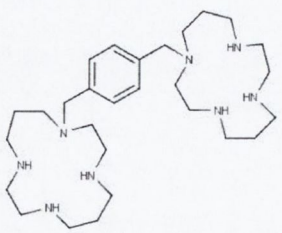
c.

d. CCR5 antagonist (investigational)

23. bicyclam JM-2987 (hydrobromide salt of AMD-3100)

a. 1,1'-[1,4-phenylene-bis(methylene)]-bis(1,4,8,11-tetra-azacyclotetradecane) octahydrochloride dehydrate

b. $C_{30}H_{70}Br_8N_8O_4$



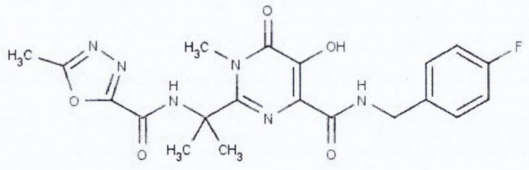
c.

d. CXCR4 antagonist (investigational)

24. Raltegravir (RAL)

a. N-[(4-Fluorophenyl)methyl]-1,6-dihydro-5-hydroxy-1-methyl-2-[1-methyl-1-[[(5-methyl-1,3,4-oxadiazol-2-yl)carbonyl]amino]ethyl]-6-oxo-4-pyrimidinecarboxamide

b. $C_{20}H_{20}FKN_6O_5$

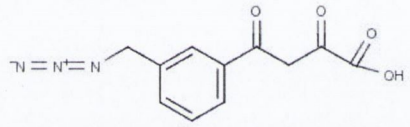
c. 

d. Integrase inhibitor

25. 118-D-24

a. 4-[3-(azidomethyl)phenyl]-2-hydroxy-4-oxo-2-butenoic acid

b. $C_{11}H_9N_3O_4$

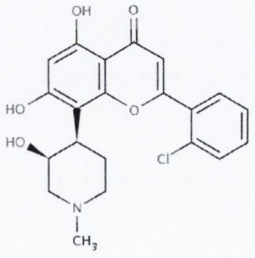
c. 

d. Integrase inhibitor (investigational)

26. Flavopiridol (FLAV)

a. (-) cis-5, 7-dihydroxy-2-(2-chlorophenyl)-8-(4-(3-hydroxy-1-methyl) piperidinyl)-4H-1-benzopyran-4-one

b. $C_{21}H_{20}O_5NCl$

c. 

d. Cyclin-dependent kinase inhibitor

References

1. Gottlieb MS, Schroff R, Schanker HM et al. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med 1981;305(24):1425-1431.

2. Barre-Sinoussi F, Chermann JC, Rey F et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 1983;220(4599):868-871.

3. Gallo RC, Salahuddin SZ, Popovic M et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 1984;224(4648):500-503.

4. Popovic M, Sarngadharan MG, Read E, Gallo RC. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 1984;224(4648):497-500.

5. Sarngadharan MG, Popovic M, Bruch L, Schupbach J, Gallo RC. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. Science 1984;224(4648):506-508.

6. Schupbach J, Popovic M, Gilden RV, Gonda MA, Sarngadharan MG, Gallo RC. Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. Science 1984;224(4648):503-505.

7. Clavel F, Guetard D, Brun-Vezinet F et al. Isolation of a new human retrovirus from West African patients with AIDS. Science 1986;233(4761):343-346.

8. Grant AD, Djomand G, De Cock KM. Natural history and spectrum of disease in adults with HIV/AIDS in Africa. AIDS 1997;11 Suppl B:S43-S54.

9. Witvrouw M, Pannecouque C, Switzer WM, Folks TM, De CE, Heneine W. Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. Antivir Ther 2004;9(1):57-65.

10. Alexaki A, Liu Y, Wigdahl B. Cellular reservoirs of HIV-1 and their role in viral persistence. Curr HIV Res 2008;6(5):388-400.

11. De CE. Strategies in the design of antiviral drugs. Nat Rev Drug Discov 2002;1(1):13-25.

12. Fenyo EM, Albert J, Asjo B. Replicative capacity, cytopathic effect and cell tropism of HIV. AIDS 1989;3 Suppl 1:S5-12.

13. Jurriaans S, Van GB, Weverling GJ et al. The natural history of HIV-1 infection: virus load and virus phenotype independent determinants of clinical course? Virology 1994;204(1):223-233.

14. Musey L, Hughes J, Schacker T, Shea T, Corey L, McElrath MJ. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N Engl J Med 1997;337(18):1267-1274.

15. de WF, Spijkerman I, Schellekens PT et al. AIDS prognosis based on HIV-1 RNA, CD4+ T-cell count and function: markers with reciprocal predictive value over time after seroconversion. AIDS 1997;11(15):1799-1806.

16. Gougeon ML, Olivier R, Garcia S et al. [Demonstration of an engagement process towards cell death by apoptosis in lymphocytes of HIV infected patients]. C R Acad Sci III 1991;312(11):529-537.

17. Douek D. HIV disease progression: immune activation, microbes, and a leaky gut. Top HIV Med 2007;15(4):114-117.

18. Zaunders JJ, Munier ML, Kaufmann DE et al. Early proliferation of CCR5(+) CD38(+++) antigen-specific CD4(+) Th1 effector cells during primary HIV-1 infection. Blood 2005;106(5):1660-1667.

19. Conlon CP. Clinical aspects of HIV infection in developing countries. Br Med Bull 1988;44(1):101-114.

20. Montagnier L. 25 years after HIV discovery: prospects for cure and vaccine (Nobel lecture). Angew Chem Int Ed Engl 2009;48(32):5815-5826.

21. Porter K, Babiker A, Bhaskaran K et al. Determinants of survival following HIV-1 seroconversion after the introduction of HAART. Lancet 2003;362(9392):1267-1274.

22. Mitsuya H, Weinhold KJ, Furman PA et al. 3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc Natl Acad Sci U S A 1985;82(20):7096-7100.

23. Peter K, Gambertoglio JG. Intracellular phosphorylation of zidovudine (ZDV) and other nucleoside reverse transcriptase inhibitors (RTI) used for human immunodeficiency virus (HIV) infection. Pharm Res 1998;15(6):819-825.

24. Balzarini J, Holy A, Jindrich J et al. Differential antiherpesvirus and antiretrovirus effects of the (S) and (R) enantiomers of acyclic nucleoside phosphonates: potent and selective in vitro and in vivo antiretrovirus activities of (R)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. Antimicrob Agents Chemother 1993;37(2):332-338.

25. Hammer SM, Squires KE, Hughes MD et al. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N Engl J Med 1997;337(11):725-733.

26. Gulick RM, Mellors JW, Havlir D et al. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 1997;337(11):734-739.

27. Murphy EL, Collier AC, Kalish LA et al. Highly active antiretroviral therapy decreases mortality and morbidity in patients with advanced HIV disease. Ann Intern Med 2001;135(1):17-26.

28. Moore RD. Cost effectiveness of combination HIV therapy: 3 years later. Pharmacoeconomics 2000;17(4):325-330.

29. Sendi PP, Bucher HC, Harr T et al. Cost effectiveness of highly active antiretroviral therapy in HIV-infected patients. Swiss HIV Cohort Study. AIDS 1999;13(9):1115-1122.

30. Clumeck N, Pozniak A, Raffi F. European AIDS Clinical Society (EACS) guidelines for the clinical management and treatment of HIV-infected adults. HIV Med 2008;9(2):65-71.

31. Wilkinson RA, Pincus SH, Shepard JB et al. Novel compounds containing multiple guanide groups that bind the HIV coreceptor CXCR4. Antimicrob Agents Chemother 2011;55(1):255-263.

32. Matthews T, Salgo M, Greenberg M, Chung J, DeMasi R, Bolognesi D. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat Rev Drug Discov 2004;3(3):215-225.

33. Lalezari JP, Henry K, O'Hearn M et al. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N Engl J Med 2003;348(22):2175-2185.

34. Lazzarin A, Clotet B, Cooper D et al. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N Engl J Med 2003;348(22):2186-2195.

35. Grinsztejn B, Nguyen BY, Katlama C et al. Safety and efficacy of the HIV-1 integrase inhibitor raltegravir (MK-0518) in treatment-experienced patients with multidrug-resistant virus: a phase II randomised controlled trial. Lancet 2007;369(9569):1261-1269.

36. Steigbigel RT, Cooper DA, Kumar PN et al. Raltegravir with optimized background therapy for resistant HIV-1 infection. N Engl J Med 2008;359(4):339-354.

37. Ghosh RK, Ghosh SM, Chawla S. Recent advances in antiretroviral drugs. Expert Opin Pharmacother 2011;12(1):31-46.

38. Kuller LH, Tracy R, Belloso W et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. PLoS Med 2008;5(10):e203.

39. Gras L, Kesselring AM, Griffin JT et al. CD4 cell counts of 800 cells/mm3 or greater after 7 years of highly active antiretroviral therapy are feasible in most patients starting with 350 cells/mm3 or greater. J Acquir Immune Defic Syndr 2007;45(2):183-192.

40. Kitahata MM, Gange SJ, Abraham AG et al. Effect of early versus deferred antiretroviral therapy for HIV on survival. N Engl J Med 2009;360(18):1815-1826.

41. Uy J, Armon C, Buchacz K, Wood K, Brooks JT. Initiation of HAART at higher CD4 cell counts is associated with a lower frequency of antiretroviral drug resistance mutations at virologic failure. J Acquir Immune Defic Syndr 2009;51(4):450-453.

42. Neuhaus J, Jacobs DR, Jr., Baker JV et al. Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection. J Infect Dis 2010;201(12):1788-1795.

43. Musicco M, Lazzarin A, Nicolosi A et al. Antiretroviral treatment of men infected with human immunodeficiency virus type 1 reduces the incidence of heterosexual transmission. Italian Study Group on HIV Heterosexual Transmission. Arch Intern Med 1994;154(17):1971-1976.

44. Castilla J, Del RJ, Hernando V, Marincovich B, Garcia S, Rodriguez C. Effectiveness of highly active antiretroviral therapy in reducing heterosexual transmission of HIV. J Acquir Immune Defic Syndr 2005;40(1):96-101.

45. Thompson MA, Aberg JA, Cahn P et al. Antiretroviral treatment of adult HIV infection: 2010 recommendations of the International AIDS Society-USA panel. JAMA 2010;304(3):321-333.

46. Davidson I, Beardsell H, Smith B et al. The frequency and reasons for antiretroviral switching with specific antiretroviral associations: the SWITCH study. Antiviral Res 2010;86(2):227-229.

47. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu Rev Microbiol 1997;51:151-178.

48. Harrigan PR, Bloor S, Larder BA. Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. J Virol 1998;72(5):3773-3778.

49. Rooke R, Tremblay M, Soudeyns H et al. Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. Canadian Zidovudine Multi-Centre Study Group. AIDS 1989;3(7):411-415.

50. Richman DD. Susceptibility to nucleoside analogues of zidovudine-resistant isolates of human immunodeficiency virus. Am J Med 1990;88(5B):8S-10S.

51. Tupinambas U, Aleixo A, Greco D. HIV-1 genotypes related to failure of nelfinavir as the first protease inhibitor treatment. Braz J Infect Dis 2005;9(4):324-329.

52. Johnson VA, Brun-Vezinet F, Clotet B et al. Update of the drug resistance mutations in HIV-1: December 2010. Top HIV Med 2010;18(5):156-163.

53. Paredes R, Clotet B. Clinical management of HIV-1 resistance. Antiviral Res 2010;85(1):245-265.

54. Schackman BR, Ribaudo HJ, Krambrink A, Hughes V, Kuritzkes DR, Gulick RM. Racial differences in virologic failure associated with adherence and quality of life on efavirenz-containing regimens for initial HIV therapy: results of ACTG A5095. J Acquir Immune Defic Syndr 2007;46(5):547-554.

55. Haas DW, Smeaton LM, Shafer RW et al. Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: an Adult Aids Clinical Trials Group Study. J Infect Dis 2005;192(11):1931-1942.

56. Elzi L, Marzolini C, Furrer H et al. Treatment modification in human immunodeficiency virus-infected individuals starting combination antiretroviral therapy between 2005 and 2008. Arch Intern Med 2010;170(1):57-65.

57. Rivero A, Mira JA, Pineda JA. Liver toxicity induced by non-nucleoside reverse transcriptase inhibitors. J Antimicrob Chemother 2007;59(3):342-346.

58. Hetherington S, McGuirk S, Powell G et al. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. Clin Ther 2001;23(10):1603-1614.

59. Mallal S, Nolan D, Witt C et al. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet 2002;359(9308):727-732.

60. Hughes DA, Vilar FJ, Ward CC, Alfirevic A, Park BK, Pirmohamed M. Cost-effectiveness analysis of HLA B*5701 genotyping in preventing abacavir hypersensitivity. Pharmacogenetics 2004;14(6):335-342.

61. Rauch A, Nolan D, Martin A, McKinnon E, Almeida C, Mallal S. Prospective genetic screening decreases the incidence of abacavir hypersensitivity reactions in the Western Australian HIV cohort study. Clin Infect Dis 2006;43(1):99-102.

62. Maagaard A, Kvale D. Long term adverse effects related to nucleoside reverse transcriptase inhibitors: clinical impact of mitochondrial toxicity. Scand J Infect Dis 2009;41(11-12):808-817.

63. Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. Clin Ther 2000;22(6):685-708.

64. Lee H, Hanes J, Johnson KA. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry 2003;42(50):14711-14719.

65. Moyle G. Toxicity of antiretroviral nucleoside and nucleotide analogues: is mitochondrial toxicity the only mechanism? Drug Saf 2000;23(6):467-481.

66. Carr A, Samaras K, Burton S et al. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. AIDS 1998;12(7):F51-F58.

67. Saint-Marc T, Partisani M, Poizot-Martin I et al. A syndrome of peripheral fat wasting (lipodystrophy) in patients receiving long-term nucleoside analogue therapy. AIDS 1999;13(13):1659-1667.

68. Mallal SA, John M, Moore CB, James IR, McKinnon EJ. Contribution of nucleoside analogue reverse transcriptase inhibitors to subcutaneous fat wasting in patients with HIV infection. AIDS 2000;14(10):1309-1316.

69. Carr A, Emery S, Law M, Puls R, Lundgren JD, Powderly WG. An objective case definition of lipodystrophy in HIV-infected adults: a case-control study. Lancet 2003;361(9359):726-735.

70. Haugaard SB. Toxic metabolic syndrome associated with HAART. Expert Opin Drug Metab Toxicol 2006;2(3):429-445.

71. van LF, Phanuphak P, Stroes E et al. Nevirapine and efavirenz elicit different changes in lipid profiles in antiretroviral-therapy-naive patients infected with HIV-1. PLoS Med 2004;1(1):e19.

72. Calza L, Manfredi R, Farneti B, Chiodo F. Incidence of hyperlipidaemia in a cohort of 212 HIV-infected patients receiving a protease inhibitor-based antiretroviral therapy. Int J Antimicrob Agents 2003;22(1):54-59.

73. Walli R, Herfort O, Michl GM et al. Treatment with protease inhibitors associated with peripheral insulin resistance and impaired oral glucose tolerance in HIV-1-infected patients. AIDS 1998;12(15):F167-F173.

74. Brambilla AM, Novati R, Calori G et al. Stavudine or indinavir-containing regimens are associated with an increased risk of diabetes mellitus in HIV-infected individuals. AIDS 2003;17(13):1993-1995.

75. Wand H, Calmy A, Carey DL et al. Metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus after initiation of antiretroviral therapy in HIV infection. AIDS 2007;21(18):2445-2453.

76. Lorenz MW, Stephan C, Harmjanz A et al. Both long-term HIV infection and highly active antiretroviral therapy are independent risk factors for early carotid atherosclerosis. Atherosclerosis 2008;196(2):720-726.

77. Sabin CA, Worm SW, Weber R et al. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients enrolled in the D:A:D study: a multi-cohort collaboration. Lancet 2008;371(9622):1417-1426.

78. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients. AIDS 2008;22(14):F17-F24.

79. Hsue PY, Hunt PW, Wu Y et al. Association of abacavir and impaired endothelial function in treated and suppressed HIV-infected patients. AIDS 2009;23(15):2021-2027.

80. Brothers CH, Hernandez JE, Cutrell AG et al. Risk of myocardial infarction and abacavir therapy: no increased risk across 52 GlaxoSmithKline-sponsored clinical trials in adult subjects. J Acquir Immune Defic Syndr 2009;51(1):20-28.

81. Justman JE, Benning L, Danoff A et al. Protease inhibitor use and the incidence of diabetes mellitus in a large cohort of HIV-infected women. J Acquir Immune Defic Syndr 2003;32(3):298-302.

82. Robertson KR, Smurzynski M, Parsons TD et al. The prevalence and incidence of neurocognitive impairment in the HAART era. AIDS 2007;21(14):1915-1921.

83. Smurzynski M, Wu K, Letendre S et al. Effects of central nervous system antiretroviral penetration on cognitive functioning in the ALLRT cohort. AIDS 2011;25(3):357-365.

84. Letendre SL, Ellis RJ, Ances BM, McCutchan JA. Neurologic complications of HIV disease and their treatment. Top HIV Med 2010;18(2):45-55.

85. Cespedes MS, Aberg JA. Neuropsychiatric complications of antiretroviral therapy. Drug Saf 2006;29(10):865-874.

86. Hawkins T, Geist C, Young B et al. Comparison of neuropsychiatric side effects in an observational cohort of efavirenz- and protease inhibitor-treated patients. HIV Clin Trials 2005;6(4):187-196.

87. Munoz-Moreno JA, Fumaz CR, Ferrer MJ et al. Neuropsychiatric symptoms associated with efavirenz: prevalence, correlates, and management. A neurobehavioral review. AIDS Rev 2009;11(2):103-109.

88. Spire B, Carrieri P, Garzot MA, L'henaff M, Obadia Y. Factors associated with efavirenz discontinuation in a large community-based sample of patients. AIDS Care 2004;16(5):558-564.

89. van LM, Bannister WP, Mocroft A et al. Absence of a relation between efavirenz plasma concentrations and toxicity-driven efavirenz discontinuations in the EuroSIDA study. Antivir Ther 2009;14(1):75-83.

90. Paton NI, Macallan DC, Griffin GE, Pazianas M. Bone mineral density in patients with human immunodeficiency virus infection. Calcif Tissue Int 1997;61(1):30-32.

91. Fernandez-Rivera J, Garcia R, Lozano F et al. Relationship between low bone mineral density and highly active antiretroviral therapy including protease inhibitors in HIV-infected patients. HIV Clin Trials 2003;4(5):337-346.

92. Grund B, Peng G, Gibert CL et al. Continuous antiretroviral therapy decreases bone mineral density. AIDS 2009;23(12):1519-1529.

93. Mora S, Sala N, Bricalli D, Zuin G, Chiumello G, Vigano A. Bone mineral loss through increased bone turnover in HIV-infected children treated with highly active antiretroviral therapy. AIDS 2001;15(14):1823-1829.

94. Brown TT, Qaqish RB. Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: a meta-analytic review. AIDS 2006;20(17):2165-2174.

95. Cazanave C, Dupon M, Lavignolle-Aurillac V et al. Reduced bone mineral density in HIV-infected patients: prevalence and associated factors. AIDS 2008;22(3):395-402.

96. Garcia Aparicio AM, Munoz FS, Gonzalez J et al. Abnormalities in the bone mineral metabolism in HIV-infected patients. Clin Rheumatol 2006;25(4):537-539.

97. Conesa-Botella A, Florence E, Lynen L, Colebunders R, Menten J, Moreno-Reyes R. Decrease of vitamin D concentration in patients with HIV infection on a non nucleoside reverse transcriptase inhibitor-containing regimen. AIDS Res Ther 2010;7:40.

98. Ellfolk M, Norlin M, Gyllensten K, Wikvall K. Regulation of human vitamin D(3) 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells. Mol Pharmacol 2009;75(6):1392-1399.

99. Cozzolino M, Vidal M, Arcidiacono MV, Tebas P, Yarasheski KE, Dusso AS. HIV-protease inhibitors impair vitamin D bioactivation to 1,25-dihydroxyvitamin D. AIDS 2003;17(4):513-520.

100. Mikulak J, Singhal PC. HIV-1 and kidney cells: better understanding of viral interaction. Nephron Exp Nephrol 2010;115(2):e15-e21.

101. El-Sadr WM, Lundgren JD, Neaton JD et al. CD4+ count-guided interruption of antiretroviral treatment. N Engl J Med 2006;355(22):2283-2296.

102. Kopp JB, Falloon J, Filie A et al. Indinavir-associated interstitial nephritis and urothelial inflammation: clinical and cytologic findings. Clin Infect Dis 2002;34(8):1122-1128.

103. Chan-Tack KM, Truffa MM, Struble KA, Birnkrant DB. Atazanavir-associated nephrolithiasis: cases from the US Food and Drug Administration's Adverse Event Reporting System. AIDS 2007;21(9):1215-1218.

104. Cooper RD, Wiebe N, Smith N, Keiser P, Naicker S, Tonelli M. Systematic review and meta-analysis: renal safety of tenofovir disoproxil fumarate in HIV-infected patients. Clin Infect Dis 2010;51(5):496-505.

105. Vidal F, Domingo JC, Guallar J et al. In vitro cytotoxicity and mitochondrial toxicity of tenofovir alone and in combination with other antiretrovirals in human renal proximal tubule cells. Antimicrob Agents Chemother 2006;50(11):3824-3832.

106. Thorne C, Newell ML. Safety of agents used to prevent mother-to-child transmission of HIV: is there any cause for concern? Drug Saf 2007;30(3):203-213.

107. Jackson JB, Musoke P, Fleming T et al. Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: 18-month follow-up of the HIVNET 012 randomised trial. Lancet 2003;362(9387):859-868.

108. Watts DH. Teratogenicity risk of antiretroviral therapy in pregnancy. Curr HIV /AIDS Rep 2007;4(3):135-140.

109. Taylor GP, O'Shea S, Mercey D, de RA. Zidovudine monotherapy in pregnancy: is it state of the art? HIV Med 2009;10(2):129-130.

110. Townsend CL, Willey BA, Cortina-Borja M, Peckham CS, Tookey PA. Antiretroviral therapy and congenital abnormalities in infants born to HIV-infected women in the UK and Ireland, 1990-2007. AIDS 2009;23(4):519-524.

111. Thorne C, Newell ML. Safety of agents used to prevent mother-to-child transmission of HIV: is there any cause for concern? Drug Saf 2007;30(3):203-213.

112. Mofenson LM. Prevention in neglected subpopulations: prevention of mother-to-child transmission of HIV infection. Clin Infect Dis 2010;50 Suppl 3:S130-S148.

113. Kakuda TN, Williams LA, Hsu AF. The 2nd International Workshop on Clinical Pharmacology of HIV Therapy. April 2nd-4th 2001, Noordwijk, The Netherlands. Expert Opin Pharmacother 2001;2(8):1339-1345.

114. Back DJ, Khoo SH, Gibbons SE, Merry C. The role of therapeutic drug monitoring in treatment of HIV infection. Br J Clin Pharmacol 2001;51(4):301-308.

115. Merry C, Barry MG, Mulcahy F et al. Saquinavir pharmacokinetics alone and in combination with ritonavir in HIV-infected patients. AIDS 1997;11(4):F29-F33.

116. Isaac A, Taylor S, Cane P et al. Lopinavir/ritonavir combined with twice-daily 400 mg indinavir: pharmacokinetics and pharmacodynamics in blood, CSF and semen. J Antimicrob Chemother 2004;54(2):498-502.

117. Seminari E, Maggiolo F, Villani P et al. Efavirenz, nelfinavir, and stavudine rescue combination therapy in HIV-1-positive patients heavily pretreated with nucleoside analogues and protease inhibitors. J Acquir Immune Defic Syndr 1999;22(5):453-460.

118. Castro A. Adherence to antiretroviral therapy: merging the clinical and social course of AIDS. PLoS Med 2005;2(12):e338.

119. Yuen GJ, Lou Y, Thompson NF et al. Abacavir/lamivudine/zidovudine as a combined formulation tablet: bioequivalence compared with each component administered concurrently and the effect of food on absorption. J Clin Pharmacol 2001;41(3):277-288.

120. Stohr W, Back D, Dunn D et al. Factors influencing efavirenz and nevirapine plasma concentration: effect of ethnicity, weight and co-medication. Antivir Ther 2008;13(5):675-685.

121. Stohr W, Back D, Dunn D et al. Factors influencing lopinavir and atazanavir plasma concentration. J Antimicrob Chemother 2010;65(1):129-137.

122. Moyle G, Boffito M, Fletcher C et al. Steady-state pharmacokinetics of abacavir in plasma and intracellular carbovir triphosphate following administration of abacavir at 600 milligrams once daily and 300 milligrams twice daily in human immunodeficiency virus-infected subjects. Antimicrob Agents Chemother 2009;53(4):1532-1538.

123. Chesney MA. Factors affecting adherence to antiretroviral therapy. Clin Infect Dis 2000;30 Suppl 2:S171-S176.

124. Pence BW. The impact of mental health and traumatic life experiences on antiretroviral treatment outcomes for people living with HIV/AIDS. J Antimicrob Chemother 2009;63(4):636-640.

125. Boffito M, Back DJ, Blaschke TF et al. Protein binding in antiretroviral therapies. AIDS Res Hum Retroviruses 2003;19(9):825-835.

126. Eagling VA, Back DJ, Barry MG. Differential inhibition of cytochrome P450 isoforms by the protease inhibitors, ritonavir, saquinavir and indinavir. Br J Clin Pharmacol 1997;44(2):190-194.

127. Granfors MT, Wang JS, Kajosaari LI, Laitila J, Neuvonen PJ, Backman JT. Differential inhibition of cytochrome P450 3A4, 3A5 and 3A7 by five human immunodeficiency virus (HIV) protease inhibitors in vitro. Basic Clin Pharmacol Toxicol 2006;98(1):79-85.

128. Hesse LM, von Moltke LL, Shader RI, Greenblatt DJ. Ritonavir, efavirenz, and nelfinavir inhibit CYP2B6 activity in vitro: potential drug interactions with bupropion. Drug Metab Dispos 2001;29(2):100-102.

129. Walsky RL, Astuccio AV, Obach RS. Evaluation of 227 drugs for in vitro inhibition of cytochrome P450 2B6. J Clin Pharmacol 2006;46(12):1426-1438.

130. Hsu A, Granneman GR, Bertz RJ. Ritonavir. Clinical pharmacokinetics and interactions with other anti-HIV agents. Clin Pharmacokinet 1998;35(4):275-291.

131. Wrighton SA, Schuetz EG, Thummel KE, Shen DD, Korzekwa KR, Watkins PB. The human CYP3A subfamily: practical considerations. Drug Metab Rev 2000;32(3-4):339-361.

132. von RO, Burk O, Fromm MF, Thon KP, Eichelbaum M, Kivisto KT. Cytochrome P450 3A4 and P-glycoprotein expression in human small intestinal enterocytes and hepatocytes: a comparative analysis in paired tissue specimens. Clin Pharmacol Ther 2004;75(3):172-183.

133. Kempf DJ, Marsh KC, Kumar G et al. Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. Antimicrob Agents Chemother 1997;41(3):654-660.

134. Zeldin RK, Petruschke RA. Pharmacological and therapeutic properties of ritonavir-boosted protease inhibitor therapy in HIV-infected patients. J Antimicrob Chemother 2004;53(1):4-9.

135. Busti AJ, Hall RG, Margolis DM. Atazanavir for the treatment of human immunodeficiency virus infection. Pharmacotherapy 2004;24(12):1732-1747.

136. Decker CJ, Laitinen LM, Bridson GW, Raybuck SA, Tung RD, Chaturvedi PR. Metabolism of amprenavir in liver microsomes: role of CYP3A4 inhibition for drug interactions. J Pharm Sci 1998;87(7):803-807.

137. Srinivas RV, Middlemas D, Flynn P, Fridland A. Human immunodeficiency virus protease inhibitors serve as substrates for multidrug transporter proteins MDR1 and MRP1 but retain antiviral efficacy in cell lines expressing these transporters. Antimicrob Agents Chemother 1998;42(12):3157-3162.

138. Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J Histochem Cytochem 1990;38(9):1277-1287.

139. Ye ZW, Camus S, Augustijns P, Annaert P. Interaction of eight HIV protease inhibitors with the canalicular efflux transporter ABCC2 (MRP2) in sandwich-cultured rat and human hepatocytes. Biopharm Drug Dispos 2010;31(2-3):178-188.

140. Kis O, Zastre JA, Ramaswamy M, Bendayan R. pH dependence of organic anion-transporting polypeptide 2B1 in Caco-2 cells: potential role in antiretroviral drug oral bioavailability and drug-drug interactions. J Pharmacol Exp Ther 2010;334(3):1009-1022.

141. Dussault I, Lin M, Hollister K, Wang EH, Synold TW, Forman BM. Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. J Biol Chem 2001;276(36):33309-33312.

142. 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(6):1329-1339.

143. 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(3):427-431.

144. Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001;276(18):14581-14587.

145. Kast HR, Goodwin B, Tarr PT et al. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 2002;277(4):2908-2915.

146. Gupta A, Mugundu GM, Desai PB, Thummel KE, Unadkat JD. Intestinal human colon adenocarcinoma cell line LS180 is an excellent model to study pregnane X receptor, but not constitutive androstane receptor, mediated CYP3A4 and multidrug resistance transporter 1 induction: studies with anti-human immunodeficiency virus protease inhibitors. Drug Metab Dispos 2008;36(6):1172-1180.

147. Hariparsad N, Nallani SC, Sane RS, Buckley DJ, Buckley AR, Desai PB. Induction of CYP3A4 by efavirenz in primary human hepatocytes: comparison with rifampin and phenobarbital. J Clin Pharmacol 2004;44(11):1273-1281.

148. Rendic S. Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev 2002;34(1-2):83-448.

149. Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. J Pharmacol Exp Ther 2003;306(1):287-300.

150. Lee CG, Gottesman MM, Cardarelli CO et al. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. Biochemistry 1998;37(11):3594-3601.

151. Yu L, Bridgers A, Polli J et al. Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. Pharm Res 1999;16(12):1812-1817.

152. Agarwal S, Pal D, Mitra AK. Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor. Int J Pharm 2007;339(1-2):139-147.

153. Shaik N, Giri N, Pan G, Elmquist WF. P-glycoprotein-mediated active efflux of the anti-HIV1 nucleoside abacavir limits cellular accumulation and brain distribution. Drug Metab Dispos 2007;35(11):2076-2085.

154. Walker DK, Abel S, Comby P, Muirhead GJ, Nedderman AN, Smith DA. Species differences in the disposition of the CCR5 antagonist, UK-427,857, a new potential treatment for HIV. Drug Metab Dispos 2005;33(4):587-595.

155. Zembruski NC, Buchel G, Jodicke L, Herzog M, Haefeli WE, Weiss J. Potential of novel antiretrovirals to modulate expression and function of drug transporters in vitro. J Antimicrob Chemother 2011;66(4):802-812.

156. Marzolini C, Elzi L, Gibbons S et al. Prevalence of comedications and effect of potential drug-drug interactions in the Swiss HIV Cohort Study. Antivir Ther 2010;15(3):413-423.

157. Clarke SM, Mulcahy FM, Tjia J et al. The pharmacokinetics of methadone in HIV-positive patients receiving the non-nucleoside reverse transcriptase inhibitor efavirenz. Br J Clin Pharmacol 2001;51(3):213-217.

158. Kigen G, Kimaiyo S, Nyandiko W et al. Prevalence of potential drug-drug interactions involving antiretroviral drugs in a large Kenyan cohort. PLoS One 2011;6(2):e16800.

159. Aberg J, Powderly W. HIV: primary and secondary prophylaxis for opportunistic infections. Clin Evid (Online) 2010;2010.

160. Chu J, Sloan CE, Freedberg KA, Yazdanpanah Y, Losina E. Drug efficacy by direct and adjusted indirect comparison to placebo: An illustration by Mycobacterium avium Complex prophylaxis in HIV. AIDS Res Ther 2011;8(1):14.

161. Pienaar ED, Young T, Holmes H. Interventions for the prevention and management of oropharyngeal candidiasis associated with HIV infection in adults and children. Cochrane Database Syst Rev 2010;(11):CD003940.

162. Gillum JG, Israel DS, Polk RE. Pharmacokinetic drug interactions with antimicrobial agents. Clin Pharmacokinet 1993;25(6):450-482.

163. Khan FA, Minion J, Pai M et al. Treatment of active tuberculosis in HIV-coinfected patients: a systematic review and meta-analysis. Clin Infect Dis 2010;50(9):1288-1299.

164. Bertilsson G, Heidrich J, Svensson K et al. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. Proc Natl Acad Sci U S A 1998;95(21):12208-12213.

165. Takara K, Tsujimoto M, Kokufu M, Ohnishi N, Yokoyama T. Up-regulation of MDR1 function and expression by cisplatin in LLC-PK1 cells. Biol Pharm Bull 2003;26(2):205-209.

166. Blazy A, Hennequin C, Gornet JM et al. Anal carcinomas in HIV-positive patients: high-dose chemoradiotherapy is feasible in the era of highly active antiretroviral therapy. Dis Colon Rectum 2005;48(6):1176-1181.

167. Bi J, Espina BM, Tulpule A, Boswell W, Levine AM. High-dose cytosine-arabinoside and cisplatin regimens as salvage therapy for refractory or relapsed AIDS-related non-Hodgkin's lymphoma. J Acquir Immune Defic Syndr 2001;28(5):416-421.

168. Moore LB, Goodwin B, Jones SA et al. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. Proc Natl Acad Sci U S A 2000;97(13):7500-7502.

169. Hennessy M, Kelleher D, Spiers JP et al. St Johns wort increases expression of P-glycoprotein: implications for drug interactions. Br J Clin Pharmacol 2002;53(1):75-82.

170. Mills E, Foster BC, van HR et al. Impact of African herbal medicines on antiretroviral metabolism. AIDS 2005;19(1):95-97.

171. Monera TG, Wolfe AR, Maponga CC, Benet LZ, Guglielmo J. Moringa oleifera leaf extracts inhibit 6beta-hydroxylation of testosterone by CYP3A4. J Infect Dev Ctries 2008;2(5):379-383.

172. van den Bout-van den Beukel CJ, Hamza OJ, Moshi MJ et al. Evaluation of cytotoxic, genotoxic and CYP450 enzymatic competition effects of Tanzanian plant extracts traditionally used for treatment of fungal infections. Basic Clin Pharmacol Toxicol 2008;102(6):515-526.

173. Ito M, Nakashima H, Baba M et al. Inhibitory effect of glycyrrhizin on the in vitro infectivity and cytopathic activity of the human immunodeficiency virus [HIV (HTLV-III/LAV)]. Antiviral Res 1987;7(3):127-137.

174. Mu Y, Zhang J, Zhang S et al. Traditional Chinese medicines Wu Wei Zi (Schisandra chinensis Baill) and Gan Cao (Glycyrrhiza uralensis Fisch) activate pregnane X receptor and increase warfarin clearance in rats. J Pharmacol Exp Ther 2006;316(3):1369-1377.

175. nantawat W, Phonrat B, Dhitavat J et al. Safety and efficacy of CKBM-A01, a Chinese herbal medicine, among asymptomatic HIV patients. Southeast Asian J Trop Med Public Health 2009;40(3):494-501.

176. van Sighem AI, Gras LA, Reiss P, Brinkman K, de WF. Life expectancy of recently diagnosed asymptomatic HIV-infected patients approaches that of uninfected individuals. AIDS 2010;24(10):1527-1535.

177. Orlando G, Meraviglia P, Cordier L et al. Antiretroviral treatment and age-related comorbidities in a cohort of older HIV-infected patients. HIV Med 2006;7(8):549-557.

178. Shah SS, McGowan JP, Smith C, Blum S, Klein RS. Comorbid conditions, treatment, and health maintenance in older persons with human immunodeficiency virus infection in New York City. Clin Infect Dis 2002;35(10):1238-1243.

179. Molto J, Blanco A, Miranda C et al. Variability in non-nucleoside reverse transcriptase and protease inhibitors concentrations among HIV-infected adults in routine clinical practice. Br J Clin Pharmacol 2007;63(6):715-721.

180. Fabbiani M, Di GS, Bracciale L et al. Pharmacokinetic variability of antiretroviral drugs and correlation with virological outcome: 2 years of experience in routine clinical practice. J Antimicrob Chemother 2009;64(1):109-117.

181. Jones AE, Brown KC, Werner RE et al. Variability in drug metabolizing enzyme activity in HIV-infected patients. Eur J Clin Pharmacol 2010;66(5):475-485.

182. Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. Pharmacol Ther 2007;116(3):496-526.

183. Rodriguez-Antona C, Sayi JG, Gustafsson LL, Bertilsson L, Ingelman-Sundberg M. Phenotype-genotype variability in the human CYP3A locus as assessed by the probe drug quinine and analyses of variant CYP3A4 alleles. Biochem Biophys Res Commun 2005;338(1):299-305.

184. Bertrand J, Treluyer JM, Panhard X et al. Influence of pharmacogenetics on indinavir disposition and short-term response in HIV patients initiating HAART. Eur J Clin Pharmacol 2009;65(7):667-678.

185. Arab-Alameddine M, Di IJ, Buclin T et al. Pharmacogenetics-based population pharmacokinetic analysis of efavirenz in HIV-1-infected individuals. Clin Pharmacol Ther 2009;85(5):485-494.

186. Fellay J, Marzolini C, Meaden ER et al. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet 2002;359(9300):30-36.

187. Motsinger AA, Ritchie MD, Shafer RW et al. Multilocus genetic interactions and response to efavirenz-containing regimens: an adult AIDS clinical trials group study. Pharmacogenet Genomics 2006;16(11):837-845.

188. Dai D, Tang J, Rose R et al. Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. J Pharmacol Exp Ther 2001;299(3):825-831.

189. Tsuchiya K, Gatanaga H, Tachikawa N et al. Homozygous CYP2B6 *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens. Biochem Biophys Res Commun 2004;319(4):1322-1326.

190. Lamba V, Lamba J, Yasuda K et al. Hepatic CYP2B6 expression: gender and ethnic differences and relationship to CYP2B6 genotype and CAR (constitutive androstane receptor) expression. J Pharmacol Exp Ther 2003;307(3):906-922.

191. Rotger M, Tegude H, Colombo S et al. Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. Clin Pharmacol Ther 2007;81(4):557-566.

192. Mahungu T, Smith C, Turner F et al. Cytochrome P450 2B6 516G-->T is associated with plasma concentrations of nevirapine at both 200 mg twice daily and 400 mg once daily in an ethnically diverse population. HIV Med 2009;10(5):310-317.

193. Ramachandran G, Ramesh K, Hemanth Kumar AK et al. Association of high T allele frequency of CYP2B6 G516T polymorphism among ethnic south Indian HIV-infected patients with elevated plasma efavirenz and nevirapine. J Antimicrob Chemother 2009;63(4):841-843.

194. Haas DW, Ribaudo HJ, Kim RB et al. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS 2004;18(18):2391-2400.

195. Hasse B, Gunthard HF, Bleiber G, Krause M. Efavirenz intoxication due to slow hepatic metabolism. Clin Infect Dis 2005;40(3):e22-e23.

196. Hustert E, Haberl M, Burk O et al. The genetic determinants of the CYP3A5 polymorphism. Pharmacogenetics 2001;11(9):773-779.

197. Anderson PL, Aquilante CL, Gardner EM et al. Atazanavir pharmacokinetics in genetically determined CYP3A5 expressors versus non-expressors. J Antimicrob Chemother 2009;64(5):1071-1079.

198. Anderson PL, Lamba J, Aquilante CL, Schuetz E, Fletcher CV. Pharmacogenetic characteristics of indinavir, zidovudine, and lamivudine therapy in HIV-infected adults: a pilot study. J Acquir Immune Defic Syndr 2006;42(4):441-449.

199. Josephson F, Allqvist A, Janabi M et al. CYP3A5 genotype has an impact on the metabolism of the HIV protease inhibitor saquinavir. Clin Pharmacol Ther 2007;81(5):708-712.

200. Hartkoorn RC, Kwan WS, Shallcross V et al. HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms. Pharmacogenet Genomics 2010;20(2):112-120.

201. Langmann T, Mauerer R, Zahn A et al. Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. Clin Chem 2003;49(2):230-238.

202. Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. Br J Pharmacol 2009;158(3):693-705.

203. Hennessy M, Clarke S, Spiers JP et al. Intracellular accumulation of nelfinavir and its relationship to P-glycoprotein expression and function in HIV-infected patients. Antivir Ther 2004;9(1):115-122.

204. Giraud C, Manceau S, Treluyer JM. ABC transporters in human lymphocytes: expression, activity and role, modulating factors and consequences for antiretroviral therapies. Expert Opin Drug Metab Toxicol 2010;6(5):571-589.

205. Wang D, Johnson AD, Papp AC, Kroetz DL, Sadee W. Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability. Pharmacogenet Genomics 2005;15(10):693-704.

206. Ma Q, Brazeau D, Zingman BS et al. Multidrug resistance 1 polymorphisms and trough concentrations of atazanavir and lopinavir in patients with HIV. Pharmacogenomics 2007;8(3):227-235.

207. Verstuyft C, Marcellin F, Morand-Joubert L et al. Absence of association between MDR1 genetic polymorphisms, indinavir pharmacokinetics and response to highly active antiretroviral therapy. AIDS 2005;19(18):2127-2131.

208. Winzer R, Langmann P, Zilly M et al. No influence of the P-glycoprotein genotype (MDR1 C3435T) on plasma levels of lopinavir and efavirenz during antiretroviral treatment. Eur J Med Res 2003;8(12):531-534.

209. Haas DW, Wu H, Li H et al. MDR1 gene polymorphisms and phase 1 viral decay during HIV-1 infection: an adult AIDS Clinical Trials Group study. J Acquir Immune Defic Syndr 2003;34(3):295-298.

210. la Porte CJ, Li Y, Beique L et al. The effect of ABCB1 polymorphism on the pharmacokinetics of saquinavir alone and in combination with ritonavir. Clin Pharmacol Ther 2007;82(4):389-395.

211. Janneh O, Chandler B, Hartkoorn R et al. Intracellular accumulation of efavirenz and nevirapine is independent of P-glycoprotein activity in cultured CD4 T cells and primary human lymphocytes. J Antimicrob Chemother 2009;64(5):1002-1007.

212. Dirson G, Fernandez C, Hindlet P et al. Efavirenz does not interact with the ABCB1 transporter at the blood-brain barrier. Pharm Res 2006;23(7):1525-1532.

213. Solas C, Simon N, Drogoul MP et al. Minimal effect of MDR1 and CYP3A5 genetic polymorphisms on the pharmacokinetics of indinavir in HIV-infected patients. Br J Clin Pharmacol 2007;64(3):353-362.

214. Paintsil E, Dutschman GE, Hu R et al. Determinants of Individual Variation in Intracellular Accumulation of Anti-HIV Nucleoside Analog Metabolites. Antimicrob Agents Chemother 2011;55(2):895-903.

215. Gifford AL, Cunningham WE, Heslin KC et al. Participation in research and access to experimental treatments by HIV-infected patients. N Engl J Med 2002;346(18):1373-1382.

216. Gwadz MV, Colon P, Ritchie AS et al. Increasing and supporting the participation of persons of color living with HIV/AIDS in AIDS clinical trials. Curr HIV /AIDS Rep 2010;7(4):194-200.

217. Siccardi M, D'Avolio A, Baietto L et al. 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):1222-1225.

218. Schipani A, Siccardi M, D'Avolio A et al. Population pharmacokinetic modeling of the association between 63396C->T pregnane X receptor polymorphism and unboosted atazanavir clearance. Antimicrob Agents Chemother 2010;54(12):5242-5250.

219. Lamba V, Panetta JC, Strom S, Schuetz EG. Genetic predictors of interindividual variability in hepatic CYP3A4 expression. J Pharmacol Exp Ther 2010;332(3):1088-1099.

220. Evans RM. The nuclear receptor superfamily: a rosetta stone for physiology. Mol Endocrinol 2005;19(6):1429-1438.

221. Jensen EV. Estrogen receptor: ambiguities in the use of this term. Science 1968;159(820):1261.

222. Payvar F, Wrange O, Carlstedt-Duke J, Okret S, Gustafsson JA, Yamamoto KR. Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo. Proc Natl Acad Sci U S A 1981;78(11):6628-6632.

223. Hollenberg SM, Weinberger C, Ong ES et al. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985;318(6047):635-641.

224. Lander ES, Linton LM, Birren B et al. Initial sequencing and analysis of the human genome. Nature 2001;409(6822):860-921.

225. Venter JC, Adams MD, Myers EW et al. The sequence of the human genome. Science 2001;291(5507):1304-1351.

226. Willson TM, Moore JT. Genomics versus orphan nuclear receptors--a half-time report. Mol Endocrinol 2002;16(6):1135-1144.

227. A unified nomenclature system for the nuclear receptor superfamily. Cell 1999;97(2):161-163.

228. Nebert DW, Adesnik M, Coon MJ et al. The P450 gene superfamily: recommended nomenclature. DNA 1987;6(1):1-11.

229. Nagy L, Schwabe JW. Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci 2004;29(6):317-324.

230. Mangelsdorf DJ, Thummel C, Beato M et al. The nuclear receptor superfamily: the second decade. Cell 1995;83(6):835-839.

231. Oro AE, McKeown M, Evans RM. Relationship between the product of the Drosophila ultraspiracle locus and the vertebrate retinoid X receptor. Nature 1990;347(6290):298-301.

232. Krasowski MD, Ni A, Hagey LR, Ekins S. Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR. Mol Cell Endocrinol 2010.

233. Zhang Z, Burch PE, Cooney AJ et al. Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. Genome Res 2004;14(4):580-590.

234. Moore LB, Maglich JM, McKee DD et al. Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. Mol Endocrinol 2002;16(5):977-986.

235. di MA, De ME, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. Mol Aspects Med 2009;30(5):297-343.

236. Jones SA, Moore LB, Shenk JL et al. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. Mol Endocrinol 2000;14(1):27-39.

237. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. Physiol Rev 2001;81(3):1269-1304.

238. Leo C, Chen JD. The SRC family of nuclear receptor coactivators. Gene 2000;245(1):1-11.

239. Umesono K, Evans RM. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 1989;57(7):1139-1146.

240. Forman BM, Evans RM. Nuclear hormone receptors activate direct, inverted, and everted repeats. Ann N Y Acad Sci 1995;761:29-37.

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

242. Rastinejad F, Perlmann T, Evans RM, Sigler PB. Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature 1995;375(6528):203-211.

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

244. Liu YY, Brent GA. Thyroid hormone crosstalk with nuclear receptor signaling in metabolic regulation. Trends Endocrinol Metab 2010;21(3):166-173.

245. Makishima M, Lu TT, Xie W et al. Vitamin D receptor as an intestinal bile acid sensor. Science 2002;296(5571):1313-1316.

246. Shulman AI, Mangelsdorf DJ. Retinoid x receptor heterodimers in the metabolic syndrome. N Engl J Med 2005;353(6):604-615.

247. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 1990;345(6272):224-229.

248. Laudet V, Hanni C, Coll J, Catzeflis F, Stehelin D. Evolution of the nuclear receptor gene superfamily. EMBO J 1992;11(3):1003-1013.

249. Leid M, Kastner P, Lyons R et al. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 1992;68(2):377-395.

250. Mader S, Chen JY, Chen Z, White J, Chambon P, Gronemeyer H. The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificites of the DNA binding domains. EMBO J 1993;12(13):5029-5041.

251. Mangelsdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES, Evans RM. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell 1991;66(3):555-561.

252. Chen JD, Umesono K, Evans RM. SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. Proc Natl Acad Sci U S A 1996;93(15):7567-7571.

253. Moore DD, Kato S, Xie W et al. International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. Pharmacol Rev 2006;58(4):742-759.

254. Kalaany NY, Mangelsdorf DJ. LXRS and FXR: the yin and yang of cholesterol and fat metabolism. Annu Rev Physiol 2006;68:159-191.

255. Lu TT, Repa JJ, Mangelsdorf DJ. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. J Biol Chem 2001;276(41):37735-37738.

256. Peet DJ, Turley SD, Ma W et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell 1998;93(5):693-704.

257. Shearer BG, Billin AN. The next generation of PPAR drugs: do we have the tools to find them? Biochim Biophys Acta 2007;1771(8):1082-1093.

258. Wilson TE, Fahrner TJ, Milbrandt J. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol Cell Biol 1993;13(9):5794-5804.

259. Kliewer SA, Moore JT, Wade L et al. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 1998;92(1):73-82.

260. Zhou C, Verma S, Blumberg B. The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. Nucl Recept Signal 2009;7:e001.

261. Watkins RE, Wisely GB, Moore LB et al. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 2001;292(5525):2329-2333.

262. Gillam EM. The PXR ligand-binding domain: how to be picky and promiscuous at the same time. Trends Pharmacol Sci 2001;22(9):448.

263. 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(5):1016-1023.

264. Pascussi JM, Gerbal-Chaloin S, Duret C, Daujat-Chavanieu M, Vilarem MJ, Maurel P. The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. Annu Rev Pharmacol Toxicol 2008;48:1-32.

265. Xie W, Evans RM. Orphan nuclear receptors: the exotics of xenobiotics. J Biol Chem 2001;276(41):37739-37742.

266. Li AP, Rasmussen A, Xu L, Kaminski DL. Rifampicin induction of lidocaine metabolism in cultured human hepatocytes. J Pharmacol Exp Ther 1995;274(2):673-677.

267. Crawford P, Chadwick DJ, Martin C, Tjia J, Back DJ, Orme M. The interaction of phenytoin and carbamazepine with combined oral contraceptive steroids. Br J Clin Pharmacol 1990;30(6):892-896.

268. Ogg MS, Gray TJ, Gibson GG. Development of an in vitro reporter gene assay to assess xenobiotic induction of the human CYP3A4 gene. Eur J Drug Metab Pharmacokinet 1997;22(4):311-313.

269. Barry M, Mulcahy F, Merry C, Gibbons S, Back D. Pharmacokinetics and potential interactions amongst antiretroviral agents used to treat patients with HIV infection. Clin Pharmacokinet 1999;36(4):289-304.

270. Madan A, Graham RA, Carroll KM et al. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. Drug Metab Dispos 2003;31(4):421-431.

271. Gervot L, Rochat B, Gautier JC et al. Human CYP2B6: expression, inducibility and catalytic activities. Pharmacogenetics 1999;9(3):295-306.

272. Halwachs S, Schafer I, Seibel P, Honscha W. Antiepileptic drugs reduce efficacy of methotrexate chemotherapy by downregulation of Reduced folate carrier transport activity. Leukemia 2009;23(6):1087-1097.

273. Xie W, Uppal H, Saini SP et al. Orphan nuclear receptor-mediated xenobiotic regulation in drug metabolism. Drug Discov Today 2004;9(10):442-449.

274. Edwards PA, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. J Lipid Res 2002;43(1):2-12.

275. Nies AT, Keppler D. The apical conjugate efflux pump ABCC2 (MRP2). Pflugers Arch 2007;453(5):643-659.

276. Meyer zu Schwabedissen HE, Tirona RG, Yip CS, Ho RH, Kim RB. Interplay between the nuclear receptor pregnane X receptor and the uptake transporter organic anion transporter polypeptide 1A2 selectively enhances estrogen effects in breast cancer. Cancer Res 2008;68(22):9338-9347.

277. Janneh O, Jones E, Chandler B, Owen A, Khoo SH. Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4 T cells and primary human lymphocytes. J Antimicrob Chemother 2007;60(5):987-993.

278. Bousquet L, Pruvost A, Didier N, Farinotti R, Mabondzo A. Emtricitabine: Inhibitor and substrate of multidrug resistance associated protein. Eur J Pharm Sci 2008;35(4):247-256.

279. Zhang J, Huang W, Qatanani M, Evans RM, Moore DD. The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. J Biol Chem 2004;279(47):49517-49522.

280. Xie W, Yeuh MF, Radominska-Pandya A et al. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proc Natl Acad Sci U S A 2003;100(7):4150-4155.

281. Zhou J, Zhai Y, Mu Y et al. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. J Biol Chem 2006;281(21):15013-15020.

282. Savill J, Hogg N, Ren Y, Haslett C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J Clin Invest 1992;90(4):1513-1522.

283. Feng J, Han J, Pearce SF et al. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. J Lipid Res 2000;41(5):688-696.

284. Zhou C, Tabb MM, Nelson EL et al. Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. J Clin Invest 2006;116(8):2280-2289.

285. Pascussi JM, Gerbal-Chaloin S, Pichard-Garcia L et al. Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. Biochem Biophys Res Commun 2000;274(3):707-713.

286. Dring MM, Goulding CA, Trimble VI et al. The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. Gastroenterology 2006;130(2):341-348.

287. Zhai Y, Pai HV, Zhou J, Amico JA, Vollmer RR, Xie W. Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. Mol Endocrinol 2007;21(1):138-147.

288. El-Sankary W, Plant NJ, Gibson GG, Moore DJ. Regulation of the CYP3A4 gene by hydrocortisone and xenobiotics: role of the glucocorticoid and pregnane X receptors. Drug Metab Dispos 2000;28(5):493-496.

289. Pascussi JM, Robert A, Nguyen M et al. Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia. J Clin Invest 2005;115(1):177-186.

290. Konno Y, Kodama S, Moore R, Kamiya N, Negishi M. Nuclear xenobiotic receptor pregnane X receptor locks corepressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) onto the CYP24A1 promoter to attenuate vitamin D3 activation. Mol Pharmacol 2009;75(2):265-271.

291. Igarashi M, Yogiashi Y, Mihara M, Takada I, Kitagawa H, Kato S. Vitamin K induces osteoblast differentiation through pregnane X receptor-mediated transcriptional control of the Msx2 gene. Mol Cell Biol 2007;27(22):7947-7954.

292. Zhang B, Xie W, Krasowski MD. PXR: a xenobiotic receptor of diverse function implicated in pharmacogenetics. Pharmacogenomics 2008;9(11):1695-1709.

293. Zhang J, Kuehl P, Green ED et al. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. Pharmacogenetics 2001;11(7):555-572.

294. Kuehl P, Zhang J, Lin Y et al. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nat Genet 2001;27(4):383-391.

295. Lown KS, Mayo RR, Leichtman AB et al. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. Clin Pharmacol Ther 1997;62(3):248-260.

296. Hustert E, Zibat A, Presecan-Siedel E et al. Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. Drug Metab Dispos 2001;29(11):1454-1459.

297. 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):169-181.

298. Gorny M, Rohm S, Laer S, Morali N, Niehues T. Pharmacogenomic adaptation of antiretroviral therapy: overcoming the failure of lopinavir in an African infant with CYP2D6 ultrarapid metabolism. Eur J Clin Pharmacol 2010;66(1):107-108.

299. Tedaldi EM, Absalon J, Thomas AJ, Shlay JC, Berg-Wolf M. Ethnicity, race, and gender. Differences in serious adverse events among participants in an antiretroviral initiation trial: results of CPCRA 058 (FIRST Study). J Acquir Immune Defic Syndr 2008;47(4):441-448.

300. Luo G, Cunningham M, Kim S et al. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. Drug Metab Dispos 2002;30(7):795-804.

301. Geyeregger R, Zeyda M, Stulnig TM. Liver X receptors in cardiovascular and metabolic disease. Cell Mol Life Sci 2006;63(5):524-539.

302. Carcamo-Orive I, Gaztelumendi A, Delgado J et al. Regulation of human bone marrow stromal cell proliferation and differentiation capacity by glucocorticoid receptor and AP-1 crosstalk. J Bone Miner Res 2010;25(10):2115-2125.

303. Okazaki R, Inoue D, Shibata M et al. Estrogen promotes early osteoblast differentiation and inhibits adipocyte differentiation in mouse bone marrow stromal cell lines that express estrogen receptor (ER) alpha or beta. Endocrinology 2002;143(6):2349-2356.

304. Commerford SR, Vargas L, Dorfman SE et al. Dissection of the insulin-sensitizing effect of liver X receptor ligands. Mol Endocrinol 2007;21(12):3002-3012.

305. Fantus IG, Ryan J, Hizuka N, Gorden P. The effect of glucocorticoids on the insulin receptor: an in vivo and in vitro study. J Clin Endocrinol Metab 1981;52(5):953-960.

306. Kanaya AM, Herrington D, Vittinghoff E et al. Glycemic effects of postmenopausal hormone therapy: the Heart and Estrogen/progestin Replacement Study. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 2003;138(1):1-9.

307. Margolis KL, Bonds DE, Rodabough RJ et al. Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. Diabetologia 2004;47(7):1175-1187.

308. Dahlman-Wright K, Cavailles V, Fuqua SA et al. International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev 2006;58(4):773-781.

309. Vaya J, Schipper HM. Oxysterols, cholesterol homeostasis, and Alzheimer disease. J Neurochem 2007;102(6):1727-1737.

310. Wolkowitz OM, Burke H, Epel ES, Reus VI. Glucocorticoids. Mood, memory, and mechanisms. Ann N Y Acad Sci 2009;1179:19-40.

311. Wang L, Schuster GU, Hultenby K, Zhang Q, Andersson S, Gustafsson JA. Liver X receptors in the central nervous system: from lipid homeostasis to neuronal degeneration. Proc Natl Acad Sci U S A 2002;99(21):13878-13883.

312. Kim WS, Chan SL, Hill AF, Guillemin GJ, Garner B. Impact of 27-hydroxycholesterol on amyloid-beta peptide production and ATP-binding cassette transporter expression in primary human neurons. J Alzheimers Dis 2009;16(1):121-131.

313. Koldamova RP, Lefterov IM, Staufenbiel M et al. The liver X receptor ligand T0901317 decreases amyloid beta production in vitro and in a mouse model of Alzheimer's disease. J Biol Chem 2005;280(6):4079-4088.

314. Sun Y, Yao J, Kim TW, Tall AR. Expression of liver X receptor target genes decreases cellular amyloid beta peptide secretion. J Biol Chem 2003;278(30):27688-27694.

315. Lue LF, Kuo YM, Roher AE et al. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 1999;155(3):853-862.

316. Burns MP, Vardanian L, Pajoohesh-Ganji A et al. The effects of ABCA1 on cholesterol efflux and Abeta levels in vitro and in vivo. J Neurochem 2006;98(3):792-800.

317. Corder EH, Saunders AM, Strittmatter WJ et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 1993;261(5123):921-923.

318. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 1993;342(8873):697-699.

319. Gutierrez F, Navarro A, Padilla S et al. Prediction of neuropsychiatric adverse events associated with long-term efavirenz therapy, using plasma drug level monitoring. Clin Infect Dis 2005;41(11):1648-1653.

320. Lochet P, Peyriere H, Lotthe A, Mauboussin JM, Delmas B, Reynes J. Long-term assessment of neuropsychiatric adverse reactions associated with efavirenz. HIV Med 2003;4(1):62-66.

321. Dooley KE, Flexner C, Andrade AS. Drug interactions involving combination antiretroviral therapy and other anti-infective agents: repercussions for resource-limited countries. J Infect Dis 2008;198(7):948-961.

322. Li T, Chen W, Chiang JY. PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine. J Lipid Res 2007;48(2):373-384.

323. Moreau A, Vilarem MJ, Maurel P, Pascussi JM. Xenoreceptors CAR and PXR activation and consequences on lipid metabolism, glucose homeostasis, and inflammatory response. Mol Pharm 2008;5(1):35-41.

324. Dussault I, Lin M, Hollister K, Wang EH, Synold TW, Forman BM. Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. J Biol Chem 2001;276(36):33309-33312.

325. Luo G, Cunningham M, Kim S et al. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. Drug Metab Dispos 2002;30(7):795-804.

326. Huang L, Wring SA, Woolley JL, Brouwer KR, Serabjit-Singh C, Polli JW. Induction of P-glycoprotein and cytochrome P450 3A by HIV protease inhibitors. Drug Metab Dispos 2001;29(5):754-760.

327. Gupta A, Mugundu GM, Desai PB, Thummel KE, Unadkat JD. Intestinal human colon adenocarcinoma cell line LS180 is an excellent model to study pregnane X receptor, but not constitutive androstane receptor, mediated CYP3A4 and multidrug resistance transporter 1 induction: studies with anti-human immunodeficiency virus protease inhibitors. Drug Metab Dispos 2008;36(6):1172-1180.

328. Hariparsad N, Nallani SC, Sane RS, Buckley DJ, Buckley AR, Desai PB. Induction of CYP3A4 by efavirenz in primary human hepatocytes: comparison with rifampin and phenobarbital. J Clin Pharmacol 2004;44(11):1273-1281.

329. Fellay J, Marzolini C, Decosterd L et al. Variations of CYP3A activity induced by antiretroviral treatment in HIV-1 infected patients. Eur J Clin Pharmacol 2005;60(12):865-873.

330. Zhang J, Kuehl P, Green ED et al. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. Pharmacogenetics 2001;11(7):555-572.

331. King CR, Xiao M, Yu J et al. Identification of NR1I2 genetic variation using resequencing. Eur J Clin Pharmacol 2007;63(6):547-554.

332. 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):169-181.

333. Hustert E, Zibat A, Presecan-Siedel E et al. Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. Drug Metab Dispos 2001;29(11):1454-1459.

334. Siccardi M, D'Avolio A, Baietto L et al. 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):1222-1225.

335. Haas DW, Ribaudo HJ, Kim RB et al. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS 2004;18(18):2391-2400.

336. Saitoh A, Singh KK, Powell CA et al. An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005;19(4):371-380.

337. Fellay J, Marzolini C, Meaden ER et al. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet 2002;359(9300):30-36.

338. Haas DW, Smeaton LM, Shafer RW et al. Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: an Adult Aids Clinical Trials Group Study. J Infect Dis 2005;192(11):1931-1942.

339. Rodriguez-Novoa S, Barreiro P, Rendon A, Jimenez-Nacher I, Gonzalez-Lahoz J, Soriano V. Influence of 516G>T polymorphisms at the gene encoding the CYP450-2B6 isoenzyme on efavirenz plasma concentrations in HIV-infected subjects. Clin Infect Dis 2005;40(9):1358-1361.

340. Rotger M, Colombo S, Furrer H et al. Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. Pharmacogenet Genomics 2005;15(1):1-5.

341. Hitzl M, Drescher S, van der KH et al. The C3435T mutation in the human MDR1 gene is associated with altered efflux of the P-glycoprotein substrate rhodamine 123 from CD56+ natural killer cells. Pharmacogenetics 2001;11(4):293-298.

342. Colombo S, Soranzo N, Rotger M et al. Influence of ABCB1, ABCC1, ABCC2, and ABCG2 haplotypes on the cellular exposure of nelfinavir in vivo. Pharmacogenet Genomics 2005;15(9):599-608.

343. Fellay J, Marzolini C, Meaden ER et al. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. Lancet 2002;359(9300):30-36.

344. Saitoh A, Singh KK, Powell CA et al. An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005;19(4):371-380.

345. Zhu D, Taguchi-Nakamura H, Goto M et al. Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy. Antivir Ther 2004;9(6):929-935.

346. Haas DW, Ribaudo HJ, Kim RB et al. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS 2004;18(18):2391-2400.

347. Haas DW, Smeaton LM, Shafer RW et al. Pharmacogenetics of long-term responses to antiretroviral regimens containing Efavirenz and/or Nelfinavir: an Adult Aids Clinical Trials Group Study. J Infect Dis 2005;192(11):1931-1942.

348. Ikeda S, Kurose K, Jinno H et al. Functional analysis of four naturally occurring variants of human constitutive androstane receptor. Mol Genet Metab 2005;86(1-2):314-319.

349. Dring MM, Goulding CA, Trimble VI et al. The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. Gastroenterology 2006;130(2):341-348.

350. Cascorbi I, Gerloff T, Johne A et al. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clin Pharmacol Ther 2001;69(3):169-174.

351. Roy JN, Barama A, Poirier C, Vinet B, Roger M. Cyp3A4, Cyp3A5, and MDR-1 genetic influences on tacrolimus pharmacokinetics in renal transplant recipients. Pharmacogenet Genomics 2006;16(9):659-665.

352. Lang T, Klein K, Fischer J et al. Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. Pharmacogenetics 2001;11(5):399-415.

353. von HN, Babacan E, Lennemann T et al. The steady-state pharmacokinetics of atazanavir/ritonavir in HIV-1-infected adult outpatients is not affected by gender-related co-factors. J Antimicrob Chemother 2008;62(3):579-582.

354. Sekar VJ, Lefebvre E, De PE et al. Pharmacokinetic interaction between darunavir boosted with ritonavir and omeprazole or ranitidine in human immunodeficiency virus-negative healthy volunteers. Antimicrob Agents Chemother 2007;51(3):958-961.

355. Luber AD, Brower R, Kim D, Silverman R, Peloquin CA, Frank I. Steady-state pharmacokinetics of once-daily fosamprenavir/ritonavir and atazanavir/ritonavir alone and in combination with 20 mg omeprazole in healthy volunteers. HIV Med 2007;8(7):457-464.

356. Saah AJ, Winchell GA, Nessly ML, Seniuk MA, Rhodes RR, Deutsch PJ. Pharmacokinetic profile and tolerability of indinavir-ritonavir combinations in healthy volunteers. Antimicrob Agents Chemother 2001;45(10):2710-2715.

357. Klein CE, Chiu YL, Cai Y et al. Effects of acid-reducing agents on the pharmacokinetics of lopinavir/ritonavir and ritonavir-boosted atazanavir. J Clin Pharmacol 2008;48(5):553-562.

358. Justesen US, Hansen IM, Andersen AB et al. The long-term pharmacokinetics and safety of adding low-dose ritonavir to a nelfinavir 1,250 mg twice-daily regimen in HIV-infected patients. HIV Med 2005;6(5):334-340.

359. Kearney BP, Mathias A, Mittan A, Sayre J, Ebrahimi R, Cheng AK. Pharmacokinetics and safety of tenofovir disoproxil fumarate on coadministration with lopinavir/ritonavir. J Acquir Immune Defic Syndr 2006;43(3):278-283.

360. Bittner B, Riek M, Holmes B, Grange S. Saquinavir 500 mg film-coated tablets demonstrate bioequivalence to saquinavir 200 mg hard capsules when boosted with twice-daily ritonavir in healthy volunteers. Antivir Ther 2005;10(7):803-810.

361. McCallister S, Valdez H, Curry K et al. A 14-day dose-response study of the efficacy, safety, and pharmacokinetics of the nonpeptidic protease inhibitor tipranavir in treatment-naive HIV-1-infected patients. J Acquir Immune Defic Syndr 2004;35(4):376-382.

362. Yuen GJ, Weller S, Pakes GE. A review of the pharmacokinetics of abacavir. Clin Pharmacokinet 2008;47(6):351-371.

363. Narang VS, Lulla A, Malhotra G, Purandare S. Pharmacokinetic profiling and bioequivalence evaluation of 2 lamivudine tablet formulations after single oral administration in healthy human Indian volunteers. J Acquir Immune Defic Syndr 2005;38(5):566-569.

364. Droste JA, Verweij-van Wissen CP, Kearney BP et al. Pharmacokinetic study of tenofovir disoproxil fumarate combined with rifampin in healthy volunteers. Antimicrob Agents Chemother 2005;49(2):680-684.

365. Marier JF, Manthos H, Kebir S et al. Comparative bioavailability study of zidovudine administered as two different tablet formulations in healthy adult subjects. Int J Clin Pharmacol Ther 2006;44(5):240-246.

366. Liu P, Foster G, LaBadie RR, Gutierrez MJ, Sharma A. Pharmacokinetic interaction between voriconazole and efavirenz at steady state in healthy male subjects. J Clin Pharmacol 2008;48(1):73-84.

367. Tarinas A, Tapanes RD, Gonzalez D, Ferrer G, Abreu D, Perez J. Bioequivalence study of two nevirapine tablet formulations in human-immunodeficiency-virus-infected patients. Farm Hosp 2007;31(3):165-168.

368. MacArthur RD, Novak RM. Reviews of anti-infective agents: maraviroc: the first of a new class of antiretroviral agents. Clin Infect Dis 2008;47(2):236-241.

369. Haan C, Behrmann I. A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. J Immunol Methods 2007;318(1-2):11-19.

370. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21(2):263-265.

371. Halperin E, Eskin E. Haplotype reconstruction from genotype data using Imperfect Phylogeny. Bioinformatics 2004;20(12):1842-1849.

372. Leger P, Dillingham R, Beauharnais CA et al. CYP2B6 variants and plasma efavirenz concentrations during antiretroviral therapy in Port-au-Prince, Haiti. J Infect Dis 2009;200(6):955-964.

373. Haas DW, Gebretsadik T, Mayo G et al. Associations between CYP2B6 polymorphisms and pharmacokinetics after a single dose of nevirapine or efavirenz in African americans. J Infect Dis 2009;199(6):872-880.

374. Allabi AC, Horsmans Y, Issaoui B, Gala JL. Single nucleotide polymorphisms of ABCB1 (MDR1) gene and distinct haplotype profile in a West Black African population. Eur J Clin Pharmacol 2005;61(2):97-102.

375. Roy JN, Barama A, Poirier C, Vinet B, Roger M. Cyp3A4, Cyp3A5, and MDR-1 genetic influences on tacrolimus pharmacokinetics in renal transplant recipients. Pharmacogenet Genomics 2006;16(9):659-665.

376. Lang T, Klein K, Fischer J et al. Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. Pharmacogenetics 2001;11(5):399-415.

377. Bosch TM, Deenen M, Pruntel R et al. Screening for polymorphisms in the PXR gene in a Dutch population. Eur J Clin Pharmacol 2006;62(5):395-399.

378. Takane H, Kobayashi D, Hirota T et al. Haplotype-oriented genetic analysis and functional assessment of promoter variants in the MDR1 (ABCB1) gene. J Pharmacol Exp Ther 2004;311(3):1179-1187.

379. Colombo S, Soranzo N, Rotger M et al. Influence of ABCB1, ABCC1, ABCC2, and ABCG2 haplotypes on the cellular exposure of nelfinavir in vivo. Pharmacogenet Genomics 2005;15(9):599-608.

380. Matsumura K, Saito T, Takahashi Y et al. Identification of a novel polymorphic enhancer of the human CYP3A4 gene. Mol Pharmacol 2004;65(2):326-334.

381. Bertrand J, Treluyer JM, Panhard X et al. Influence of pharmacogenetics on indinavir disposition and short-term response in HIV patients initiating HAART. Eur J Clin Pharmacol 2009;65(7):667-678.

382. Lamba JK, Lin YS, Thummel K et al. Common allelic variants of cytochrome P4503A4 and their prevalence in different populations. Pharmacogenetics 2002;12(2):121-132.

383. Murayama N, Nakamura T, Saeki M et al. CYP3A4 gene polymorphisms influence testosterone 6beta-hydroxylation. Drug Metab Pharmacokinet 2002;17(2):150-156.

384. Dai D, Tang J, Rose R et al. Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. J Pharmacol Exp Ther 2001;299(3):825-831.

385. GeneCards Human Gene Database. Available from http://www.genecards.org/cgi-bin/carddisp.pl?gene=CYP3A4&search=rs4987159&snp=322&snp_sort_mode=140#snp. (Accessed 17 November 2009). 1-11-2009.
Ref Type: Online Source

386. Kang YS, Park SY, Yim CH et al. The CYP3A4*18 genotype in the cytochrome P450 3A4 gene, a rapid metabolizer of sex steroids, is associated with low bone mineral density. Clin Pharmacol Ther 2009;85(3):312-318.

387. Eiselt R, Domanski TL, Zibat A et al. Identification and functional characterization of eight CYP3A4 protein variants. Pharmacogenetics 2001;11(5):447-458.

388. Rotger M, Tegude H, Colombo S et al. Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. Clin Pharmacol Ther 2007;81(4):557-566.

389. Rotger M, Colombo S, Furrer H, Decosterd L, Buclin T, Telenti A. Does tenofovir influence efavirenz pharmacokinetics? Antivir Ther 2007;12(1):115-118.

390. Gatanaga H, Hayashida T, Tsuchiya K et al. Successful efavirenz dose reduction in HIV type 1-infected individuals with cytochrome P450 2B6 *6 and *26. Clin Infect Dis 2007;45(9):1230-1237.

391. Saitoh A, Singh KK, Powell CA et al. An MDR1-3435 variant is associated with higher plasma nelfinavir levels and more rapid virologic response in HIV-1 infected children. AIDS 2005;19(4):371-380.

392. Garsa AA, McLeod HL, Marsh S. CYP3A4 and CYP3A5 genotyping by Pyrosequencing. BMC Med Genet 2005;6:19.

393. Rodriguez-Antona C, Sayi JG, Gustafsson LL, Bertilsson L, Ingelman-Sundberg M. Phenotype-genotype variability in the human CYP3A locus as assessed by the probe drug quinine and analyses of variant CYP3A4 alleles. Biochem Biophys Res Commun 2005;338(1):299-305.

394. Wyen C, Hendra H, Vogel M et al. Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. J Antimicrob Chemother 2008;61(4):914-918.

395. Hoffmeyer S, Burk O, von RO et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A 2000;97(7):3473-3478.

396. Zhu D, Taguchi-Nakamura H, Goto M et al. Influence of single-nucleotide polymorphisms in the multidrug resistance-1 gene on the cellular export of nelfinavir and its clinical implication for highly active antiretroviral therapy. Antivir Ther 2004;9(6):929-935.

397. Kosoy R, Nassir R, Tian C et al. Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. Hum Mutat 2009;30(1):69-78.

398. Stohr W, Back D, Dunn D et al. Factors influencing efavirenz and nevirapine plasma concentration: effect of ethnicity, weight and co-medication. Antivir Ther 2008;13(5):675-685.

399. Tedaldi EM, Absalon J, Thomas AJ, Shlay JC, Berg-Wolf M. Ethnicity, race, and gender. Differences in serious adverse events among participants in an antiretroviral initiation trial: results of CPCRA 058 (FIRST Study). J Acquir Immune Defic Syndr 2008;47(4):441-448.

400. Watkins RE, Wisely GB, Moore LB et al. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 2001;292(5525):2329-2333.

401. Wang J, Sonnerborg A, Rane A et al. Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. Pharmacogenet Genomics 2006;16(3):191-198.

402. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M. Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. Mol Cell Biol 1999;19(9):6318-6322.

403. Colombo S, Telenti A, Buclin T et al. Are plasma levels valid surrogates for cellular concentrations of antiretroviral drugs in HIV-infected patients? Ther Drug Monit 2006;28(3):332-338.

404. Kozawa M, Honma M, Suzuki H. Quantitative prediction of in vivo profiles of CYP3A4 induction in humans from in vitro results with a reporter gene assay. Drug Metab Dispos 2009;37(6):1234-1241.

405. Molto J, Blanco A, Miranda C et al. Variability in non-nucleoside reverse transcriptase and protease inhibitors concentrations among HIV-infected adults in routine clinical practice. Br J Clin Pharmacol 2007;63(6):715-721.

406. Smirnov AN. Nuclear receptors: nomenclature, ligands, mechanisms of their effects on gene expression. Biochemistry (Mosc) 2002;67(9):957-977.

407. Svard J, Spiers JP, Mulcahy F, Hennessy M. Nuclear Receptor-Mediated Induction of CYP450 by Antiretrovirals: Functional Consequences of NR1I2 (PXR) Polymorphisms and Differential Prevalence in Whites and Sub-Saharan Africans. J Acquir Immune Defic Syndr 2010.

408. Lehmann JM, Kliewer SA, Moore LB et al. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem 1997;272(6):3137-3140.

409. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest 2006;116(3):607-614.

410. Repa JJ, Liang G, Ou J et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 2000;14(22):2819-2830.

411. Seo JB, Moon HM, Kim WS et al. Activated liver X receptors stimulate adipocyte differentiation through induction of peroxisome proliferator-activated receptor gamma expression. Mol Cell Biol 2004;24(8):3430-3444.

412. McDevitt MA, Glidewell-Kenney C, Jimenez MA et al. New insights into the classical and non-classical actions of estrogen: evidence from estrogen receptor knock-out and knock-in mice. Mol Cell Endocrinol 2008;290(1-2):24-30.

413. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. N Engl J Med 1999;340(23):1801-1811.

414. Foryst-Ludwig A, Kintscher U. Metabolic impact of estrogen signalling through ERalpha and ERbeta. J Steroid Biochem Mol Biol 2010;122(1-3):74-81.

415. Lu NZ, Wardell SE, Burnstein KL et al. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 2006;58(4):782-797.

416. Baschant U, Tuckermann J. The role of the glucocorticoid receptor in inflammation and immunity. J Steroid Biochem Mol Biol 2010;120(2-3):69-75.

417. Simpson GM, El SA, Loza N et al. An 8-week open-label trial of a 6-day course of mifepristone for the treatment of psychotic depression. J Clin Psychiatry 2005;66(5):598-602.

418. Keenan PA, Jacobson MW, Soleymani RM, Mayes MD, Stress ME, Yaldoo DT. The effect on memory of chronic prednisone treatment in patients with systemic disease. Neurology 1996;47(6):1396-1402.

419. Lenhard JM, Furfine ES, Jain RG et al. HIV protease inhibitors block adipogenesis and increase lipolysis in vitro. Antiviral Res 2000;47(2):121-129.

420. Pou J, Rebollo A, Roglans N et al. Ritonavir increases CD36, ABCA1 and CYP27 expression in THP-1 macrophages. Exp Biol Med (Maywood) 2008;233(12):1572-1582.

421. Nguyen AT, Gagnon A, Angel JB, Sorisky A. Ritonavir increases the level of active ADD-1/SREBP-1 protein during adipogenesis. AIDS 2000;14(16):2467-2473.

422. Riddle TM, Kuhel DG, Woollett LA, Fichtenbaum CJ, Hui DY. HIV protease inhibitor induces fatty acid and sterol biosynthesis in liver and adipose tissues due to the accumulation of activated sterol regulatory element-binding proteins in the nucleus. J Biol Chem 2001;276(40):37514-37519.

423. Caron M, Auclair M, Vigouroux C, Glorian M, Forest C, Capeau J. The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. Diabetes 2001;50(6):1378-1388.

424. Bastard JP, Caron M, Vidal H et al. Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. Lancet 2002;359(9311):1026-1031.

425. Mitro N, Vargas L, Romeo R, Koder A, Saez E. T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. FEBS Lett 2007;581(9):1721-1726.

426. Duniec-Dmuchowski Z, Ellis E, Strom SC, Kocarek TA. Regulation of CYP3A4 and CYP2B6 expression by liver X receptor agonists. Biochem Pharmacol 2007;74(10):1535-1540.

427. Shenoy SD, Spencer TA, Mercer-Haines NA et al. CYP3A induction by liver x receptor ligands in primary cultured rat and mouse hepatocytes is mediated by the pregnane X receptor. Drug Metab Dispos 2004;32(1):66-71.

428. Sporstol M, Tapia G, Malerod L, Mousavi SA, Berg T. Pregnane X receptor-agonists down-regulate hepatic ATP-binding cassette transporter A1 and scavenger receptor class B type I. Biochem Biophys Res Commun 2005;331(4):1533-1541.

429. Roth A, Looser R, Kaufmann M, Meyer UA. Sterol regulatory element binding protein 1 interacts with pregnane X receptor and constitutive androstane receptor and represses their target genes. Pharmacogenet Genomics 2008;18(4):325-337.

430. Li Y, Ross-Viola JS, Shay NF, Moore DD, Ricketts ML. Human CYP3A4 and murine Cyp3A11 are regulated by equol and genistein via the pregnane X receptor in a species-specific manner. J Nutr 2009;139(5):898-904.

431. Wang H, Li H, Moore LB et al. The phytoestrogen coumestrol is a naturally occurring antagonist of the human pregnane X receptor. Mol Endocrinol 2008;22(4):838-857.

432. Bujalska IJ, Walker EA, Tomlinson JW, Hewison M, Stewart PM. 11Beta-hydroxysteroid dehydrogenase type 1 in differentiating omental human preadipocytes: from de-activation to generation of cortisol. Endocr Res 2002;28(4):449-461.

433. Stulnig TM, Oppermann U, Steffensen KR, Schuster GU, Gustafsson JA. Liver X receptors downregulate 11beta-hydroxysteroid dehydrogenase type 1 expression and activity. Diabetes 2002;51(8):2426-2433.

434. Safrin S, Grunfeld C. Fat distribution and metabolic changes in patients with HIV infection. AIDS 1999;13(18):2493-2505.

435. Dube MP, Parker RA, Tebas P et al. Glucose metabolism, lipid, and body fat changes in antiretroviral-naive subjects randomized to nelfinavir or efavirenz plus dual nucleosides. AIDS 2005;19(16):1807-1818.

436. Stellbrink HJ, Orkin C, Arribas JR et al. Comparison of changes in bone density and turnover with abacavir-lamivudine versus tenofovir-emtricitabine in HIV-infected adults: 48-week results from the ASSERT study. Clin Infect Dis 2010;51(8):963-972.

437. Reddy KJ, Singh M, Bangit JR, Batsell RR. The role of insulin resistance in the pathogenesis of atherosclerotic cardiovascular disease: an updated review. J Cardiovasc Med (Hagerstown) 2010;11(9):633-647.

438. Mondy KE, de las FL, Waggoner A et al. Insulin resistance predicts endothelial dysfunction and cardiovascular risk in HIV-infected persons on long-term highly active antiretroviral therapy. AIDS 2008;22(7):849-856.

439. Podzamczer D, Ferrer E, Sanchez P et al. Less lipoatrophy and better lipid profile with abacavir as compared to stavudine: 96-week results of a randomized study. J Acquir Immune Defic Syndr 2007;44(2):139-147.

440. Schultz JR, Tu H, Luk A et al. Role of LXRs in control of lipogenesis. Genes Dev 2000;14(22):2831-2838.

441. Joseph SB, McKilligin E, Pei L et al. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. Proc Natl Acad Sci U S A 2002;99(11):7604-7609.

442. Carr A, Miller J, Law M, Cooper DA. A syndrome of lipoatrophy, lactic acidaemia and liver dysfunction associated with HIV nucleoside analogue therapy: contribution to protease inhibitor-related lipodystrophy syndrome. AIDS 2000;14(3):F25-F32.

443. Miller KK, Daly PA, Sentochnik D et al. Pseudo-Cushing's syndrome in human immunodeficiency virus-infected patients. Clin Infect Dis 1998;27(1):68-72.

444. Sutinen J, Kannisto K, Korsheninnikova E et al. In the lipodystrophy associated with highly active antiretroviral therapy, pseudo-Cushing's syndrome is associated with increased regeneration of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue. Diabetologia 2004;47(10):1668-1671.

445. Hirsch MS, Klibanski A. What price progress? Pseudo-Cushing's syndrome associated with antiretroviral therapy in patients with human immunodeficiency virus infection. Clin Infect Dis 1998;27(1):73-75.

446. Wilson ME, Sengoku T, Allred KF. Estrogen prevents cholesteryl ester accumulation in macrophages induced by the HIV protease inhibitor ritonavir. J Cell Biochem 2008;103(5):1598-1606.

447. Allred KF, Smart EJ, Wilson ME. Estrogen receptor-alpha mediates gender differences in atherosclerosis induced by HIV protease inhibitors. J Biol Chem 2006;281(3):1419-1425.

448. Brunger AT. Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. Nature 1992;355(6359):472-475.

449. Son YL, Lee YC. Molecular determinants of the interactions between LXR/RXR heterodimers and TRAP220. Biochem Biophys Res Commun 2009;384(3):389-393.

450. Oberkofler H, Schraml E, Krempler F, Patsch W. Potentiation of liver X receptor transcriptional activity by peroxisome-proliferator-activated receptor gamma co-activator 1 alpha. Biochem J 2003;371(Pt 1):89-96.

451. Tcherepanova I, Puigserver P, Norris JD, Spiegelman BM, McDonnell DP. Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1. J Biol Chem 2000;275(21):16302-16308.

452. Yanagisawa J, Kitagawa H, Yanagida M et al. Nuclear receptor function requires a TFTC-type histone acetyl transferase complex. Mol Cell 2002;9(3):553-562.

453. Brzozowski AM, Pike AC, Dauter Z et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389(6652):753-758.

454. Bledsoe RK, Montana VG, Stanley TB et al. Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell 2002;110(1):93-105.

455. Svensson S, Ostberg T, Jacobsson M et al. Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. EMBO J 2003;22(18):4625-4633.

456. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 1999;4(2):67-73.

457. Tamehiro N, Sato Y, Suzuki T et al. Riccardin C: a natural product that functions as a liver X receptor (LXR)alpha agonist and an LXRbeta antagonist. FEBS Lett 2005;579(24):5299-5304.

458. Son YL, Lee YC. Molecular determinants of the interactions between SRC-1 and LXR/RXR heterodimers. FEBS Lett 2010;584(18):3862-3866.

459. Phelan CA, Weaver JM, Steger DJ et al. Selective partial agonism of liver X receptor alpha is related to differential corepressor recruitment. Mol Endocrinol 2008;22(10):2241-2249.

460. Albers M, Blume B, Schlueter T et al. A novel principle for partial agonism of liver X receptor ligands. Competitive recruitment of activators and repressors. J Biol Chem 2006;281(8):4920-4930.

461. Hu X, Li S, Wu J, Xia C, Lala DS. Liver X receptors interact with corepressors to regulate gene expression. Mol Endocrinol 2003;17(6):1019-1026.

462. Lenasi H, Breskvar K. Specific interactions of steroids, arylhydrocarbons and flavonoids with progesterone receptors from the cytosol of the fungus Rhizopus nigricans. J Steroid Biochem Mol Biol 2004;91(4-5):273-284.

463. Lund EG, Peterson LB, Adams AD et al. Different roles of liver X receptor alpha and beta in lipid metabolism: effects of an alpha-selective and a dual agonist in mice deficient in each subtype. Biochem Pharmacol 2006;71(4):453-463.

464. Luo G, Guenthner T, Gan LS, Humphreys WG. CYP3A4 induction by xenobiotics: biochemistry, experimental methods and impact on drug discovery and development. Curr Drug Metab 2004;5(6):483-505.

465. Olsavsky KM, Page JL, Johnson MC, Zarbl H, Strom SC, Omiecinski CJ. Gene expression profiling and differentiation assessment in primary human hepatocyte cultures, established hepatoma cell lines, and human liver tissues. Toxicol Appl Pharmacol 2007;222(1):42-56.

466. Pineda T, I, Freedman LP, Garabedian MJ. Identification of DRIP205 as a coactivator for the Farnesoid X receptor. J Biol Chem 2004;279(35):36184-36191.

467. Oberkofler H, Schraml E, Krempler F, Patsch W. Restoration of sterol-regulatory-element-binding protein-1c gene expression in HepG2 cells by peroxisome-proliferator-activated receptor-gamma co-activator-1alpha. Biochem J 2004;381(Pt 2):357-363.

468. Richmond SR, Carper MJ, Lei X, Zhang S, Yarasheski KE, Ramanadham S. HIV-protease inhibitors suppress skeletal muscle fatty acid oxidation by reducing CD36 and CPT1 fatty acid transporters. Biochim Biophys Acta 2010;1801(5):559-566.

469. Zhang Y, Mangelsdorf DJ. LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing. Mol Interv 2002;2(2):78-87.

470. Danner SA, Carr A, Leonard JM et al. A short-term study of the safety, pharmacokinetics, and efficacy of ritonavir, an inhibitor of HIV-1 protease. European-Australian Collaborative Ritonavir Study Group. N Engl J Med 1995;333(23):1528-1533.

471. Markowitz M, Saag M, Powderly WG et al. A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. N Engl J Med 1995;333(23):1534-1539.

472. Carey D, Amin J, Boyd M, Petoumenos K, Emery S. Lipid profiles in HIV-infected adults receiving atazanavir and atazanavir/ritonavir: systematic review and meta-analysis of randomized controlled trials. J Antimicrob Chemother 2010;65(9):1878-1888.

473. Hill A, Sawyer W, Gazzard B. Effects of first-line use of nucleoside analogues, efavirenz, and ritonavir-boosted protease inhibitors on lipid levels. HIV Clin Trials 2009;10(1):1-12.

474. Tomaka F, Lefebvre E, Sekar V et al. Effects of ritonavir-boosted darunavir vs. ritonavir-boosted atazanavir on lipid and glucose parameters in HIV-negative, healthy volunteers. HIV Med 2009;10(5):318-327.

475. Ceccarelli G, d'Ettorre G, Marchetti F et al. Development of Buffalo Hump in the course of antiretroviral therapy including raltegravir and unboosted atazanavir: a case report and review of the literature. J Med Case Reports 2011;5(1):70.

476. El HK, Glorian M, Monsempes C et al. In vitro suppression of the lipogenic pathway by the nonnucleoside reverse transcriptase inhibitor efavirenz in 3T3 and human preadipocytes or adipocytes. J Biol Chem 2004;279(15):15130-15141.

477. Fumaz CR, Munoz-Moreno JA, Molto J et al. Long-term neuropsychiatric disorders on efavirenz-based approaches: quality of life, psychologic issues, and adherence. J Acquir Immune Defic Syndr 2005;38(5):560-565.

478. O'Mahony SM, Myint AM, Steinbusch H, Leonard BE. Efavirenz induces depressive-like behaviour, increased stress response and changes in the immune response in rats. Neuroimmunomodulation 2005;12(5):293-298.

479. Lubbers LS, Zafian PT, Gautreaux C et al. Estrogen receptor (ER) subtype agonists alter monoamine levels in the female rat brain. J Steroid Biochem Mol Biol 2010;122(5):310-317.

480. Lund TD, Rovis T, Chung WC, Handa RJ. Novel actions of estrogen receptor-beta on anxiety-related behaviors. Endocrinology 2005;146(2):797-807.

481. Graff J, von HN, Kuczka K et al. Significant effects of tipranavir on platelet aggregation and thromboxane B2 formation in vitro and in vivo. J Antimicrob Chemother 2008;61(2):394-399.

482. Stewart KG, Zhang Y, Davidge ST. Estrogen decreases prostaglandin H synthase products from endothelial cells. J Soc Gynecol Investig 1999;6(6):322-327.

483. Arnal JF, Douin-Echinard V, Brouchet L et al. Understanding the oestrogen action in experimental and clinical atherosclerosis. Fundam Clin Pharmacol 2006;20(6):539-548.

484. Nilsson S, Gustafsson JA. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 2011;89(1):44-55.

485. Lindberg MK, Erlandsson M, Alatalo SL et al. Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the OPG/RANKL (osteoprotegerin/receptor activator of NF-kappa B ligand) ratio and serum interleukin-6 in male mice. J Endocrinol 2001;171(3):425-433.

486. Gibellini D, Borderi M, de CE et al. Analysis of the effects of specific protease inhibitors on OPG/RANKL regulation in an osteoblast-like cell line. New Microbiol 2010;33(2):109-115.

487. Mora S, Zamproni I, Cafarelli L et al. Alterations in circulating osteoimmune factors may be responsible for high bone resorption rate in HIV-infected children and adolescents. AIDS 2007;21(9):1129-1135.

488. Rahim S, Ortiz O, Maslow M, Holzman R. A case-control study of gynecomastia in HIV-1-infected patients receiving HAART. AIDS Read 2004;14(1):23-32, 35.

489. Kegg S, Lau R. Tamoxifen in antiretroviral-associated gynaecomastia. Int J STD AIDS 2002;13(8):582-583.

490. Sikora MJ, Rae JM, Johnson MD, Desta Z. Efavirenz directly modulates the oestrogen receptor and induces breast cancer cell growth. HIV Med 2010;11(9):603-607.

491. Chao SH, Fujinaga K, Marion JE et al. Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. J Biol Chem 2000;275(37):28345-28348.

492. Terzolo M, Borretta G, Ali A et al. Misdiagnosis of Cushing's syndrome in a patient receiving rifampicin therapy for tuberculosis. Horm Metab Res 1995;27(3):148-150.

493. Tontonoz P, Hu E, Spiegelman BM. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor gamma. Curr Opin Genet Dev 1995;5(5):571-576.

494. Viccica G, Francucci CM, Marcocci C. The role of PPARgamma for the osteoblastic differentiation. J Endocrinol Invest 2010;33(7 Suppl):9-12.

495. Moller DE, Berger JP. Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. Int J Obes Relat Metab Disord 2003;27 Suppl 3:S17-S21.

496. Boccara F, Auclair M, Cohen A et al. HIV protease inhibitors activate the adipocyte renin angiotensin system. Antivir Ther 2010;15(3):363-375.

497. Holick MF. Vitamin D deficiency. N Engl J Med 2007;357(3):266-281.

498. Martins D, Wolf M, Pan D et al. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey. Arch Intern Med 2007;167(11):1159-1165.

499. Welz T, Childs K, Ibrahim F et al. Efavirenz is associated with severe vitamin D deficiency and increased alkaline phosphatase. AIDS 2010;24(12):1923-1928.

500. Makishima M, Okamoto AY, Repa JJ et al. Identification of a nuclear receptor for bile acids. Science 1999;284(5418):1362-1365.

501. Hartman HB, Gardell SJ, Petucci CJ, Wang S, Krueger JA, Evans MJ. Activation of farnesoid X receptor prevents atherosclerotic lesion formation in LDLR-/- and apoE-/- mice. J Lipid Res 2009;50(6):1090-1100.

502. Gazzard BG, Anderson J, Babiker A et al. British HIV Association Guidelines for the treatment of HIV-1-infected adults with antiretroviral therapy 2008. HIV Med 2008;9(8):563-608.

503. Tashima KT, Caliendo AM, Ahmad M et al. Cerebrospinal fluid human immunodeficiency virus type 1 (HIV-1) suppression and efavirenz drug concentrations in HIV-1-infected patients receiving combination therapy. J Infect Dis 1999;180(3):862-864.

504. Kainu T, Kononen J, Enmark E, Gustafsson JA, Pelto-Huikko M. Localization and ontogeny of the orphan receptor OR-1 in the rat brain. J Mol Neurosci 1996;7(1):29-39.

505. Whitney KD, Watson MA, Collins JL et al. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. Mol Endocrinol 2002;16(6):1378-1385.

506. Repa JJ, Turley SD, Lobaccaro JA et al. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 2000;289(5484):1524-1529.

507. Dietschy JM, Turley SD. Cholesterol metabolism in the brain. Curr Opin Lipidol 2001;12(2):105-112.

508. Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 1998;8(5):571-575.

509. Delacourte A. Pathological Tau proteins of Alzheimer's disease as a biochemical marker of neurofibrillary degeneration. Biomed Pharmacother 1994;48(7):287-295.

510. Zhang YW, Thompson R, Zhang H, Xu H. APP processing in Alzheimer's disease. Mol Brain 2011;4:3.

511. Fukumoto H, Deng A, Irizarry MC, Fitzgerald ML, Rebeck GW. Induction of the cholesterol transporter ABCA1 in central nervous system cells by liver X receptor agonists increases secreted Abeta levels. J Biol Chem 2002;277(50):48508-48513.

512. Czech C, Burns MP, Vardanian L et al. Cholesterol independent effect of LXR agonist TO-901317 on gamma-secretase. J Neurochem 2007;101(4):929-936.

513. Donkin JJ, Stukas S, Hirsch-Reinshagen V et al. ATP-binding cassette transporter A1 mediates the beneficial effects of the liver X receptor agonist GW3965 on object recognition memory and amyloid burden in amyloid precursor protein/presenilin 1 mice. J Biol Chem 2010;285(44):34144-34154.

514. Loane DJ, Washington PM, Vardanian L et al. Modulation of ABCA1 by an LXR Agonist Reduces Beta-Amyloid Levels and Improves Outcome after Traumatic Brain Injury. J Neurotrauma 2011;28(2):225-236.

515. Lee SJ, Liyanage U, Bickel PE, Xia W, Lansbury PT, Jr., Kosik KS. A detergent-insoluble membrane compartment contains A beta in vivo. Nat Med 1998;4(6):730-734.

516. Riddell DR, Christie G, Hussain I, Dingwall C. Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. Curr Biol 2001;11(16):1288-1293.

517. Wada S, Morishima-Kawashima M, Qi Y et al. Gamma-secretase activity is present in rafts but is not cholesterol-dependent. Biochemistry 2003;42(47):13977-13986.

518. Burns MP, Rebeck GW. Intracellular cholesterol homeostasis and amyloid precursor protein processing. Biochim Biophys Acta 2010;1801(8):853-859.

519. Simons M, Keller P, De SB, Beyreuther K, Dotti CG, Simons K. Cholesterol depletion inhibits the generation of beta-amyloid in hippocampal neurons. Proc Natl Acad Sci U S A 1998;95(11):6460-6464.

520. Ehehalt R, Keller P, Haass C, Thiele C, Simons K. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol 2003;160(1):113-123.

521. Kim WS, Bhatia S, Elliott DA et al. Increased ATP-binding cassette transporter A1 expression in Alzheimer's disease hippocampal neurons. J Alzheimers Dis 2010;21(1):193-205.

522. Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. Lancet 2000;356(9242):1627-1631.

523. Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. Arch Neurol 2000;57(10):1439-1443.

524. Rockwood K, Kirkland S, Hogan DB et al. Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. Arch Neurol 2002;59(2):223-227.

525. Refolo LM, Pappolla MA, LaFrancois J et al. A cholesterol-lowering drug reduces beta-amyloid pathology in a transgenic mouse model of Alzheimer's disease. Neurobiol Dis 2001;8(5):890-899.

526. Pitas RE, Boyles JK, Lee SH, Hui D, Weisgraber KH. Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. J Biol Chem 1987;262(29):14352-14360.

527. O'Hara R, Luzon A, Hubbard J, Zeitzer JM. Sleep apnea, apolipoprotein epsilon 4 allele, and TBI: mechanism for cognitive dysfunction and development of dementia. J Rehabil Res Dev 2009;46(6):837-850.

528. Leoni V, Solomon A, Kivipelto M. Links between ApoE, brain cholesterol metabolism, tau and amyloid beta-peptide in patients with cognitive impairment. Biochem Soc Trans 2010;38(4):1021-1025.

529. Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci 2006;7(11):850-859.

530. Beffert U, Nematollah FF, Masiulis I et al. ApoE receptor 2 controls neuronal survival in the adult brain. Curr Biol 2006;16(24):2446-2452.

531. Marzolo MP, Bu G. Lipoprotein receptors and cholesterol in APP trafficking and proteolytic processing, implications for Alzheimer's disease. Semin Cell Dev Biol 2009;20(2):191-200.

532. Jiang Q, Lee CY, Mandrekar S et al. ApoE promotes the proteolytic degradation of Abeta. Neuron 2008;58(5):681-693.

533. Spector SA, Singh KK, Gupta S et al. APOE epsilon4 and MBL-2 O/O genotypes are associated with neurocognitive impairment in HIV-infected plasma donors. AIDS 2010;24(10):1471-1479.

534. Koldamova R, Fitz NF, Lefterov I. The role of ATP-binding cassette transporter A1 in Alzheimer's disease and neurodegeneration. Biochim Biophys Acta 2010;1801(8):824-830.

535. Cao G, Liang Y, Jiang XC, Eacho PI. Liver X receptors as potential therapeutic targets for multiple diseases. Drug News Perspect 2004;17(1):35-41.

536. Green DA, Masliah E, Vinters HV, Beizai P, Moore DJ, Achim CL. Brain deposition of beta-amyloid is a common pathologic feature in HIV positive patients. AIDS 2005;19(4):407-411.

537. Anthony IC, Ramage SN, Carnie FW, Simmonds P, Bell JE. Accelerated Tau deposition in the brains of individuals infected with human immunodeficiency virus-1 before and after the advent of highly active anti-retroviral therapy. Acta Neuropathol 2006;111(6):529-538.

538. Tanic N, Perovic M, Mladenovic A, Ruzdijic S, Kanazir S. Effects of aging, dietary restriction and glucocorticoid treatment on housekeeping gene expression in rat cortex and hippocampus-evaluation by real time RT-PCR. J Mol Neurosci 2007;32(1):38-46.

539. Haan C, Behrmann I. A cost effective non-commercial ECL-solution for Western blot detections yielding strong signals and low background. J Immunol Methods 2007;318(1-2):11-19.

540. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.

541. Cheng X, Klaassen CD. Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. Drug Metab Dispos 2006;34(11):1863-1867.

542. Li T, Chen W, Chiang JY. PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine. J Lipid Res 2007;48(2):373-384.

543. Abildayeva K, Jansen PJ, Hirsch-Reinshagen V et al. 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. J Biol Chem 2006;281(18):12799-12808.

544. Wang JM, Irwin RW, Brinton RD. Activation of estrogen receptor alpha increases and estrogen receptor beta decreases apolipoprotein E expression in hippocampus in vitro and in vivo. Proc Natl Acad Sci U S A 2006;103(45):16983-16988.

545. Bang OY, Hong HS, Kim DH et al. Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. Neurobiol Dis 2004;16(1):21-28.

546. Liang Y, Lin S, Beyer TP et al. A liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein E expression, secretion and cholesterol homeostasis in astrocytes. J Neurochem 2004;88(3):623-634.

547. Goodwin B, Watson MA, Kim H, Miao J, Kemper JK, Kliewer SA. Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha. Mol Endocrinol 2003;17(3):386-394.

548. Rigamonti E, Helin L, Lestavel S et al. Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. Circ Res 2005;97(7):682-689.

549. Gilardi F, Viviani B, Galmozzi A et al. Expression of sterol 27-hydroxylase in glial cells and its regulation by liver X receptor signaling. Neuroscience 2009;164(2):530-540.

550. Boyles JK, Pitas RE, Wilson E, Mahley RW, Taylor JM. Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. J Clin Invest 1985;76(4):1501-1513.

551. Lefterov I, Bookout A, Wang Z, Staufenbiel M, Mangelsdorf D, Koldamova R. Expression profiling in APP23 mouse brain: inhibition of Abeta amyloidosis and inflammation in response to LXR agonist treatment. Mol Neurodegener 2007;2:20.

552. Brown J, III, Theisler C, Silberman S et al. Differential expression of cholesterol hydroxylases in Alzheimer's disease. J Biol Chem 2004;279(33):34674-34681.

553. Hoe HS, Cooper MJ, Burns MP et al. The metalloprotease inhibitor TIMP-3 regulates amyloid precursor protein and apolipoprotein E receptor proteolysis. J Neurosci 2007;27(40):10895-10905.

554. Roberts SB, Ripellino JA, Ingalls KM, Robakis NK, Felsenstein KM. Non-amyloidogenic cleavage of the beta-amyloid precursor protein by an integral membrane metalloendopeptidase. J Biol Chem 1994;269(4):3111-3116.

555. Farris W, Mansourian S, Chang Y et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc Natl Acad Sci U S A 2003;100(7):4162-4167.

556. Tamboli IY, Prager K, Barth E, Heneka M, Sandhoff K, Walter J. Inhibition of glycosphingolipid biosynthesis reduces secretion of the beta-amyloid precursor protein and amyloid beta-peptide. J Biol Chem 2005;280(30):28110-28117.

557. Sanfeliu C, Cristofol R, Toran N, Rodriguez-Farre E, Kim SU. Use of Human Central Nervous System Cell Cultures in Neurotoxicity Testing. Toxicol In Vitro 1999;13(4-5):753-759.

558. Xie HR, Hu LS, Li GY. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chin Med J (Engl) 2010;123(8):1086-1092.

559. Samadi A, Marco-Contelles J, Soriano E et al. Multipotent drugs with cholinergic and neuroprotective properties for the treatment of Alzheimer and neuronal vascular diseases. I. Synthesis, biological assessment, and molecular modeling of simple and readily available 2-aminopyridine-, and 2-chloropyridine-3,5-dicarbonitriles. Bioorg Med Chem 2010;18(16):5861-5872.

560. Reed JC, Meister L, Tanaka S et al. Differential expression of bcl2 protooncogene in neuroblastoma and other human tumor cell lines of neural origin. Cancer Res 1991;51(24):6529-6538.

561. Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC, Cleary P. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science 1988;239(4840):586-592.

562. Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 2001;410(6831):988-994.

563. Blasko I, Veerhuis R, Stampfer-Kountchev M, Saurwein-Teissl M, Eikelenboom P, Grubeck-Loebenstein B. Costimulatory effects of interferon-gamma and interleukin-1beta or tumor necrosis factor alpha on the synthesis of Abeta1-40 and Abeta1-42 by human astrocytes. Neurobiol Dis 2000;7(6 Pt B):682-689.

564. Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-drug interactions. J Neuroimmune Pharmacol 2006;1(3):323-339.

565. Wang J, Sonnerborg A, Rane A et al. Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. Pharmacogenet Genomics 2006;16(3):191-198.

566. Ma X, Cheung C, Krausz KW et al. A double transgenic mouse model expressing human pregnane X receptor and cytochrome P450 3A4. Drug Metab Dispos 2008;36(12):2506-2512.

567. Fellay J, Marzolini C, Decosterd L et al. Variations of CYP3A activity induced by antiretroviral treatment in HIV-1 infected patients. Eur J Clin Pharmacol 2005;60(12):865-873.

568. Mannu J, Jenardhanan P, Mathur PP. A computational study of CYP3A4 mediated drug interaction profiles for anti-HIV drugs. J Mol Model 2010.

569. Mahungu TW, Johnson MA, Owen A, Back DJ. The impact of pharmacogenetics on HIV therapy. Int J STD AIDS 2009;20(3):145-151.

570. Moyle G. Efavirenz: practicalities, considerations and new issues. Int J Clin Pract Suppl 1999;103:30-34.

571. Staszewski S, Morales-Ramirez J, Tashima KT et al. Efavirenz plus zidovudine and lamivudine, efavirenz plus indinavir, and indinavir plus zidovudine and lamivudine in the treatment of HIV-1 infection in adults. Study 006 Team. N Engl J Med 1999;341(25):1865-1873.

572. Repa JJ, Li H, Frank-Cannon TC et al. Liver X receptor activation enhances cholesterol loss from the brain, decreases neuroinflammation, and increases survival of the NPC1 mouse. J Neurosci 2007;27(52):14470-14480.

573. Pan Y, Li L, Kim G, Ekins S, Wang H, Swaan PW. Identification and validation of novel human pregnane X receptor activators among prescribed drugs via ligand-based virtual screening. Drug Metab Dispos 2011;39(2):337-344.

574. Zhou C, King N, Chen KY, Breslow JL. Activation of PXR induces hypercholesterolemia in wild-type and accelerates atherosclerosis in apoE deficient mice. J Lipid Res 2009;50(10):2004-2013.

575. Khogali AM, Chazan BI, Metcalf VJ, Ramsay JH. Hyperlipidaemia as a complication of rifampicin treatment. Tubercle 1974;55(3):231-233.

576. Rezen T, Tamasi V, Lovgren-Sandblom A, Bjorkhem I, Meyer UA, Rozman D. Effect of CAR activation on selected metabolic pathways in normal and hyperlipidemic mouse livers. BMC Genomics 2009;10:384.

577. Yamazaki Y, Kakizaki S, Horiguchi N et al. The role of the nuclear receptor constitutive androstane receptor in the pathogenesis of non-alcoholic steatohepatitis. Gut 2007;56(4):565-574.