Nuclear receptor-mediated induction of drug metabolizing enzymes and transporters by anticancer drugs

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Nucleaire receptor gemedieerde inductie van geneesmiddel metaboliserende enzymen en transporters door antikanker middelen
(met een samenvatting in het Nederlands)

Proefschrift

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Introduction

The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology

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Abstract

Drug-drug interactions can have a major impact on treatment outcome in cancer patients. These patients are at high risk of such interactions, because they are treated with combinations of multiple cytotoxic anticancer drugs or hormonal agents often co-administered with prophylactic antiemetics and analgesics to provide palliation. Interactions between drugs can affect the pharmacokinetics of concomitantly administered chemotherapeutic agents. Especially due to the specific properties of anticancer drugs such as a narrow therapeutic index and steep dose-toxicity curve, small pharmacokinetic changes can have significant clinical consequences like decreased therapeutic efficacy or increased toxicity. An important mechanism that underlies these interactions is the induction of enzymes or efflux transporters involved in the biotransformation and clearance of anticancer drugs. Several nuclear receptors, like the pregnane X receptor (PXR) and the constitutively androstane receptor (CAR) have been shown to regulate induction. Activation of these receptors will lead to induction of important enzymes like cytochrome P450 3A4 (CYP3A4), which is involved in the biotransformation of more than 50 percent of all clinically used drugs. Therefore, concomitant administration of agents that activate PXR will therefore affect the pharmacokinetics of drugs that are substrate for PXRs target genes, which include CYP3A4 and P-glycoprotein. Understanding of the molecular mechanisms that underlie enzyme induction and the identification of (new) drugs involved in pharmacokinetic drug-drug interactions may contribute to the predictability of drug-drug interactions and eventually help to develop safer anticancer regimens.

Introduction

One of the major obstacles in predicting the treatment outcome in cancer patients in terms of efficacy and toxicity is the large intra- and interindividual pharmacokinetic variability of chemotherapy. Because of the specific properties of anticancer drugs, like a narrow therapeutic index and a steep dose-toxicity curve, small changes in the pharmacokinetic profile can significantly alter the clinical response to these drugs [1, 2]. Multiple factors are known to be responsible for the interindividual differences in the pharmacokinetic profile of antineoplastic agents such as age, sex, genetic polymorphisms in biotransformation and drug transport, disease status and environmental determinants. However, a substantial part of the pharmacokinetic variability in oncology is caused by drug-drug interactions. Overall, drug-drug interactions are estimated to be responsible for 20-30% of all adverse drug reactions [3], and the risk of clinically significant drug-drug interactions increases with the number of concomitantly administered agents. This is especially relevant in oncology, where the treatment of cancer often constitutes combinations of multiple cytotoxic anticancer drugs. To reduce side effects or to provide palliation, anticancer treatment is also regularly supplemented with supportive medication like prophylactic antiemetics, corticosteroids, anticoagulants, analgesics, antibiotics and anticonvulsants. Furthermore, elderly cancer patients often use drugs for the treatment of other comorbidities, such as diabetes, cardiovascular diseases, mood disturbances, and peptic complaints. In addition, up to 63% of all cancer patients have been reported to use over-the-counter drugs, nutritional supplements and complementary alternative medicines (CAM) [4], which further increases the risk of clinically relevant drug interactions [5].

Many pharmacokinetic drug-drug interactions are not recognized as such, because they are mistaken for symptoms of the disease, or, due to the specific properties of anticancer drugs, a certain amount of toxicity is accepted [6]. In addition, due to the use of combination regimens, the specific agent(s) causing the interaction often cannot be identified. This review explores the clinical implications of pharmacokinetic drug-drug interactions in oncology and discusses the molecular mechanisms involved in these interactions, with a special focus on nuclear receptors.

Understanding of the mechanisms underlying nuclear receptor mediated drug-drug interactions may eventually help to predict and manage adverse drug reactions, which may lead to safer and more efficious anticancer regimens.

Pharmacokinetic drug-drug interactions

Drug-drug interactions are generally categorized into pharmacokinetic, pharmacodynamic and pharmaceutical interactions. In oncology these interactions can occur between anticancer drugs, when administered as a combination, or between anticancer drugs and other concomitantly administered drugs, herbs or food components. When one drug alters the absorption, distribution, metabolism or excretion (ADME) of another drug, this interaction is defined as a pharmacokinetic interaction. One of the main mechanisms by which compounds cause pharmacokinetic interactions is the modulation of the activity of enzymes involved in the clearance of anticancer drugs, either by inhibition or induction of the(se) specific enzyme(s).
Enzyme inhibition impairs the biotransformation or clearance of the substrates of a specific enzyme. For instance, inhibition of cytochrome P450 (CYP) 3A4, an enzyme involved in the biotransformation of >50% of all clinically used drugs, including many anticancer agents (table 1), may have important implications for the treatment outcome. As an example, the antimycotic agent ketoconazole, a known CYP3A4 inhibitor, reduces the clearance of concomitantly administered docetaxel, a substrate of CYP3A4 [7]. Reduced clearance of this cytotoxic agent results in higher plasma levels of docetaxel, thereby increasing the risk for neutropenia at standard doses several-fold. To prevent toxicities, caution must be taken and substantial dose reductions are required when docetaxel is administered together with ketoconazole or other potent inhibitors of CYP3A4 [7]. Inhibition can also occur at the level of drug transport, which involves members of the ATP-binding cassette (ABC) drug transporters like P-glycoprotein (Pgp; ABCB1). Inhibition of intestinal Pgp has been shown to enhance the bioavailability of orally administered taxanes [8].
In contrast, enzyme induction enhances the clearance of chemotherapeutic agents, which might result in undertreatment or even therapeutic failure.

TABLE 1

Anticancer agents and related drug-metabolizing enzymes and drug transporters

Enzyme	Substrate	Inhibitor	Inducer
CYP1A1	dacarbazine, docetaxel, erlotinib, tamoxifen, toremifene		
CYP1A2	dacarbazine, erlotinib, etoposide, flutamide, imatinib, tamoxifen, toremifene	anastrazole	
CYP2A6	cyclophosphamide, ifosfamide, letrozole, tegafur	letrozole	
CYP2B6	altretamine, cyclophosphamide, ifosfamide, tamoxifen	thiotepa	
CYP2C8	cyclophosphamide, docetaxel, ifosfamide, paclitaxel, tegafur, tretinoin	anastrazole	
CYP2C9	bexarotene, cyclophosphamide, ifosfamide, imatinib, tamoxifen, targretin, tegafur, toremifene, tretinoin	anastrazole, imatinib, teniposide	
CYP2C19	cyclophosphamide, ifosfamide, imatinib, tamoxifen, teniposide, thalidomide	letrozole	
CYP2D6	imatinib, tamoxifen, vinorelbine	doxorubicin, imatinib, lomustine, vinblastine vincristine	
CYP2E1	etoposide, tamoxifen, tretinoin, vinorelbine		
CYP3A4/5	bexarotene, busulfan, cyclophosphamide, cytarabine, dexamethasone, docetaxel, doxorubicin, erlotinib, etoposide, exemestane, flutamide, fulvestrant, gefitinib, ifosfamide, imatinib, irinotecan, letrozole, medroxyprogresterone acetate, mitoxantrone, paclitaxel, tamoxifen, targretin, teniposide, topotecan, toremifene, tretinoin, vinblastine, vincristine, vindesine, vinorelbine		paclitaxel, cyclophosphamide-dexamethasone

TABLE 1

Anticancer agents and related drug-metabolizing enzymes and drug transporters

Enzyme	Substrate	Inhibitor	Inducer
UGT	doxorubicin, epirubicin, etoposide, irinotecan, topotecan, tamoxifen		
SULT	tamoxifen		
GST	busulfan, chlorambucil, cyclophosphamide, doxorubicin, ifosfamide, melphalan, nitrosurea		
P-gp	daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, idarubicin, methotrexate, mitoxantrone, paclitaxel, teniposide, vinblastine, vincristine	tamoxifen	
MRP1	arsenic trioxide, chlorambucil, daunorubicin, doxorubicin, epirubicin, etoposide, melphalan, methotrexate, mitoxantrone, paclitaxel, vinblastine, vincristine		
MRP	cisplatin, irinotecan doxorubicin, etoposide, methotrexate, SN-38, vinblastine, vincristine		
MRP3	doxorubicin, epirubicin,etoposide, methotrexate, teniposide, vinblastine,vincristine		
MRP4	cyclic nucleotide analogs, methotrexate		
MRP5	doxorubicin, methotrexate, nucleotide analogs, topotecan		
MRP6	doxorubicin, etoposide, teniposide		
MRP8	5-fluorouracil and its metabolites		
BCRP	imatinib, methotrexate, mitoxantrone, SN-38, topotecan		

For example, pre- or cotreatment of the novel tyrosine kinase inhibitor erlotinib with the classic CYP3A4 inducer rifampicin results in increased clearance of erlotinib by 3-fold and reduces the AUC of erlotinib by 66% [9]. However, enzyme induction might also lead to toxicities, when the metabolizing enzymes are involved in the bioactivation of anticancer prodrugs, such as the oxazaphosphorines cyclophosphamide and ifosfamide. These agents are subject to 4-hydroxylation by CYP2B6 and CYP3A4 leading to the formation of the clinically active phosporamide moiety [10]. The effect of enzyme induction on prodrug activation was illustrated in a recent case report by de Jonge *et al*. (2005) [11]. A patient with germ-cell cancer was treated with 4-day courses of high dose CTC (cyclophosphamide, thiotepa, carboplatin) chemotherapy. Prior to the second course, the patient was treated for regimen-related seizures with phenytoin; a known inducer of both CYP2B6 and CYP3A4. Co-administration with phenytoin decreased the levels of cyclo phosphamide and increased the plasma levels of its cytotoxic metabolite 4-hydroxycyclophosphamide significantly. In this case, however, timely dose-reduction prevented the occurrence of severe systemic toxicity.
Therefore, to prevent intoxications or loss of therapeutic efficacy as a result of pharmacokinetic drug-drug interactions at the level of induction, it is important to understand the molecular basis and the individual factors involved in the mechanisms that underlie these interactions.

Molecular mechanism of induction

Until recently, the mechanisms behind enzyme induction were unclear. However, in the late 1990s several new orphan receptors like the pregnane X receptor (PXR; NR1I2) [12] and the constitutive androstane receptor (CAR; NR1I3) [13] were discovered. These receptors belong to the family of ligand-activated transcription factors, known as nuclear receptors, that also include other important drug targets such as the glucocorticoid receptor (GR; NR3C1) and the vitamin D_3 receptor (VDR, NR1I1).
The pregnane X receptor was named after a class of naturally occurring steroids, the pregnanes, which activate this receptor [12]. Shortly after its discovery, PXR was shown to be expressed in the liver, small intestines and in the brain. PXR is located in the cytoplasm, but in response to activation by a wide variety of structurally unrelated compounds, including

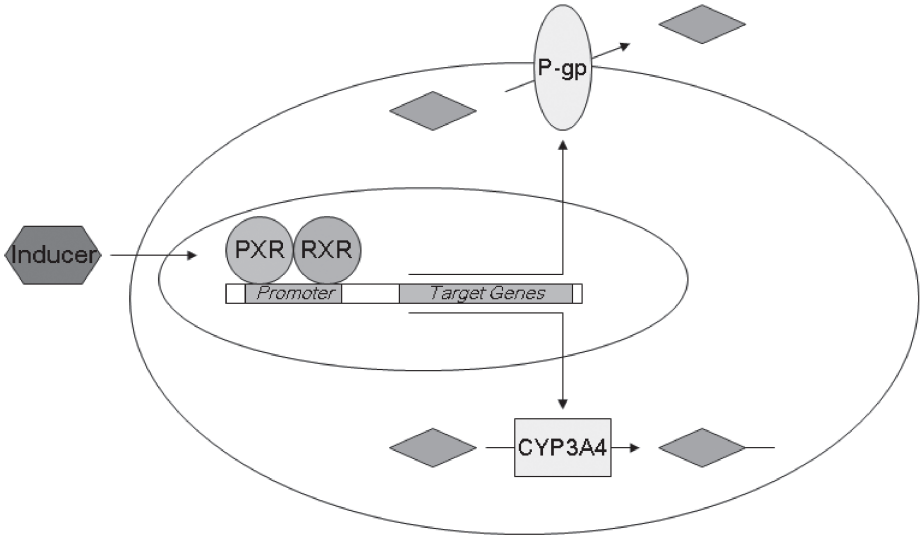

Figure 1 Mechanism of PXR mediated gene induction
Exposure of cells to inducing agents leads to upregulation of important drug metabolizing enzymes or drug efflux transporters such as CYP3A4 and Pgp respectively which leads to enhanced biotransformation and/or clearance of drugs. Induction of such enzymes is mediated by nuclear receptors such as PXR. Upon ligand binding, PXR heterodimerizes with RXR and binds to specific response elements within the promoter area of its target genes, which triggers enzyme induction.

established CYP3A4 inducers such as rifampicin, dexamethasone, and hyperforin (table 2), it translocates to the nucleus and forms a heterodimer with the retinoid X receptor (RXR; NR2B1) [14]. The PXR-RXR heterodimer then binds to the promoter region of its target genes such as CYP3A4 [14] and MDR1 [15], thereby increasing transcription (figure 1). The development of *in vitro* screening assays and the establishment of transgenic and knockout mouse models have revealed the involvement of PXR in the transcription of several other important drug metabolizing enzymes and drug transporters as well (reviewed by Dai *et al*. (2005) [16]). Treatment of wild-type mice with the specific rodent PXR-agonist pregnenolone 16α-carbonitrile (PCN) resulted in increased expression of several important metabolic enzymes such as cyp3a11 [17], cyp2c37 [18], ugt1a9 [19], gstm2 [17], mrp2 [17] and mdr1 [17]. This could not be achieved in PXR null mice, confirming the role of PXR as a mediator of xenobiotic metabolism. However, comparison of human PXR and murine PXR also revealed that there is a marked distinction in the amino acid sequence of both receptors, resulting in large interspecies differences with respect to ligand

TABLE 2

Ligands of the various nuclear receptors

Nuclear Receptor	Agonists	Inverse agents
PXR	amprenavir, avasimibe, bile acids derivatives and precursors, bosentan, carbamazepine, ciglitazone, clotrimazole, cortisone, corticosterone, cyclophosphamide, dexamethasone, efavirenz, exemestane, guggulsterone, hydrocortisone, hyperforin, lovastatin, mifepristone, nelfinavir, nifedipine, omeprazole, paclitaxel, phenobarbital, phenytoin, rifabutin, rifampicin, ritonavir, simvastatin, spironolactone, SR12813, tamoxifen and 4-hydroxytamoxifen, troglitazone, troleandomycin, vitamin E, vitamin K2	ET-743
CAR	direct: CITCO indirect: clotrimazole, phenobarbital, phenytoin, bilirubin	androstane, androstene
VDR	calcitriol, lithocholic acid	

affinity and specificity [14]. For example, while PCN is a strong inducer of cyp3a11 (the murine ortholog of CYP3A4) in mice, it does not induce human CYP3A4. Reciprocally, rifampicin does not affect the expression of murine cyp3a11 in wild-type mice [20]. However, rifampicin caused a significant increase in cyp3a11 expression in humanized PXR mice, while PCN failed to induce cyp3a11 in these animals [21]. So, PXR has been established as a key regulator of CYP3A4 and Pgp, thereby protecting vital organs such as liver and brain against many xenobiotics and toxic endogenous compounds such as bile acids. For example, PXR activation tightened the blood-brain barrier by induction of Pgp resulted in a lower penetration of methadone, a CNS-acting drug [22]. Other enzymes that are regulated by PXR include CYP2B6 [23], CYP2C8 [24], CYP2C9 [25], sulfotransferase (SULT) [26], uridinediphosphate(UDP)-glucuronosyltransferase (UGT) 1A1 [27], and MRP2 [28] (table 3).

The other orphan receptor, CAR, is also expressed in the liver [13] and small intestine [29]. CAR resides in the cytoplasm where it is unable to activate gene transcription. Upon exposure to phenobarbital (PB) or PB-

like inducers (e.g. phenytoin), CAR translocates to the nucleus where it activates CYP2B6 transcription [30]. Remarkably, many inducers do not bind directly to CAR, but modulate a phosphorylation dependent mechanism leading to the translocation of CAR [31, 32]. However, the exact mechanism of the nuclear translocation is still not fully understood. While PXR is a very promiscuous receptor in terms of ligand affinity and specificity, CAR has a more selective set of ligands (table 2) [33]. Activation of CAR in wild-type mice increased the expression of sultn [17], mrp1 [17], mrp2 [17], and oatp2 [34], while this could not be observed in CAR knock-out mice. This clearly illustrates the involvement of CAR in the transcriptional regulation of important drug metabolizing enzymes and drug transporters. Currently, CAR has been adopted as a xenosensor and is implicated in the regulation of drug metabolizing enzymes and transporters such as CYP2B6 [23], CYP2C9 [35], CYP2C19 [36], CYP3A4 [37], UGT1A [27, 38], SULT [39], Pgp [40], MRP2 [28] (table 3).

TABLE 3
Nuclear Receptor and their target genes important in metabolism and disposition

Nuclear Receptor	Dimerization Partner	Target Gene	Binding Motif
PXR	Dimerization Partner RXR	CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5, CYP3A7, UGT1A1, UGT1A3, UGT1A6, SULT MDR1, MRP2	DR3, ER6 (DR4)
CAR	RXR	CYP2B6, CYP2C9, CYP2C19, CYP3A4, UGT1A1, UGT2B1, SULT MRP2	DR4 (DR3)
VDR	RXR	CYP2B6, CYP2C9, CYP3A4	ER6 (DR3, DR4)

In general the nuclear receptors share a common structure, mainly consisting of two functional domains: a variable ligand-binding domain (LBD) and a highly conserved DNA-binding domain (DBD). The LBD is found at the carboxy-terminal portion of the receptor. It harbors a hydrophobic ligand-binding pocket, but also two activation factor domains (AF) 1 and 2, involved in respectively heterodimerization to RXR and co-regulator recruitment. Especially in PXR the hydrophobic ligand-binding pocket is large and flexible, which explains the promiscuity of this receptor. Ligand-binding triggers a conformational change in the LBD allowing the heterodimerization of the receptor with RXR. In addition, depending on the nature of the ligand, the AF-2 domain can be changed in a way that it allows the binding of either a co-activator protein or a co-repressor protein [41]. Activation of PXR results in the dissociation of a co-repressor protein such as the silencing mediator of retinoid and thyroid receptor (SMRT) and the nuclear receptor co-repressor (NCoR), allowing the binding of co-activator proteins such as glucocorticoid receptor interacting protein (GRIP) and steroid receptor coactivator-1 (SRC-1). While SMRT and NCoR stabilize chromatin and consequently repress transcription, SRC-1 and GRIP destabilize chromatin eventually leading to recruitment of the transcription machinery [42].

The DBD mediates the recognition and binding of the nuclear receptors to specific response elements within the promoter regions of a target gene. These response elements are arranged as direct (DRn), everted (ERn) or inverted (IRn) repeats of the related consensus AG(G/T)TCA, separated by a spacer containing n (0-9) number of base pairs. The PXR-RXR heterodimer binds to a specific response element within the CYP3A4 promoter known as PXRE, consisting of DR3 or ER6 type motifs [14]. In contrast, CAR-RXR mediates CYP2B6 induction via binding to an enhancer module known as the PB-responsive enhancer module (PBREM), which contains two nuclear receptor-binding sites (NR1 and 2) that have a DR4 type motif [43]. Although the CYP3A4 promoter only contains DR3, and CYP2B6 only contains DR4 type motifs, cross regulation occurs because both PXR and CAR are able to recognize mutual response elements [23, 44]. In addition, VDR is also able to cross-regulate genes that contain DR3 motifs [45, 46].

Other nuclear receptors have also been linked to the regulation of drug metabolizing enzymes and transporters. These receptors include the vitamin D_3 receptor (VDR; NR1I1), farnesoid X receptor (FXR; NR1H4), liver

X receptor (LXR; NR1H2/3), glucocorticoid receptor (GR; NR3C1) and hepatocyte nuclear factor 4α (HNF4α; NR2A1). The vitamin D_3 receptor was already known to have an important function in maintaining calcium and phosphate homeostasis, inhibiting cell proliferation, and regulating parathyroid hormone synthesis. However, Schmiedlin-Ren *et al*. (1997) showed that, in addition to PXR and CAR, VDR is also involved in the regulation of CYP3A4, CYP2C9 and CYP2B6 induction [46, 47]. In contrast to PXR and CAR, VDR has a narrow substrate specificity and only two sets of compounds were shown to have affinity for this receptor: vitamin D derivatives and bile acids [48]. Consequently, VDR is less important in drug-drug interactions, because it is mainly activated by endogenous compounds such as bile acids[49]. Although two other nuclear receptors, FXR and LXR, were shown to regulate drug-metabolizing enzymes and drug transporters such as CYP3A4 [50, 51], MRP2 [28], and even other nuclear receptors like PXR [52]. These receptors are only activated by endogenous substances such as bile acids and lipids, and not by widely used (anticancer) drugs. As there is no evidence that FXR and LXR contribute to clinically relevant drug-drug interactions, these receptors are beyond the scope of this review.

In contrast, the role of GR in enzyme induction was already implicated, because a specific ligand of this receptor, dexamethasone, is known to potentiate the induction of CYP3A4 by established inducers such as phenobarbital and rifampicin. Until recently, the mechanisms behind this potentiation were not understood, Pascussi *et al*. [53, 54] showed that activation of GR by dexamethasone results in upregulation of both PXR and CAR. Therefore, concomitant use of glucocorticoids like dexamethasone and PXR/CAR activators may result in additive or even synergistic induction of CYP3A4 and other target genes. HNF4α is considered to play a key role in the tissue specific expression of hepatic drug metabolizing enzymes [55] and binding of this nuclear receptor to the promoter area of CYP2C8, CYP2C9, CYP2C19 and CYP3A4 results in increased basal and inducible levels of their gene products [24, 56].

Methods

Before the discovery of the X receptors, the inductive capacity of compounds was based on data obtained from animal tests and primary cultures of human hepatocytes. However, both models have major draw

backs. An important factor that compromises the use of induction data obtained from *in vivo* or *in vitro* animal tests is the marked interspecies differences. The PXR-LBDs of common laboratory animals only have an approximate homology of about 70% with human PXR-LBD, resulting in a high interspecies variation in terms of ligand affinity and specificity. This complicates the extrapolation to the human *in vivo* situation. To tackle the difficulties that arise from induction studies in animals, primary cultures of human hepatocytes can be used, because of their resemblance to the *in vivo* situation. Several approaches are applied to determine the inductive capacity of a compound in hepatocytes, such as protein and mRNA analysis, and assessment of enzyme activity by measuring the rate of probe substrate metabolism (e.g. testosterone 6β-hydroxylation for CYP3A4) by the microsomal fractions of these cells. Unfortunately, the use of human hepatocytes has major drawbacks such as low availability, high cost, high interindividual donor variation and a rapid decline of metabolic enzyme expression [57].

After the discovery of the regulation of CYP3A4 by PXR and CAR, cell-based reporter gene assays have been developed that allow rapid screening of (new) drugs for their ability to cause enzyme induction [58]. These assays are based on the transient transfection of a cell line with a reporter construct containing a heterologous or intact promoter region of a target gene coupled to a reporter gene such as a firefly luciferase, green fluorescent protein, or alkaline phosphatase. In addition, an expression plasmid of the nuclear receptor of interest is co-transfected into the cell. Although nuclear receptor levels in these reporter gene assays are raised artificially, a good correlation between PXR-mediated CYP3A4 induction measured in a reporter gene assay, and CYP3A4 mRNA [59] and protein [60] expression in primary cultures of human hepatocytes was reported. Furthermore, by comparing data obtained from a PXR reporter assay and known data on CYP3A4 induction *in vivo*, Persson *et al*. (2006) concluded that a PXR reporter gene assay is a reliable screening method for the assessment of CYP3A4 induction [61]. In contrast to PXR, CAR-mediated induction studies are more difficult due to the lack of good models. In human liver, hCAR is retained in the cytoplasm and upon exposure to PB-like inducers, it translocates to the nucleus where hCAR activates gene transcription in a ligand-independent fashion. Although it is important to screen (anticancer) drugs for their ability to activate translocation of CAR, transfection of hCAR in cell lines results in spontaneous accumula-

tion in the nucleus [31] and marked upregulation of its target genes [56]. However, direct activation of hCAR by the only known agonist 6-(4-chlorophenyl)imidazo[2,1-b]-[1,3]-thiazole-5-carbaldehyde-O-3,4-dichlorobenzyl)-oxime (CITCO) resulted in an additive increase in target gene induction [62]. This indicates that a CAR-reporter gene assay can be used to identify agonists of CAR, but that it is unable to identify indirect activators of CAR that activate translocation. Elucidation of the mechanism behind translocation may eventually provide good models to investigate the involvement of this receptor in drug-drug interactions to the full extend.

Another valuable tool to study enzyme induction and related drug-drug interactions or the physiological roles of nuclear receptors *in vivo*, is the use of genetically altered animal models. For instance, PXR and CAR knock-out mice already have provided insights into the physiological roles of these receptors in the body, like xenobiotic and bilirubin metabolism [16]. In addition, the establishment of "humanized" PXR and CAR mouse models further could help to elucidate the involvement of these nuclear receptors in pharmacokinetic drug-drug interactions.

Although all these approaches may help to identify potential inducers or provide insights into the mechanism behind drug-drug interactions, pharmacokinetic studies in patients still are the most ideal model.

Nuclear receptors in oncological drug-drug interactions

It has become clear that the nuclear receptors PXR and CAR regulate the induction of many drug-metabolizing enzymes and drug transporters, many of which are involved in the biotransformation and clearance of widely used anticancer drugs. Activation of PXR and CAR has been associated with clinically important drug-drug interactions. The best-characterized examples that illustrate the clinical significance of both PXR and CAR in oncological drug-drug interactions are those that involve prototypical PXR activators such as rifampicin or hyperforin, or prototypical CAR activators such as phenobarbital and phenytoin.

Rifampicin is involved in many clinically significant drug-drug interactions, and has been shown to affect the plasma levels of many drugs including anticoagulants, contraceptives and glucocorticoids. Treatment of primary cultures of human hepatocytes with rifampicin revealed that

in addition to CYP3A4 and Pgp also other drug metabolizing enzymes (CYP2C8 [63], CYP2C9 [63], CYP2C19 [63], UGT1A1 [64]) and transporters (MRP2 [65]) are upregulated by this drug as well. Furthermore, Oswald *et al*. (2006) studied the protein and mRNA contents of intestinal Pgp, MRP2 and UGT1A1 in 12 healthy subjects after rifampicin administration. After 8 days of treatment Pgp, MRP2 and UGT1A1 mRNA and protein content increased significantly [66]. As a consequence, rifampicin has been shown to increase the rate of drug clearance of, for example warfarin, which is widely used in cancer treatment to prevent (treatment-induced) coagulation. Already in 1974 O'Reilly reported that concomitant treatment of rifampicin and warfarin reduced the AUC of warfarin by 58% [67]. This effect has now been ascribed to PXR mediated CYP2C9 induction by rifampicin [25]. Moreover, rifampicin was shown to cause sub-therapeutic doses of cyclosporine, a CYP3A4 substrate, in a pediatric patient with chronic myeloid leukaemia after bone marrow transplantation. The prolonged sub-therapeutic dose of cyclosporine as a result of rifampicin cotreatment eventually led to failure of the bone marrow transplantation [68, 69].

Because enzymes such as CYP3A4 and CYP2C9 are also involved in the biotransformation and clearance of many widely used antineoplastic agents (table 1), rifampicin can affect the bioavailability of concomitantly administered anticancer drugs as well. Indeed, in a study by Kerbusch *et al.* (2001) it was shown that rifampicin doubled the clearance of ifosfamide. The fraction of ifosfamide metabolized to the dechloroethylated metabolites was also increased, whereas exposure to these metabolites was decreased as a result of enhanced elimination [70]. Furthermore, Bolton *et al*. (2003) found a decrease of the maximum observed concentration (C_{max}) and area under the concentration-time curves (AUC) of imatinib with respectively 54% and 68% when imatinib was coadministered with rifampicin [71]. Other drug-drug interactions involving rifampicin are reviewed by M.Niemi *et al* (2003) [72]. Another example of a potent PXR activator is hyperforin, which is the active constituent of the herbal antidepressant St.John's wort. Healthy volunteers receiving oral imatinib before and after a two-week co-administration of the herbal antidepressant St.John's wort showed a significant alteration of the pharmacokinetics of imatinib. The AUC and Cmax were reduced by 32% and 29%, respectively [73]. In addition, hyperforin was also shown to enhance the clearance of irinote can and its active metabolite SN-38 with respectively 12% and 42% [74].

A similar influence on the plasma levels of several commonly used anticancer agents was observed when these agents were combined with anticonvulsant medications such as phenobarbital and phenytoin. In patients receiving phenobarbital in combination with irinotecan, both the AUC of irinotecan and SN-38 were decreased by respectively 27% and 75% [75]. Moreover, SN-38G/SN-38 AUC ratios in these patients increased, which reflects the induction of UGT1A1. Patients who received irinotecan in combination with phenytoin were also shown to have a decreased AUC of both irinotecan and SN-38 by 63% and 60%, respectively [76], most likely due to CYP3A4 induction, which is involved in the conversion of irinotecan to its inactive metabolite 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxy-camptothecin (APC). Other studies further support the influence of anticonvulsants on the pharmacokinetics of other anticancer drugs such as paclitaxel [77], topotecan [78], methotrexate [79] teniposide [79, 80] and imatinib [81]. The clinical consequences of these observations are far reaching. For instance, low systemic exposure to anticancer drugs as a result of increased clearance has an adverse effect on the efficacy of chemotherapy and has been associated with an increased risk of relapse [79].

In addition to established enzyme inducers such as rifampicin, phenobarbital and phenytoin, and herbal constituents (reviewed by Meijerman *et al*. (2006) [5]), also anticancer drugs have been shown to activate these receptors. In 2001, Synold *et al*. reported that PXR is activated by clinically relevant concentrations of the widely used microtubule stabilizing agent paclitaxel [15]. Nallani *et al*. (2003) showed that testosterone 6β-hydroxylase activity of PCN or paclitaxel-treated wild-type mice increased 30- and 15- fold, respectively, compared to the control [82]. The cyp3a induction response was completely abolished in PCN- and paclitaxel-treated PXR-null mice [82], indicating that paclitaxel exerts its effects on metabolism via activation of PXR. Comparison of testosterone 6β-hydroxylation activity in primary cultures of human hepatocytes, which were exposed to clinically relevant concentrations of paclitaxel or the model PXR agonist rifampicin (48h-96h), resulted in a similar 8-fold increase in enzyme activity [83]. While several studies indicate that paclitaxel is a potent CYP3A4 inducer in human hepatocytes [15, 83-85] and in a cell-based reporter gene assay [15, 85], clinically relevant drug-drug interactions with respect to CYP3A4 induction have not been reported yet. A possible explanation for the lack of clinical evidence is that due to

the pharmacological properties of paclitaxel, drug-drug interactions can be mistaken for side effects attributed to this drug.
Interestingly, docetaxel, which is structurally very related to paclitaxel, only induces CYP3A4 and Pgp to a minor extent [15]. Nallani *et al*. (2004) evaluated the capacity of both paclitaxel and docetaxel to induce CYP3A4 in primary cultures of human hepatocytes and also found that in contrast to paclitaxel, docetaxel did not increase testosterone 6β-hydroxylation [85]. This can be explained by the inability of docetaxel to cause the conformational change of the ligand binding domain of PXR that enables the displacement of a co-repressor protein with a co-activator protein (figure 2) [15]. Therefore, docetaxel might have superior pharmacokinetic characteristics compared to paclitaxel. However, there is no clinical evidence for this assumption (yet).
In addition to the taxane microtubule stabilizing agents, also nontaxane anti-tubulin drugs were tested for their capacity to activate PXR. Two novel anti-tubulin agents, discodermolide and ixabepilone (BMS247550), were shown to increase CYP3A4-reporter gene activity via activation of PXR [86]. The increase in reporter activity was comparable with that of paclitaxel. Mani *et al*. (2005) further investigated the physiologic significance of PXR activation by studying the effect of ixabepilone and paclitaxel on the duration of the loss of righting reflex (LORR) in mice challenged with tribromoethanol. Both paclitaxel and ixabepilone were shown to decrease the mean duration of LORR, which indicates that enzymes involved in the metabolism of tribromoethanol were induced by these agents. The selective estrogen receptor modulator tamoxifen is widely used in the treatment and prevention of estrogen receptor positive breast cancer. Tamoxifen is mainly metabolized by CYP3A4 [87]. In a study on the inductive capacity of tamoxifen, Desai *et al*. (2002) showed that both tamoxifen, and its biologically active metabolite 4-hydroxytamoxifen, are able to activate PXR mediated CYP3A4 induction in primary cultures of human hepatocytes and in a cell-based reporter gene assay [88]. The CYP3A4 induction after exposure to 4-hydroxytamoxifen was comparable with that of rifampicin, indicating that tamoxifen has the propensity to cause clinically relevant drug-drug interactions when combined with other (anticancer) drugs. Indeed, in a clinical trial tamoxifen reduced the plasma levels of concomitantly administered aromatase inhibitors letrozole and anastrazole, which are substrates for CYP3A4, by respectively 37% and 27% [89].

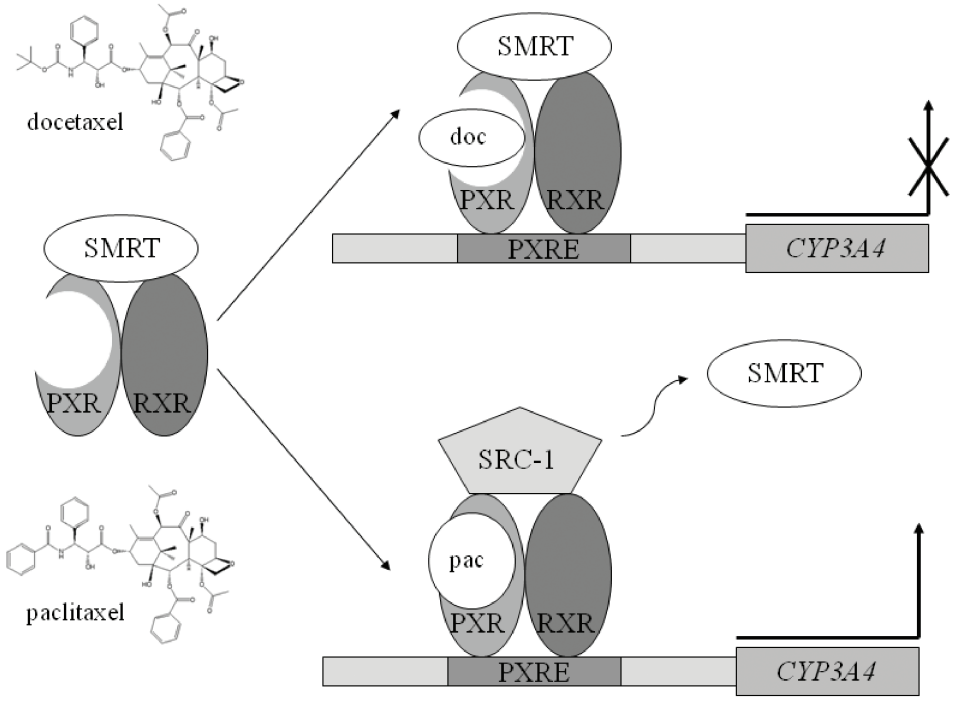

Figure 2 Mechanism behind the differential effects of two structurally related anticancer drugs on the induction of CYP3A4

Unliganded PXR interacts with the corepressor SMRT, which represses gene transcription. Binding of paclitaxel triggers a conformational change of the LBD, which stimulates the displacement of SMRT and simultaneously recruits coactivators such as SRC-1. In addition, coactivators recruit a battery of other proteins that are involved in transcriptional activation of CYP3A4 eventually leading to upregulation. In contrast to paclitaxel, the structurally related taxane docetaxel binds to the LBD of PXR, but is not able to displace SMRT and thus does not causes CYP3A4 induction.

In addition, tamoxifen was also shown to accelerate its own clearance as a result of autoinduction [88]. Cyclophosphamide is widely used to treat a variety of cancers. As already mentioned, this alkylating prodrug is activated predominantly by CYP2B6 and CYP3A4 to its active cytotoxic 4-hydroxycyclophosphamide metabolite [90]. Treatment of primary cultures of human hepatocytes with potent nuclear receptor agonists like phenobarbital, dexamethasone and rifampicin resulted in markedly increased 4-hydroxylation of both cyclophosphamide and its isomer ifosfamide [91]. Increased 4-hydroxylation eventually can lead to systemic toxicities as a result of higher blood levels of the cytotoxic metabolites of cyclophosphamide. Cyclophosphamide was also shown to autoinduce its own clearance in cultured human hepatocytes [91]. The molecular basis

for this observation was provided by Lindley *et al*. (2002) who reported that cyclophosphamide was able to activate PXR. On the other hand, they also found that cyclophosphamide more potently induced CYP2B6 than CYP3A4, which may imply a role for CAR, the key regulator of CYP2B6. However, this has not been confirmed yet. In contrast to anticancer drugs like paclitaxel, tamoxifen and cyclophosphamide, that are PXR agonists, the novel marine derived anticancer agent ecteinascidin (ET)-743 (Yondelis®, Trabectedin®) has been shown to inhibit the transcriptional upregulation of CYP3A4 and MDR1 by directly antagonizing PXR [15]. CYP3A4 enzyme activity was measured using 7-benzyl-4-(trifluoromethyl) coumarin in HepG2 and several osteo sarcoma cell lines and ET-743 was shown to significantly decrease CYP3A4 activity in these cell lines [92]. Furthermore, pretreatment of an osteosarcoma cell line with ET-743 significantly enhanced the cytotoxicity of doxorubicin. The increase in cytotoxicity was associated with a downregulation of Pgp [92]. Because doxorubicin is a substrate for Pgp, down-regulation of this efflux transporter will result in higher intracellular levels of this antineoplastic agent. In addition, pretreatment with ET-743 of Pgp-overexpressing cell lines, which had become resistant to both vincristine and doxorubicin, partially reversed the resistance of these cells to these agents as a result of Pgp downregulation [93]. Furthermore, since PXR has also been shown to be expressed in the human brain [94], possibly co- or pretreatment with PXR antagonists like ET-743 may improve brain-penetration of cytotoxic agents by downregulation of Pgp. However, no data have been published on this issue and this should be studied further. Recently, a second compound, the phytochemical sulforaphane which is abundantly found in cruciferous vegetable such as broccoli and sprouts [95], was shown to bind to the LBD of PXR and act as a antagonist of PXR [96]. Furthermore, sulforaphane was also shown to suppress constitutive and inducible CYP3A4-mediated midazolam clearance in primary cultures of human hepatocytes. Sulforaphane antagonizes PXR at nutrionally relevant concentrations. For example, 100 g broccoli results in a sulforaphane plasma concentration of 2.3 μM [95], while Zhou *et al*. (2006) showed that 1 μM of sulforaphane had a significant effect on the induction of CYP3A4 by rifampicin. These observations indicate that pharmacological doses of sulforaphane may have the potential to reduce or prevent adverse drug reactions that arise through PXR mediated enzyme induction.

In addition to direct antagonism of PXR via binding to the LBD, which

results in the inhibition of the inducible expression of its target genes such as CYP3A4, PXR-mediated gene induction can also be modulated by two other mechanisms. The anti-fungal agent ketoconazole was shown to directly bind to liganded PXR [97], thereby allosterically modifying the structure of PXR leading to disruption of the interaction of PXR with SRC-1. Recently, studies by Huang *et al*. (2007) confirmed the ability of ketoconazole to disrupt co-regulator binding to PXR [98] and additionally showed that ketoconazole was able to inhibit CAR-mediated gene induction as well. Moreover, by combining ketoconazole with PCN in wild type (+/+) mice, the duration of LORR compared to PCN treatment alone in these mice was prolonged. In PXR-null (-/-) mice no alteration of the LORR was observed, indicating that PXR is the key mediator of this effect [98]. The second mechanism involves GR. PXR and CAR expression is transcriptionally regulated by GR and antagonism of GR therefore will result in decreased expression of PXR and CAR. Since PXR and CAR are the primary regulators of a battery of drug metabolizing enzymes and drug transporters, GR antagonism indirectly will decrease the expression of their target genes. Recently ketoconazole, already identified as GR-antagonist [54, 99]and micoconazole were shown to abrogate the inducible properties of rifampicin and phenobarbital [100]. This may have important clinical consequences. Cotreatment with ketoconazole or ketoconazole analogs, that are pure GR-antagonists, might improve the pharmacokinetic properties of anticancer drugs, such as paclitaxel, tamoxifen and cyclophosphamide. On the other hand, cotreatment of anticancer agents with ketoconazole could also increase adverse drug reactions as a result of impaired metabolism due to inhibition of CYP3A4 or diminished inducible expression of other bodily defence mechanisms such as efflux transporters. Physicians should be aware of such interactions between ketoconazole and anticancer drugs, and are advised to monitor the plasma levels of the anticancer drugs in order to prevent intoxications, or undertreatment in the case of anticancer prodrugs.

The aforementioned studies provide a clear molecular link between the activation of PXR and CAR, induction of metabolizing enzymes and transporters, and the increased biotransformation and clearance of anticancer drugs. In addition it has become clear that not only well-known enzyme inducers such as rifampicin and phenobarbital, but also several widely used anticancer drugs activate these receptors and therefore have the propensity to exert a negative effect on the efficacy of concomitantly

administered chemotherapeutic agents. In contrast, nuclear receptor antagonists might prove valuable tools in reducing or even preventing adverse drug reactions that arise as a result of treatment with nuclear receptor activating anticancer drugs or co-medication.

Conclusion

The role of PXR and CAR as key mediators of an elaborate network of genes involved in the detoxification and clearance of (anticancer) drugs has become clear. Administration of PXR activating (anticancer) drugs can impact the pharmacokinetic profile of concomitantly given anticancer drugs due to the induction of enzymes and efflux transporters involved in their clearance. Because of the pharmacological properties of anticancer drugs altered pharmacokinetics often lead to decreased therapeutic efficacy or increased toxicities.

Over the past decade, the development of *in vitro* assays has provided useful tools to screen (new) drugs for their ability to activate PXR and subsequent enzyme induction. Further, many compounds have since then been shown to cause clinically relevant drug-drug interactions via activation of PXR. The role of CAR, however, is still unclear due to the lack of good models. It is, therefore, very important to elucidate the mechanisms that are involved in the translocation of this receptor.

In addition, possibly also other receptors are involved in the regulatory mechanisms that underlie induction, and their roles should be further investigated. Understanding the complex regulatory network underlying enzyme induction may eventually be exploited to predict or prevent drug-drug interactions. Furthermore, assessment of PXR as a drug target may lead to drugs that improve the pharmacokinetics of other drugs by inhibiting the action of PXR, or drugs can be chemically modified so that they retain their pharmacological properties, but lack the ability to activate PXR mediated gene expression. This may ultimately provide oncologic regimens with fewer and less severe side effects and possibly enhance antitumor activity.

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Chapter 2

Comparison of two immortalized human cell lines to study nuclear receptor-mediated CYP3A4 induction

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Abstract

Since cytochrome P450 3A4 (CYP3A4) is responsible for the biotransformation of over 50% of all clinically used drugs, induction results in an increased clearance of many concomitantly administered drugs, thereby decreasing treatment efficacy or, in the case of prodrugs, lead to severe intoxications. CYP3A4 induction is regulated by the pregnane X receptor, constitutive androstane receptor, and vitamin D_3 receptor. Since these nuclear receptors show large interspecies differences, accurate prediction of nuclear receptor mediated CYP3A4 induction in humans requires the use of human systems. As primary cultures of human hepatocytes or enterocytes have major drawbacks like poor availability and poor reproducibility, human cell lines are a good alternative. In this study, the widely used HepG2 cell line was compared to the LS180 cell line to serve as a model to study CYP3A4 induction. There was a clear difference between the cell lines with respect to CYP3A enzyme expression and induction. In LS180 CYP3A4 was expressed and was found to be induced by prototypical nuclear receptor agonists, while in HepG2 CYP3A4 was non-responsive to treatment with rifampicin, CITCO or calcitriol. We subsequently evaluated if these host-cell differences also have an effect on CYP3A4 reporter gene activity. We clearly show that there are differences in CYP3A4 reporter activity between the cell lines, and based on these results and those found on mRNA and protein level, we conclude that LS180 is a more suitable cell line to study CYP3A4 induction than the widely used HepG2.

Introduction

The risk of clinically relevant drug-drug interactions that involve the highly inducible cytochrome P450 (CYP) 3A enzyme CYP3A4 is significant, because CYP3A4 has a wide substrate specificity and is involved in the biotransformation of more than 50% of all clinically used drugs [1]. Therefore, several strategies to study CYP3A4 induction have been developed. Phenotyping studies, such as the erythromycin breath test, can be used directly to measure CYP induction in humans. However, this method has some serious disadvantages like the invasive administration of probe substances to humans, labor-intensive protocols, large interindividual differences and difficulties in processing and interpreting the results. Also the use of animals, for both *in vivo* and *in vitro* studies, poses problems, because there are large interspecies differences in the CYP3A4 induction capacity of compounds [2]. Therefore, CYP3A4 induction is mainly studied *in vitro* in primary cultures of human hepatocytes, which are considered to mostly resemble the *in vivo* situation. However, the interindividual donor variability, rapid loss of drug metabolizing enzyme expression, poor availability, and the costs are major drawbacks [3].
The finding that the highly promiscuous pregnane X receptor (PXR; NR1I2) [4] is one of the main regulators of CYP3A4 induction has led to the development of CYP3A4 reporter gene assays. These assays are based on the transient transfection of a cell line with a CYP3A4 reporter construct containing the response elements of PXR located in the proximal (-362/+53) and distal (-7836/-7208) promoter regions of the CYP3A4 gene linked to a firefly-luciferase or other reporter genes such as alkalinephosphatase [5]. In contrast to hepatocytes, many cell lines express lower levels or even no PXR [6-8]. Therefore cell lines used for the reporter gene assay are often co-transfected with a PXR expression plasmid to increase the levels of this nuclear receptor. Since two other nuclear receptors, the constitutive androstane receptor (CAR; NR1I3) [9] and the vitamin D_3 receptor (VDR; NR1I1) [10], are also known to regulate CYP3A4 induction, cells can also be co-transfected with CAR or VDR instead of PXR. At the moment cell-based reporter gene assays have become suitable alternatives to primary cultures of human hepatocytes to study nuclear receptor mediated CYP3A4 induction. Although nuclear receptor expression levels in these reporter gene assays are raised artificially, a good correlation between PXR-mediated CYP3A4 induction measured in a reporter gene assay,

and CYP3A4 mRNA [11] and protein [12] expression in primary cultures of human hepatocytes was reported. Furthermore, by comparing CYP3A4 reporter gene assay data and known data on CYP3A4 induction *in vivo*, Persson *et al*. (2006) showed that a CYP3A4 reporter gene assay is a reliable screening method for the assessment of drug-induced CYP3A4 expression [13].

Currently, the cell line most often used in CYP3A4 reporter gene assays is the human hepatocarcinoma-derived HepG2 cell line [12, 14-20]. However, HepG2 mainly expresses the fetal enzyme CYP3A7 instead of CYP3A4 [21]. In addition, despite the presence of endogenous PXR, CYP3A4 expression is not enhanced by rifampicin treatment in HepG2 cells [22]. Since CYP3A4 is also present in the gastrointestinal tract, and has been shown to contribute significantly to the pre-hepatic metabolism of drugs [23], other groups use the human colon carcinoma-derived LS180 cell line as a host for their CYP3A4 reporter gene assay [24, 25]. In contrast to HepG2, rifampicin was shown to increase CYP3A4 expression in LS180 cells [26] Moreover, in a comparison between three colon carcinoma cell lines (LS180, Caco-2 and TC-7) only LS180 showed inducible CYP3A4 expression [27].

The aim of this study was to investigate the suitability of LS180 cells as a model to study CYP3A4 induction in comparison with the widely used HepG2 cell line. Therefore, CYP3A mRNA and protein expression levels in both cell lines were determined after treatment with prototypical nuclear receptor agonists (rifampicin (PXR), CITCO (CAR), calcitriol (VDR)) that are known inducers of CYP3A4. Furthermore, the use of these cell lines as hosts for CYP3A4 reporter gene assays was evaluated.

Materials and methods

Materials

All cell culture media and supplements were purchased from Invitrogen (Breda, The Netherlands). 6-(4-chlorophenyl)imidazo [2,1-b] [1,3] thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)-oxime (CITCO; purity >98%) was obtained from BIOMOL International (Plymouth Meeting, PA, USA). All other chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Plasmids

The pGL3-CYP3A4-XREM (proximal (-362/+53) and distal (-7836/-7208)) luciferase reporter construct and the pEF-hCAR expression plasmid were a kind gift from Dr. Richard Kim (Vanderbilt University, Nashville, USA), the pCDG-hPXR expression vector was generously provided by Dr. Ron Evans (The Howard Hughes Medical Institute, La Jolla, USA), the pSG5-hVDR expression plasmid was kindly donated by Dr. Bandana Chatterjee (Department of Molecular Medicine/Institute of Biotechnology, University of Texas Health Science Centre, San Antonio), the pRL-TK control plasmid was obtained from Promega (Madison, WI, USA). Plasmids were checked by enzyme restriction and agarose gel electrophoresis and purified using Promega's Pureyield Midi-prep (Madison, WI, USA) according to the instructions of the manufacturer.

Cell culture

The human colon adenocarcinoma-derived cell line, LS180 (used between passage 12 to 14) and the human hepatoma-derived cell line, HepG2 (used between passage 13 and 15) were purchased from ATCC (Manassas, VA, USA). The cell- lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 ++ medium (with 25 mM HEPES and L-glutamine, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin), at 37°C under a humidified atmosphere of 5% CO_2.

Treatment

HepG2 and LS180 cells were plated at a density of $1x10^6$ cells/well in 6-well plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) in 2ml RPMI 1640 medium++. After reaching 80-90% confluency, medium was replaced with medium containing different concentrations of rifampicin (1 µM or 10 µM), CITCO (10 nM or 250 nM) or calcitriol (1 nM or 100 nM) and refreshed after 24 hours. The final solvent concentrations did not exceed 0.1%. After 48 hours cells were washed with phosphate buffered saline (PBS). The cells were further used for immunoblot analysis or qPCR as described below.

RNA Extraction and RT-PCR

Total RNA was extracted using the GeneElute Mammalian total RNA miniprep kit (SigmaAldrich, Zwijndrecht, The Netherlands). RNA integrity and quantity were determined using a Nanodrop Diode Array Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). 1 µg of total RNA was reverse transcribed according to the manufacturer guidelines concerning the random hexamer primer (RevertAid™ First Strand cDNA synthesis kit, Fermentas, St.Leon-Rot, Germany).

Quantitative RT-PCR

The CYP3A4, CYP3A5, CYP3A7, and 18S mRNA expression levels were analyzed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). All reactions were singleplexed with 18S. Oligonucleotide primers and a Taqman probe for CYP3A4 were as follows: forward, TCAATAACAGTCTTTCCATTCCTCAT; reverse, CTTCGAGGCGACTTTCTTTCA; and probe, TGTTTCCAAGAGAAGTTACAAA. The primers and probe used for 18S, CYP3A5 (Hs00241417_m1), and CYP3A7 (Hs00426361_m1) real-time PCR were commercially available Assay on Demand kits (Applied Biosystems, Foster City, CA, USA). According to manufacturer guidelines, data were expressed as threshold cycle value (ct) and used to determine dct values. Fold changes in expression were calculated according to the transformation: fold increase = $2^{-(\text{difference in dct})}$.

Immunoblot analysis

After 48 hours, cells were harvested and lysed in 250 µl PBS containing 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, and 1% protease inhibitor cocktail tablet (Roche, Basal, Switzerland). Protein concentrations were determined by a Pierce BCA protein assay (Pierce, Rockford, IL, USA) and 10 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis on NuPage Novex Bis-Tris precast gradient gels (4-12%) (Invitrogen, Breda, The Netherlands). Human CYP3A4 protein (Gentest, Becton Dickinson, Woburn, MA, USA) was used as a control. Proteins were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA, USA). After overnight blocking in 3% bovine serum albumin, the membranes were incubated with a murine monoclonal anti-human CYP3A primary antibody (1:500; Gentest, Becton Dickinson, Woburn, MA, USA). This antibody is known to cross-react with both CYP3A4 and CYP3A7, but not with CYP3A5. Next, the blot is incubated with a bovine anti-mouse IgG coupled to horse radish peroxidase (HRP) secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized by a chemiluminescence-based detection reagent (Supersignal West Femto, Pierce, Rockford, IL, USA) and the intensities of the CYP3A bands were determined on a Gel Doc Imaging system equipped with a XRS camera with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

CYP3A4-reporter gene assay

LS180 or HepG2 cells were seeded (2 x 10^5 cells/well and 5 x 10^5 cells/well respectively) in 96-well plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) in 200 µl RPMI 1640 ++ medium and incubated overnight in 5% CO_2-humidified, 37°C atmosphere. Following incubation, the cells were transfected with 75 ng/well of nuclear receptor expression vector (pCDG-hPXR, pEF-hCAR, or pSG5-hVDR), 210 ng/well of the CYP3A4 luciferase reporter construct (pGL3-CYP3A4-XREM), and 15 ng/well of the renilla luciferase expression controle vector (pRL-TK), using 0.99 µl/well Exgen500 *in vitro* transfection reagent (Fermentas, St.Leon-Rot, Germany) in 150 mM NaCl. In addition, to study the effect of endogenously expressed PXR, CAR or VDR on CYP3A4 reporter activity, transfections were performed in which the nuclear receptor expression plasmids were

replaced by an empty plasmid (pSG5; 75 ng/well). After overnight transfection, the medium was removed, cells were washed with phosphate buffered saline (PBS), and fresh medium (200 µl) containing different concentrations of the inducers rifampicin, CITCO or calcitriol, were added to the wells. Rifampicin and CITCO were dissolved in DMSO, while calcitriol was dissolved in ethanol.The final solvent concentration did not exceed 0.1%. After 48 hours, the medium was removed, cells were washed with PBS, and lysed with 20 µl/well Passive Lysis Buffer (Promega, Medison, WI, USA) for 15 minutes on a shaker. The cell lysates (10 µl) were transferred to a white half area 96-well plate (Corning BV, Schiphol-Rijk, The Netherlands) and the reporter activities of firefly luciferase and renilla luciferase were determined using the Dual-Luciferase™ Reporter (DLR™) Assay System (Promega, Medison, WI, USA) according to the manufacturer's manual, with reagent volumes adjusted to the cell lysate volume (Promega, Madison, WI, USA). Luminescence was recorded on a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). The fold induction was calculated by normalization of the firefly-luciferase signal to the renilla-luciferase signal.

Statistical analysis

Data were analyzed by using one-way ANOVA and the means were compared by applying the Bonferroni post-hoc test and were considered statistically significant when $P < 0.05$. The correlation between the data obtained from the CYP3A4 reporter gene assays and the protein expression levels were determined using the Pearson correlation coefficient. All statistical determinations were performed with SPSS v14.

Results

Inducible expression of CYP3A mRNA in LS180 and HepG2

First we considered which CYP3A enzymes are induced following treatment with rifampicin, CITCO and calcitriol in LS180 and HepG2. As shown in figure 1, in LS180 a significant increase of CYP3A4 mRNA expression was observed after exposure to rifampicin and calcitriol, while a less marked induction of CYP3A5 mRNA expression was observed following treatment with these compounds. CITCO however did not affect CYP3A4, CYP3A5 or CYP3A7 mRNA expression in LS180. In HepG2 no significant increase in CYP3A mRNA expression was detected after exposure to rifampicin, CITCO or calcitriol.

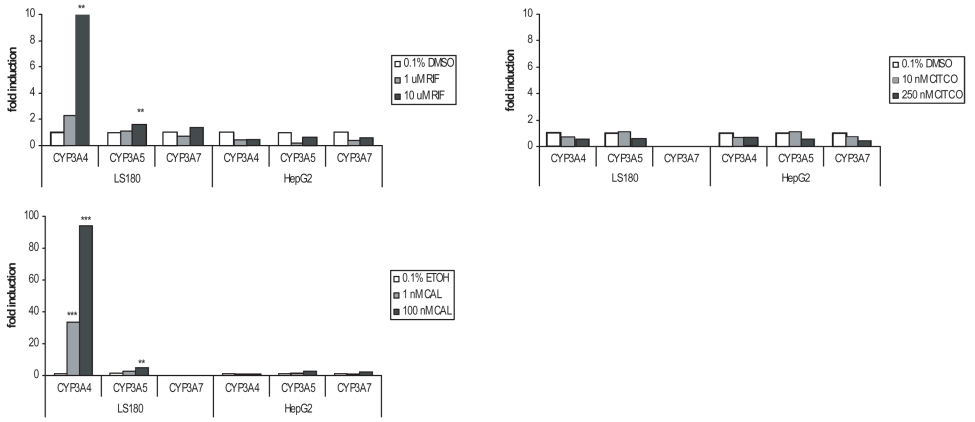

Figure 1 Inducible expression of CYP3A enzymes in HepG2 and LS180
The inducibility of the different CYP3A enzyme mRNA levels were determined after 2 × 24 hours treatment of the cells with 0.1% DMSO, rifampicin (1 μM and 10 μM), CITCO (10 nM and 250 nM), and calcitriol (1 nM and 100 nM). Total RNA was isolated and converted to cDNA, which was assessed at least in triplicate using singleplexed quantitative Taqman real-time PCR (CYP3A4, CYP3A5, CYP3A7) and normalized to 18S. The results are expressed as fold induction over the control (0.1% DMSO/EtOH). Statistical analysis on real-time PCR data were performed on mean dct values (and not fold changes) to exclude potential bias attributable to averaging data that had been transformed through the equation $2^{-(\text{difference in dct})}$ [38] (significance * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Inducible expression of CYP3A protein in LS180 and HepG2

The CYP3A protein response profile after exposure to rifampicin, CITCO or calcitriol was examined in LS180 and HepG2 using Western immunoblotting. As shown in figure 2, inducible expression of CYP3A protein was

detected after exposure to rifampicin and calcitriol in LS180. CITCO did not increase CYP3A protein expression in LS180 cells. In HepG2 the protein expression of CYP3A was very low and was not induced after treatment with the prototypical nuclear receptor agonists rifampicin, CITCO and calcitriol (data not shown).

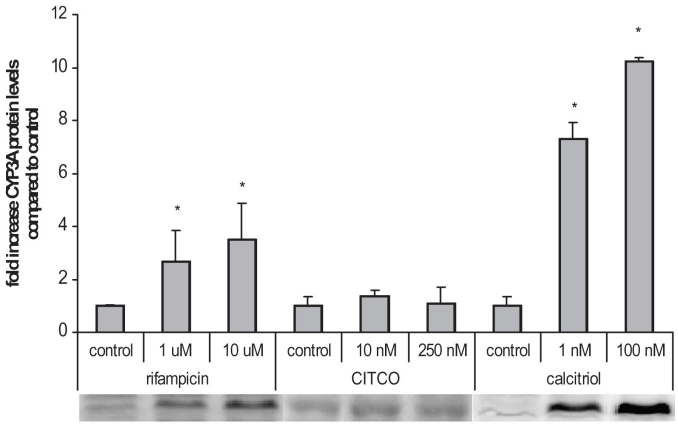

Figure 2 CYP3A induction in LS180 after treatment with prototypical nuclear receptor agonists

Proteins (10 µg) were separated using SDS-PAGE, transferred to Immobilon-P membrane and probed with mouse-antihuman CYP3A4/7 antibody. Blots were subjected to densitometric analysis. The level of induction is expressed as an increase in CYP3A protein levels. The results are derived from a representative experiment and data are the means (± SD) from three separate determinations and are expressed as the relative induction compared to the vehicle (significance * $P<0.05$)

Effect of endogenously expressed nuclear receptors on CYP3A4 reporter activity

To evaluate the effect of endogenously expressed nuclear receptors on the CYP3A4 reporter gene activity, LS180 and HepG2 cells were transiently transfected with the CYP3A4 reporter construct and an "empty" nuclear receptor expression plasmid. The transfected cells were treated with the corresponding nuclear receptor agonists. Treatment of LS180 with 100 nM calcitriol for 48 hours resulted in a major increase in CYP3A4 reporter activity (figure 3). In HepG2 also an increase in CYP3A4 reporter gene activity was observed after treatment with calcitriol, but it was much lower compared to the CYP3A4 reporter activity in LS180. This indicates that

both cell lines express functional endogenous VDR. Exposure to rifampicin led to a small, but significant ($P<0.05$) increase in CYP3A4 reporter gene activity in LS180, but not in HepG2, indicating the presence of functional endogenous PXR in LS180. CITCO did not significantly increase CYP3A4 reporter activity in both LS180 and HepG2 compared to the vehicle 0.1% DMSO, which indicates that both cell lines do not express high levels of functional CAR.

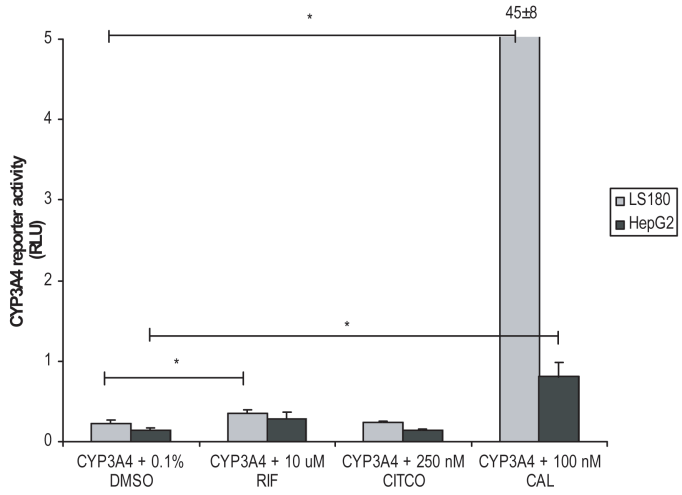

Figure 3 Effect of endogenous nuclear receptor expression on CYP3A4 reporter activity
LS180 and HepG2 cells were transfected with the CYP3A4-reporter construct and an empty nuclear receptor expression plasmid. Cells were treated for 48 hours with the nuclear receptor agonists rifampicin (RIF; 10 µM), CITCO (250 nM) or calcitriol (CAL; 100 nM). Only in LS180 calcitriol induced CYP3A4 reporter activity, which indicates that LS180 expresses high endogenous levels of VDR (significance * $P<0.05$).

CYP3A4 reporter gene activity in LS180 and HepG2

To examine the influence of the host cell line on the activity of the CYP3A4 reporter gene assay in the presence of exogenous PXR, CAR or VDR, LS180 and HepG2 were transiently transfected with the CYP3A4 reporter construct combined with PXR-, CAR- or VDR-expression plasmids, followed by exposure to different concentrations of the corresponding agonists. The CYP3A4 reporter activities in both LS180 and HepG2 as a result of nuclear receptor activation are shown in figure 4.

In LS180 rifampicin, CITCO and calcitriol were able to significantly increase CYP3A4 reporter gene activity compared to the control, while in HepG2 only rifampicin and calcitriol gave a significant increase in CYP3A4 reporter gene activity compared to the control. Moreover, the increase of PXR- and VDR-mediated CYP3A4 reporter activity following treatment with respectively rifampicin and calcitriol in HepG2 was significantly lower (~2 fold) compared to the increase of CYP3A4 reporter gene activity in LS180 after treatment with the same nuclear receptor agonists. In contrast to rifampicin and calcitriol, CITCO only increased CAR-mediated CYP3A4 reporter gene activity in LS180 and not in HepG2.

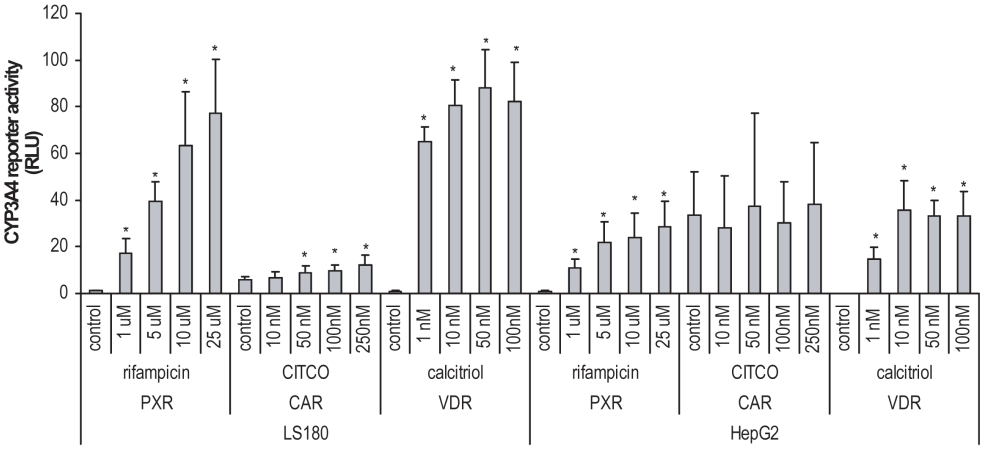

Figure 4 Differential response in CYP3A4 reporter activity upon activation of three distinct nuclear receptors in LS180 and HepG2

LS180 and HepG2 cells (2 x 10^5 cells/ml and 5 x 10^5 cells/well, respectively) were transfected with the pGL3-CYP3A4-XREM reporter construct, and the nuclear receptor expression vectors pCDG-hPXR, pEF-hCAR or pSG5-hVDR and the pRL-TK control vector. After 24 hours of transfection, cells were exposed to the corresponding nuclear receptor agonists serially diluted in DMSO (rifampicin: 1, 5, 10, 25 μM ; CITCO: 10, 50 ,100, 250 nM ; calcitriol: 1, 10, 50, 100 nM) with a final solvent concentration of 0.1%. After 48 hours, luciferase activity was measured. These results are derived from a representative experiment and data are the means (± SD) from three separate determinations and is expressed as absolute reporter activity (significance (* $P<0.05$)).

Discussion

In the present study, we explored the suitability of the colon carcinoma-derived LS180 cell line for its use in CYP3A4 induction studies compared to the hepatoma-derived HepG2 cell line. HepG2 is still the most widely used cell line in CYP3A regulation and induction studies. Although several groups have already shown that the HepG2 cell line expresses high levels of the fetal CYP3A enzyme CYP3A7 [21, 28]. In contrast, LS180 has been shown to express CYP3A4 [23, 26].

To evaluate the difference in CYP3A enzyme inducibility between HepG2 and LS180, both cells were challenged with the prototypical nuclear receptor agonists rifampicin, CITCO or calcitriol, and mRNA and protein expression were determined. mRNA analysis of the different CYP3A enzyme expression levels in both cell lines revealed that CYP3A4 and CYP3A5 were induced after treatment with rifampicin or calcitriol in LS180, while none of the CYP3A mRNA enzyme levels in HepG2 were induced after treatment with these agents. Protein expression analysis showed that the expression of CYP3A protein was inducible in LS180 only. The protein levels increased after treatment with the prototypical CYP3A4 inducers rifampicin and calcitriol. Although the antibody used could not discriminate between the CYP3A4 and CYP3A7 enzymes, it can be assumed that CYP3A4 protein expression levels in this cell line were induced based on the results of the mRNA analysis, which clearly show that only CYP3A4 mRNA expression levels are inducible following treatment with these compounds.

In HepG2, CYP3A protein was hardly detectable even after treatment with rifampicin, CITCO or calcitriol. This is consistent with the mRNA analysis data, which also show no CYP3A induction after treatment with the same compounds. Furthermore, as mentioned before HepG2 expresses CYP3A7, which is not induced by rifampicin [29, 30]. Krusekopf only observed pronounced CYP3A7 induction in HepG2 after treatment with typical human glucocorticoid receptor (hGR) agonists indicating an important role for the hGR in CYP3A7 regulation. Furthermore, the promoter sequence of the CYP3A7 gene has two mutations in the proximal ER6 repeat, which has implications for binding of nuclear receptors of the NR1I family (e.g. PXR, CAR, VDR). These mutations lead to less pronounced binding of liganded VDR to the CYP3A7 promoter resulting in a loss of gene activa-

tion after calcitriol treatment [31]. Since PXR also recognizes and binds to ER6 repeats, the mutations may affect the binding of PXR to the CYP3A7 promoter as well, which could explain the lack of response to rifampicin treatment of HepG2 cells with respect to CYP3A7 induction.

The results discussed above clearly show that HepG2 and LS180 have distinct CYP3A induction profiles. We therefore evaluated if this alternate expression pattern also affects CYP3A4 reporter activity. Indeed, in the CYP3A4 reporter gene assay, both cell lines clearly showed distinct responses after treatment with the prototypical nuclear receptor agonists. Treatment of LS180 and HepG2 that were transfected with CYP3A4 reporter constructs without contransfection of nuclear receptor expression plasmids, resulted in a significant ($P<0.05$) increase of reporter activity after treatment with rifampicin and calcitriol in the LS180 cell line. These results indicate that functional PXR and VDR are endogenously expressed in this cell line.

The most pronounced effect on CYP3A4 reporter activity was found when both cell lines were co-transfected with the nuclear receptor expression plasmids of PXR or VDR and subsequently treated with the corresponding agonists. Comparison of the CYP3A4 reporter activities in LS180 and HepG2 cells after PXR or VDR activation revealed that there was a significant difference in the increase of CYP3A4 reporter activity between the cell lines. The fold induction of CYP3A4 reporter activity in LS180 cells was about 2 fold higher for both nuclear receptors than the fold induction in HepG2.

The CYP3A4 reporter activities in the presence of PXR expression plasmid in LS180 showed a good correlation with CYP3A4 protein expression levels, which in turn were in concordance with CYP3A4 protein expression levels found in primary cultures of human hepatocytes that were treated with rifampicin [32]. In the case of calcitriol, however, the CYP3A4 protein levels were twice as high in LS180 as in human hepatocytes [10]. This difference is probably a result of the high endogenous expression of VDR in LS180. The high endogenous VDR expression may cause problems with respect to CYP3A4 reporter gene assays that are cotransfected with other nuclear receptors such as PXR, because PXR and VDR bind to the same response elements within the promoter region of the CYP3A4-luciferase construct. However, in contrast to the highly promiscuous PXR, VDR has a very narrow ligand specificity and only bile acids and vitamin D derivatives are known to activate this receptor [31, 33].

Therefore, although endogenous VDR expression is high in LS180 cells, it still is a suitable cell line to study the effect of xenobiotics on CYP3A4 induction, as most compounds exert their effect on CYP3A4 expression through the more promiscuous PXR [2, 5, 16].

In addition to PXR and VDR, CAR is also able to cross regulate CYP3A4 [34]. Therefore the role of CAR activation on the induction of CYP3A4 was investigated. CAR is, just like PXR [35] and VDR [36], located in the cytoplasm *in vivo*. In contrast to PXR and VDR, CAR translocation is stimulated in a ligand-independent manner. Phenobarbital, phenytoin and bilirubin have been shown to trigger CAR nuclear translocation without binding to the LBD of CAR. Due to the constitutive activity of CAR, translocation results in transcriptionally activation of its target genes. In HepG2 cells it has been reported that CAR spontaneously translocates to the nucleus due to a lack of cytoplasmic CAR retention protein (CCRP) [37]. As a consequence, CAR transfection may automatically result in increased nuclear accumulation and enhanced transcription of its target genes (e.g. CYP3A4) in a ligand-independent manner due to its constitutive activity. This might explain the high ligand-independent CYP3A4 reporter activation in the HepG2 cell line after co-transfection with CAR. Co-transfection of CAR in the LS180 cell line also resulted in increased CYP3A4 reporter gene activity, but additional treatment with CITCO resulted in a dose-dependent increase of the CYP3A4 reporter gene activity. CITCO is the only known agonist of CAR, and causes transcriptional activation as a result of direct binding to the ligand binding domain of CAR. This triggers ligand-dependent nuclear accumulation and results in increased CYP3A4 reporter gene activity. As a consequence, LS180 cells could be used to evaluate the potential of compounds to bind directly to the LBD of CAR and subsequently activate transcription in a ligand-dependent manner. Currently, however, no validated systems are available to screen compounds for their capacity to activate CAR either ligand-dependently or independently.

In conclusion, there is a clear difference in the inducible protein expression of CYP3A between both cell lines. We clearly show that HepG2 is inferior to LS180 with respect to CYP3A4 induction. The alternate CYP3A enzyme expression and gene regulation in HepG2 compromises the use of this cell line for CYP3A4 induction studies. Based on our results we therefore recommend the use of the LS180 cell line to study CYP3A4 induction instead of the widely used HepG2.

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Chapter 3

Nuclear receptor-mediated induction of cytochrome P450 3A4 by anticancer drugs: a key role for the pregnane X receptor

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Abstract

Induction of cytochrome P450 (CYP) 3A4, an enzyme that is involved in the biotransformation of more than 50% of all drugs, by xenobiotics is an important cause of pharmacokinetic drug-drug interactions in oncology. In addition to rifampicin and hyperforin, the anticancer drug paclitaxel has also been shown to be an inducer of CYP3A4 via activation of the pregnane X receptor. We therefore screened a panel of widely used anticancer drugs for their ability to activate PXR-mediated CYP3A4 induction. A CYP3A4 reporter gene assay was employed to identify PXR agonists among the eighteen anticancer drugs. Subsequently CYP3A4 mRNA and protein expression following treatment with these PXR agonists was assessed. Finally, the effect of pretreatment with these agents on the 1'-hydroxylation of midazolam (a specific CYP3A4 probe) was determined. Paclitaxel, erlotinib, tamoxifen, ifosfamide, flutamide and docetaxel are able to activate PXR, while only strong PXR activation leads to significant induction of CYP3A4 activity. The identified PXR agonists may have the propensity to cause clinically relevant drug-drug interactions as a result of CYP3A4 induction.

Introduction

The treatment of cancer often constitutes a combination of multiple anticancer drugs or hormonal agents, supplemented with a wide range of symptomatic therapies to treat or prevent regimen-related side effects. The risk of drug-drug interactions, however, increases with the number of concomitantly administered medication [1]. A significant part of these drug-drug interactions are pharmacokinetic interactions [2, 3]. Due to the narrow therapeutic index and steep dose-toxicity curves of chemotherapeutic agents, small changes in the pharmacokinetic profile can significantly alter the toxicity and therapeutic efficacy of these agents.

Alterations in the pharmacokinetic profile of anticancer drugs often involve inhibition or induction of drug-metabolizing enzymes or drug transporters. One of the most important drug-metabolizing enzymes is cytochrome P450 (CYP) 3A4, which represents approximately 30% of total hepatic, and up to 70% of total intestinal CYP content. Human CYP3A4 has a wide substrate specificity and is involved in the biotransformation of more than 50% of all clinically used drugs [4], including several antineoplastic agents. The expression of CYP3A4 is highly inducible and concomitant administration of CYP3A4 inducers, like phenobarbital or rifampicin, limits the oral bioavailability of drugs that are substrate for this enzyme, possibly resulting in sub therapeutic dosing or therapeutic failure. In addition, enhanced anticancer prodrug activation, due to induction, might increase morbidity and mortality as a result of increased blood levels of cytotoxic metabolites.

CYP3A4 expression is transcriptionally regulated by members of the NR1I nuclear receptor subfamily of ligand-activated transcription factors, which constitutes the human pregnane X receptor (PXR; NR1I2) [3, 5, 6], the vitamin D_3 receptor (VDR; NR1I1) [7] and the constitutive androstane receptor (CAR, NRI3) [8, 9]. Only a few agonists are known for CAR and VDR, while PXR is activated by a wide variety of structurally unrelated compounds that include rifampicin, phenobarbital and hyperforin, but also anticancer drugs like paclitaxel [10] and tamoxifen [11]. Upon agonist binding, the nuclear receptors heterodimerize to the retinoid X receptor α (RXRα; NR2B1) and bind to distinct motifs within the promoter area of CYP3A4 [12]. Nuclear receptor activation is therefore one of the major mechanisms behind drug-drug interactions due to induction of CYP3A4.

We hypothesize that also other anticancer drugs induce CYP3A4 by activation of the nuclear receptor PXR. To evaluate the potential of anticancer drugs to cause or reduce pharmacokinetic drug-drug interactions by direct binding to nuclear receptors, we examined a panel of widely used anticancer drugs for their capacity to modulate nuclear receptor mediated CYP3A4 expression in a cell-based nuclear receptor (PXR) reporter gene assay [12]. In addition, also effects on CYP3A4 mRNA and protein expression were studied, as well as midazolam 1'-hydroxylation to determine CYP3A4 activity after exposure to these anticancer agents.

Materials and methods

Materials

All cell culture media were purchased from PAA (Colbe, Germany). Cell media supplements were purchased from Invitrogen (Breda, The Netherlands). cDNA-expressed CYP3A4 + reductase and b5 supersomes and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) were purchased from BD biosciences (Alphen a/d Rijn, The Netherlands). Nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt was obtained from Alkemi (Lokeren, Belgium). All other chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Anticancer agents

Carboplatin, ifosfamide, doxorubicin hydrochloride, epirubicin hydrochloride, irinotecan hydrochloride, topotecan hydrochloride, tamoxifen citrate and etoposide were purchased form Axxora (San Diego, CA, USA). Cisplatin, cyclophosphamide hydrate, flutamide, paclitaxel, docetaxel, vinblastine, vincristine and 5-fluorouracil were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Erlotinib (Tarceva®) and imatinib mesylate (Gleevec®) were provided by the Dutch Cancer Institute / Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands).

Plasmids

The pGL3-CYP3A4-XREM luciferase reporter construct [12] was a kind gift from Dr.Christopher Liddle (Westmead Millenium Institute, Westmead, Australia), the pCDG-hPXR expression vector was generously provided by Dr. Ron Evans (Salk institute for biological studies, La Jolla, CA, USA), and the pRL-TK control plasmid was obtained from Promega (Madison, WI, USA). Plasmids were checked by enzyme restriction and agarose gel electrophoresis and purified using Promega's Pureyield Midi-prep (Madison, WI, USA) according to the instructions of the manufacturer.

Cell culture

The human colon adenocarcinoma-derived cell line LS180 was purchased from the ATCC (Manassas, VA, USA). The cell- line was maintained in Roswell Park Memorial Institute (RPMI) 1640 ++ medium (with phenolred, 25 mM HEPES and L-glutamine, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin), at 37°C under a humidified atmosphere of 5% CO_2.

Nuclear receptor-reporter gene assay

LS180 cells were seeded in 96-well plates (5 x 10^4 cells/well) in 200 µl RPMI 1640 ++ medium and incubated overnight in 5% CO_2-humidified, 37°C atmosphere. Following incubation, the cells were transfected with 75 ng/well of nuclear receptor expression vector (pCDG-hPXR), 210 ng/well of the CYP3A4 luciferase reporter construct (pGL3-CYP3A4-XREM), and 15 ng/well of the renilla luciferase expression control vector (pRL-TK), using 0.99 µl/well Exgen500 *in vitro* transfection reagent (Fermentas, St.Leon-Rot, Germany) in 150 mM NaCl. After overnight transfection the medium was removed and cells were washed with PBS. Test compounds, diluted in DMSO, were added to the plates and serially diluted in culture medium. Rifampicin (10 µM) was used as a positive control in the PXR-reporter gene assay, respectively. The final solvent concentration did not exceed 0.1%. After 48 hours, the medium was removed, cells were washed with PBS, and lysed with 20 µl/well Passive Lysis Buffer (Promega, Madison, WI, USA) for 15 minutes on a shaker.

The cell lysates (5 µl) were transferred to a white 96-well half area plate (Corning, Corning, NY, USA) and the reporter activities of firefly luciferase and renilla luciferase were determined using the Dual-Luciferase™ Reporter (DLR™) Assay System according to the manufacturer's manual, with reagent volumes adjusted to the cell lysate volume (Promega, Madison, WI, USA). Luminescence was recorded on a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). The fold induction was calculated by normalization of the firefly-luciferase signal to the renilla-luciferase signal.

Induction studies

LS180 cells were plated at a density of 1 x 10^6 cells/well in 6-well plates in 2ml RPMI 1640 ++. After reaching 80-90% confluency, medium was replaced with medium containing the different anticancer drugs cisplatin (20 µM), carboplatin (20 µM), cyclophosphamide (300 µM), ifosfamide (300 µM), docetaxel (20 µM), paclitaxel (20 µM), flutamide (20 µM), tamoxifen (20 µM), erlotinib (10 µM) and rifampicin (10 µM; positive control) and DMSO (0.1%; negative control). The concentrations chosen correspond with peak plasma concentrations of the anticancer drugs in standard anticancer regimens, while for ifosfamide and cyclophosphamide a peak plasma concentration that is reached in high-dose regimens was chosen. The cells were treated for two consecutive days with the drugs and the controls. At the end of each treatment period, the medium was removed and the cells were washed with PBS. After the washing, the cells were used to determine the fold increase of CYP3A4 mRNA by using qPCR. Protein expression levels were determined with Western blotting, and midazolam 1'-hydroxylation was used to determine the CYP3A4 activity in these drug treated cells.

RNA Extraction and RT-PCR

Total RNA was extracted using the GeneElute Mammalian total RNA miniprep kit (SigmaAldrich, Zwijndrecht, The Netherlands). RNA integrity and quantity were determined using a Nanodrop Diode Array Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). Next, 1 µg of total RNA was reverse transcribed according to the manufacturer guidelines using a random hexamer primer (RevertAid™ First Strand cDNA synthesis kit, Fermentas, St.Leon-Rot, Germany).

Quantitative RT-PCR

The CYP3A4 and housekeeping gene (18S) mRNA expression levels were analyzed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). All reactions were singleplexed with the housekeeping gene (18S). Oligonucleotide primers and a Taqman probe for CYP3A4 were as follows: forward, 5′-TCAATAACAGTCTTTC-CATTCCTCAT-3′; reverse, 5′-CTTCGAGGCGACTTTCTTTCA-3′; and probe, 5′-TGTTTCCAAGAGAAGTTACAAA-3′. The primers and probe used for 18S real-time PCR were a commercially available Assay on Demand (Applied Biosystems, Foster City, CA, USA). According to manufacturer guidelines, data were expressed as threshold cycle value (ct) values and used to determine dct values. Fold changes in expression were calculated according to the transformation: fold increase = $2^{-(\text{difference in dct})}$.

Western immunoblot analysis

The cells that were treated with the different anticancer drugs were lysed in 250 µl PBS containing 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, and 1% (w/v) complete protease inhibitor cocktail tablet EDTA-free (Roche, Basal, Switzerland). Protein concentrations were determined by a Pierce BCA protein assay (Pierce, Rockford, IL, USA) and 25 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis (10%). Proteins were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA, USA). After overnight blocking in 3% bovine serum albumin, the membranes were incubated with a murine monoclonal anti-human CYP3A primary antibody (1:500; Gentest, Becton Dickinson, Woburn, MA, USA) or β-actin (1:10000; AC-15; Abcam, Cambridge, UK) followed by incubation with a bovine anti-mouse IgG coupled to horse radish peroxidase (HRP) secondary antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized by a chemiluminescence-based detection reagent (West Femto; Pierce Biosciences) and the intensities of the CYP3A4 bands were determined on a ChemiDoc XRS Imaging system and analyzed with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Cell-based CYP3A4 activity assessment

The cells that were treated with the different anticancer drugs were incubated with 4 µg/ml midazolam (MDZ) in phenolred-free RPMI without FBS and penicillin/streptomycin for three hours. After the incubation period, the medium was aliquoted and centrifuged for 5 minutes at 10000 x g. The supernatant was further analysed using HPLC-UV.

HPLC-UV analysis

The HPLC-UV analysis was performed on a Symmetry C18 column (150 mm x 3 mm; 3.5 µm; Waters, Milford, MA, USA) protected with a MetaGaurd C18-a guard column (2.0 mm Polaris 3 µm; Varian, Palo Alto, CA, USA). Both columns were maintained at 40°C. The mobile phase consisted of 75:25 (v/v) water/acetonitrile (containing 4.35 mM perchloric acid; pH=2.4). The flow rate was 0.5 ml/min. The HPLC system comprised a LC-10AT pump (Shimadzu, Kyoto, Japan), a SIL-10AD autoinjector (Shimadzu, Kyoto, Japan), and a SCL-10A system controller (Shimadzu, Kyoto, Japan). Midazolam and its metabolite 1'-hydroxymidazolam were detected with a SPD-M10 diode array detector (220 nm; Shimadzu, Kyoto, Japan). The precision was evaluated by repeatability and intermediate precision; both were <15%.

Fluorometric enzyme activity assay

Incubations were conducted in black 96-well microtiter plates based on the method described on the GENTEST Corporation website (www.gentest.com). Each well contained recombinant human CYP3A4 protein (supersomes; 1 nM), 1 mM NADPH, 10 µM of the test compound and different concentrations that varied between 50 µM and 50 nM of the non-fluorescent CYP3A4 probe BFC, in 200 µL 100 mM potassium phosphate (pH 7.4) supplemented with 3.3 mM magnesium chloride. The final concentration of DMSO, which was the solvent for both the test compounds and the BFC, did not exceed 0.1%. The reaction mixtures were incubated at 37°C for 20 minutes. Formation of the highly fluorescent metabolite of BFC 7-hydroxy-4-trifluoromethylcoumarin (HFC) was measured on a Mithras LB940 microplate reader Berthold Technologies, Bad Wildbad, Germany).

Cell viability

Cell viability was determined based on the quantization of intracellular ATP with CellTiter-GLO® (Promega, Madison, WI, USA) according to the manufacturer's manual.

Statistical analysis

One-way ANOVA with Bonferroni and Dunnett *post hoc* testing was performed for statistical comparison of the obtained CYP3A4 reporter gene and activity results and considered statistically significant when $P<0.05$. Statistical analysis on real-time PCR data were performed on mean dct values (and not fold changes) to exclude potential bias attributable to averaging data that had been transformed through the equation $2^{-(\text{difference in dct})}$ [13]. All statistical calculations were done in SPSS (v14, SPSS inc. Chicago, IL, USA).

Results

CYP3A4 reporter gene assay

The induction potential of eighteen widely used anticancer drugs (cisplatin, carboplatin, cyclophosphamide, ifosfamide, doxorubicin, epirubicin, irinotecan, topotecan, paclitaxel, docetaxel, vinblastine, vincristine, flutamide, tamoxifen imatinib, erlotinib, etoposide and 5-fluorouracil) to activate nuclear receptor mediated CYP3A4 induction was determined in LS180 cells that were co-transfected with the hPXR expression plasmids and a CYP3A4 reporter construct. As shown in figure 1, paclitaxel is a strong activator of PXR-mediated CYP3A4 reporter gene activity, while flutamide, erlotinib, cyclophosphamide, ifosfamide, tamoxifen and docetaxel only moderately activate PXR-mediated CYP3A4 reporter gene activity. In addition, no increase in reporter gene activity was observed in the absence of the nuclear receptor expression plasmids indicating that the increase in CYP3A4 reporter activity is mediated by PXR.

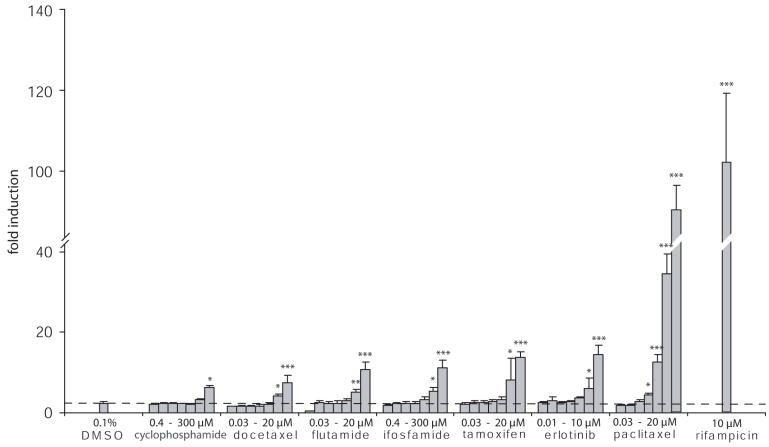

Figure 1 PXR-mediated induction of CYP3A4 by widely used anticancer drugs

LS180 cells were transfected with the pGL3-CYP3A4-XREM reporter construct, and the nuclear receptor expression vectors pCDG-hPXR, and the pRL-TK control vector. After 24 hours of transfection, cells were exposed to the anticancer drugs serially diluted in DMSO with a dilution factor of 3 from 20-0.3 µM, with the exception of erlotinib, which was serial diluted from 10-0.1 µM and ifosfamide and cyclophosphamide, which were serially diluted from 300-0.4 µM. Rifampicin (10 µM) was used as a prototypical CYP3A4 inducer and PXR agonist. After 48 hours, luciferase activity was measured. These results are derived from a representative experiment and data are the means (± SD) from three separate determinations and is expressed as absolute fold induction (significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) compared to 0.1% DMSO). (---) represents the CYP3A4 level after treatment with the vehicle 0.1 % DMSO.

CYP3A4 mRNA expression

CYP3A4 mRNA expression levels were determined in LS180 cells that were exposed for 48 hours to rifampicin (10 μM; positive control), erlotinib (10 μM), paclitaxel (20 μM), and flutamide (20 μM) strongly induced CYP3A4 mRNA expression, while ifosfamide (300 μM), docetaxel (20 μM), tamoxifen (20 μM), cyclophosphamide (300 μM) and carboplatin (20 μM) by using quantitative RT-qPCR analysis. The concentrations that were used were not cytotoxic as was determined by a cell viability assay (results not shown). As shown in figure 2, rifampicin, erlotinib, paclitaxel, and flutamide strongly induced CYP3A4 mRNA expression, while ifosfamide, docetaxel, tamoxifen and cyclophosphamide only moderately induced CYP3A4 mRNA expression compared to the control. Carboplatin did not alter CYP3A4 mRNA expression, as was expected based on the results from the CYP3A4 reporter gene assay.

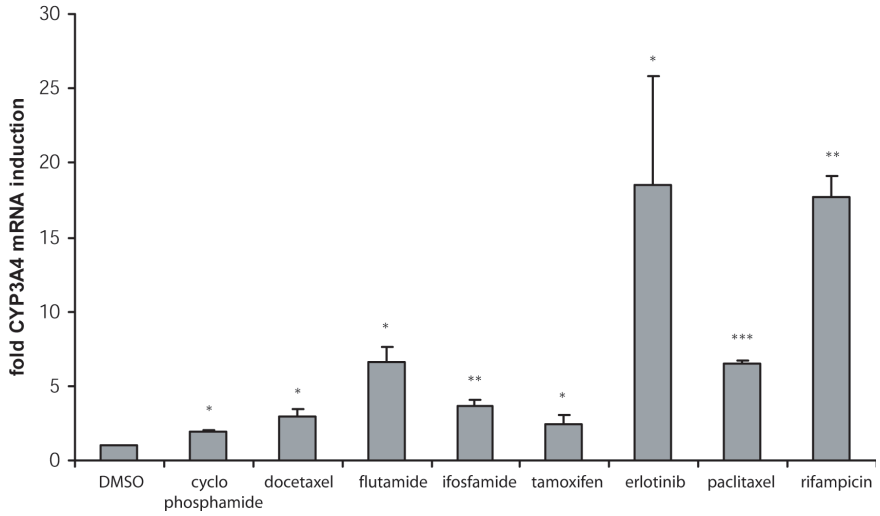

Figure 2 CYP3A4 mRNA expression levels

The CYP3A4 mRNA levels were determined after 2 × 24 hours treatment of the cells with one concentration of the anticancer drugs; cyclophosphamide (300 μM), docetaxel (20 μM), flutamide (20 μM), ifosfamide (300 μM), tamoxifen (20 μM), erlotinib (10μM) and paclitaxel (20 μM). Rifampicin (10 μM) was used as a positive control. For each anticancer drug, 3 wells of cells were grown. cDNA from each well was assessed at least in triplicate using singleplexed quantitative Taqman real-time PCR with 18S as a control gene (significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) compared to the vehicle 0.1% DMSO).

CYP3A4 immunoreactive protein expression levels

CYP3A4 protein levels were evaluated using Western immunoblotting and densitometric analysis, and plotted as fold induction. As shown in figure 3, treatment of the LS180 with rifampicin (20 μM) and paclitaxel (20 μM) resulted in significantly induced CYP3A4 protein levels, while erlotinib, ifosfamide, docetaxel, flutamide and cyclophosphamide and tamoxifen did not significantly induce CYP3A4 protein levels.

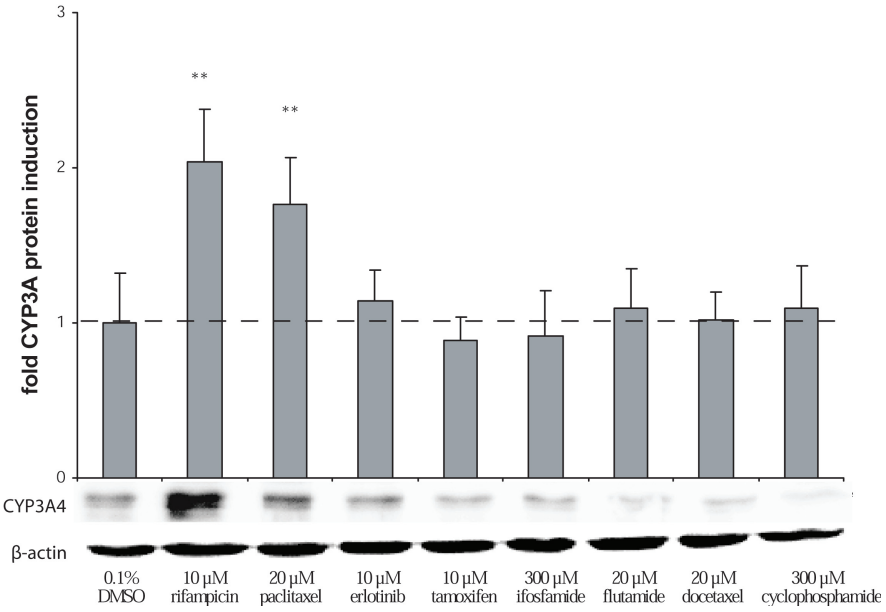

Figure 3 CYP3A4 protein expression levels

CYP3A4 protein expression was determined with Western blotting after 2 × 24 hours treatment with the anticancer drugs; cyclophosphamide (300 μM), docetaxel (20 μM), flutamide (20 μM), ifosfamide (300 μM), tamoxifen (20 μM), erlotinib (10 μM) and paclitaxel (20 μM). Rifampicin (10 μM) was used as a positive control. The CYP3A4 protein expression levels are represented as fold induction over the vehicle 0.1% DMSO. These results are derived from a representative experiment.

Cell-based CYP3A4 activity assessment

To evaluate the clinical relevance of the observations that certain anticancer drugs are able to induce CYP3A4 reporter gene activity, and mRNA and protein expression levels, the metabolic activity of CYP3A4 was determined after treatment of LS180 cells with these agents. The metabolic activity of CYP3A4 was assessed by measuring the formation of 1′-hydroxymidazolam after a 3 hours incubation of the cells with the CYP3A4 probe-substrate midazolam. As expected, based on the CYP3A4 protein expression levels, both rifampicin and paclitaxel enhanced the biotransformation of midazolam to the inactive 1′-hydroxymidazolam metabolite (figure 4). Flutamide caused a 2-fold increase in 1′-hydroxymidazolam formation, while erlotinib was shown to decrease the formation of the 1′-hydroxy metabolite. All other compounds did not significantly affect 1′-hydroxymidazolam formation compared to the vehicle.

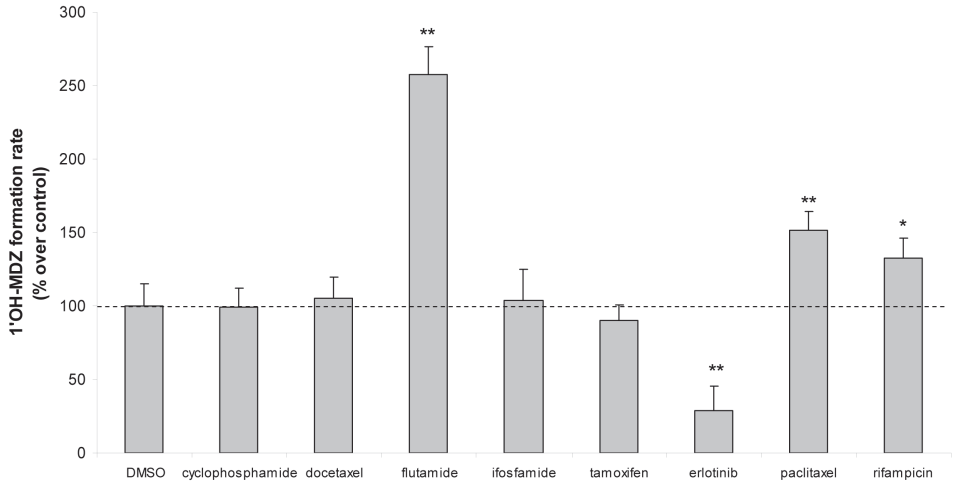

Figure 4 **Cell-based CYP3A4 activity assay**

CYP3A4 activity was assessed after 2 × 24 hours treatment with the anticancer drugs; cyclophosphamide (300 μM), docetaxel (20 μM), flutamide (20 μM), ifosfamide (300 μM), tamoxifen (20 μM), erlotinib (10 μM) and paclitaxel (20 μM). Rifampicin (10 μM) was used as a positive control. These results are derived from a representative experiment and data are the means (± SD) from three separate determinations and is expressed as fold (%) increase in 1′-hydroxymidazolam formation (significance (* $P<0.05$, ** $P<0.01$) compared to 0.1% DMSO).

Effect of erlotinib and flutamide on the activity of recombinant human CYP3A4 enzyme

Since both flutamide and erlotinib were shown to act differently on CYP3A4 activity as was expected based on the CYP3A4 reporter activity and mRNA and protein expression levels, the effect of both compounds on the enzyme kinetics of CYP3A4 was further evaluated using recombinant human CYP3A4. Co-incubations with flutamide (20μM) had no significant effect on the formation of 7-hydroxy-4-trifluoromethylcoumarin (HFC), while erlotinib (10 μM) non-competitively inhibited HFC formation (figure 5).

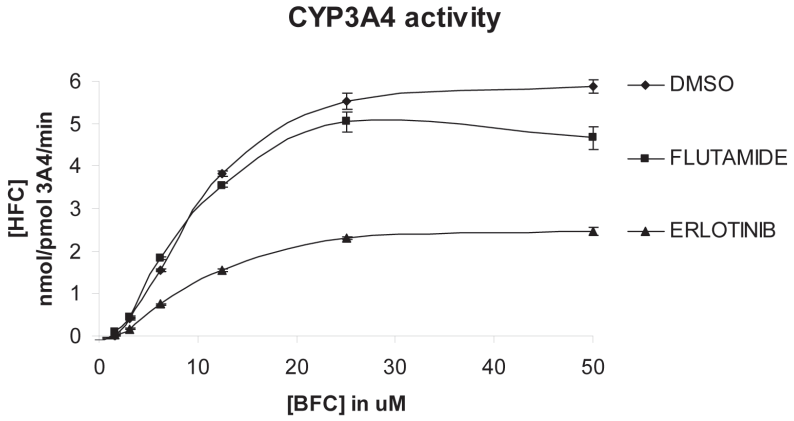

Figure 5 Effect of flutamide and erlotinib on CYP3A4 activity
Flutamide (20 μM) and erlotinib (10 μM) were co-incubated with the non-fluorescent CYP3A4 substrate BFC. Erlotinib was shown to non-competitively inhibit CYP3A4, while flutamide did not significantly alter rate of substrate conversion compared to the DMSO. These results are derived from a representative experiment and data are the means (± SD) from three separate determinations and is expressed as increase HFC formation.

Discussion

A panel of anticancer drugs were screened for their ability to modulate CYP3A4 expression and activity. These oncolytic agents were chosen to cover a broad range of mechanisms, and consisted of topoisomerase I inhibitors, microtubule-stabilizing agents, antimetabolites, antitumor antibiotics, alkylating agents, and protein tyrosine kinase inhibitors, antiestrogens and an antiandrogen.

A CYP3A4 reporter gene assay was used to rapidly assess the potential of these selected agents to induce CYP3A4. The screening was performed in the intestinal cell line LS180, because previous comparison of the CYP3A4 inducibility in both LS180 and other widely used cell lines such as Caco-2 [14] and HepG2 [15] had revealed that LS180 cells represent a better model to study CYP3A4 induction.

PXR was shown to be involved in the induction of CYP3A4 by seven of the selected anticancer drugs (figure 1). A highly significant ($P<0.001$) induction level of PXR-mediated CYP3A4 expression was observed for paclitaxel, while docetaxel, flutamide, ifosfamide, cyclophosphamide, erlotinib and tamoxifen moderately increased CYP3A4 expression compared to rifampicin, a prototypical CYP3A4 inducer. Cisplatin, carboplatin, doxorubicin, epirubicin, irinotecan, topotecan, vinblastine, vincristine, imatinib, etoposide and 5-fluorouracil all did not exert a significant effect on the PXR-mediated induction of CYP3A4.

Since the nuclear receptor expression levels in the CYP3A4 reporter gene assay described above are raised artificially, the effect of treatment with the anticancer agents on CYP3A4 mRNA expression and protein levels in non-transfected LS180 cells was evaluated. The same compounds that increased CYP3A4 reporter activity were shown to increase CYP3A4 mRNA expression levels following 48 hours incubation. Interestingly, with the exception of erlotinib, the compounds (cyclophosphamide, tamoxifen, docetaxel, ifosfamide, flutamide) that elicited a modest CYP3A4 reporter gene activation also showed a moderate induction of CYP3A4 mRNA expression (<10 fold) and protein expression (<1.5 fold) levels. Erlotinib, however, highly induced CYP3A4 mRNA expression levels (~18 fold) in comparison to other compounds that were shown to activate CYP3A4 induction. In the CYP3A4 reporter gene assay and Western blot assay, erlotinib was shown to only moderately increase both CYP3A4 reporter gene activity (<20 fold) as well as CYP3A4 protein expression levels (<1.5

fold). The discrepancy between the CYP3A4 mRNA- and the CYP3A4 protein expression levels is difficult to explain. Especially, because many different anticancer drugs were used that all have different targets within the cell by which these agents exert their cytotoxic effects. We did show that eight anticancer drugs were able to activate PXR-mediated CYP3A4 transcription of CYP3A4. However, erlotinib caused an exceptionally high fold increase in CYP3A4 mRNA expression, which can not be explained by the activation of PXR by erlotinib alone. Possibly post-transcriptional processes such as RNA stabilization are modulated by erlotinib. However, this remains to be explored.The enzymatic activity of CYP3A4 after 48 hours treatment with the anticancer drugs was assessed by measuring 1′-hydroxylation of midazolam, which is solely catalyzed by CYP3A4 [16]. It revealed that the 1′-hydroxylation of midazolam was increased by exposure to the strong PXR activators paclitaxel and rifampicin, while no significant increase could be measured after exposure to the weak PXR activators tamoxifen, docetaxel, cyclophosphamide and ifosfamide. Erlotinib inhibited midazolam 1′hydroxylation, which was confirmed by a CYP3A4 inhibition study with erlotinib on recombinant human CYP3A4 supersomes. The increase in midazolam 1′-hydroxylation after treatment with paclitaxel and rifampicin relates to the CYP3A4 protein expression levels after treatment with these agents. In contrast to paclitaxel and rifampicin, the increase of 1′-hydroxymidazolam after pretreatment with flutamide did not correlate with CYP3A4 reporter gene, CYP3A4 mRNAand CYP3A4 protein expression level data. These data showed that flutamide only moderately induced CYP3A4 compared to rifampicin, while flutamide caused major increase in the cell-based CYP3A4 activity assay. Possibly flutamide (or a metabolite of flutamide) increases 1′-hydroxymidazolam formation by allosterically activating CYP3A4. A similar effect has been described for α-naphthoflavone [17]. However, the results from an enzyme kinetics study with recombinant human CYP3A4 supersomes co-incubated with flutamide and BFC showed that flutamide does not allosterically activate CYP3A4. Possibly a metabolite of flutamide causes the increase in the cell-based assay, but this remains to be explored.

Although the weak PXR activators do not significantly increase CYP3A4 protein levels and midazolam 1′hydroxylation, these agents were shown to induce CYP3A4 reporter gene activity as well as mRNA expression levels. Indeed, several of these agents (tamoxifen, cyclophosphamide and ifosfamide) were shown to cause clinical relevant drug-drug interaction

as a result of CYP3A4 induction. Tamoxifen, for instance, reduced the plasma levels of concomitantly administered aromatase inhibitors letrozole and anastrozole, by 37% and 27%, respectively, in a clinical trial [18]. Both aromatase inhibitors are CYP3A4 substrates. Tamoxifen was also shown to autoinduce its own clearance as a result of CYP3A4 induction [11]. Our study shows that this autoinduction is most likely mediated by PXR. In addition, the oxazophosphorines, cyclophosphamide and ifosfamide, are also known to autoinduce their clearance [19]. We found that ifosfamide (>100 μM) causes PXR-mediated CYP3A4 induction. Although this concentration seems very high, it corresponds with the peak plasma concentration after intravenous administration of >1.5 g m-2 ifosfamide (depending on the infusion schedule) [20, 21]. The ability of ifosfamide to cause CYP3A4 induction at a clinically relevant concentration of >100 μM may, in part, provide an insight into the mechanism by which ifosfamide is able to autoinduce its own biotransformation [19]. However, also other mechanisms like inhibition of enzyme degradation should be taken into account [21]. Remarkably, ifosfamide had a greater capacity to induce CYP3A4 compared to cyclophosphamide, which is an isomer of ifosfamide. Lindley *et al*. [22] showed that cyclophosphamide is able to activate PXR at a concentration >100 μM *in vitro*, which is confirmed by our results.

Clinical proof for our observations that in contrast to the other anticancer drugs, paclitaxel, docetaxel, flutamide and erlotinib, have the potential to cause pharmacokinetic drug-drug interaction is not yet known and should be further investigated. This especially is remarkable for paclitaxel, since this compound has been identified as a strong activator of PXR mediated CYP3A4 induction. However, many anticancer drugs are co-administered with other antineoplastic agents and supportive care drugs (analgesics, antiemetics, etc.). Therefore it is difficult to identify the specific drugs involved in the interaction. For instance, the taxanes, paclitaxel and docetaxel, are routinely co-administered with dexamethasone, an agent used as anti-emetic and hypersensitivity prophylaxis and also a classic and potent CYP3A4 inducer [23]. In addition, non-responsiveness to chemotherapeutic agents is generally accepted as regimen related. However, non-responsiveness could also be an effect of enzyme induction, for instance due to concomitant unprescribed use of CYP3A4 inducers like hyperforin, the active component of St.John's wort [24].

In conclusion, we present the inductive capacity of a range of widely

used anticancer drugs on CYP3A4 expression and the mechanism by which these agents mediate this response. Paclitaxel [10], cyclophosphamide [22] and tamoxifen [11] were already known to induce CYP3A4 expression via activation of PXR and our data confirm these results. However, in contrast to other studies [10, 25] we observed a small, but significant CYP3A4 reporter activity after treatment with docetaxel.

We have identified three new PXR activating anticancer drugs; erlotinib, ifosfamide and flutamide, and have provided new insight into the autoinduction mechanism of ifosfamide. Both the CYP3A4 reporter gene assay and to a lesser extent the CYP3A4 mRNA determination are more accurate in predicting possible clinical relevant drug-drug interactions compared to Western blotting and the midazolam 1'-hydroxylation assay. With the latter two assays only strong PXR activators were shown to generate a response, possibly due to detection limits, while the former two assays allowed the identification of weak PXR activators that in the clinical setting are known to cause drug-drug interactions as a result of CYP3A4 induction (e.g. cyclophosphamide, ifosfamide and tamoxifen). The data on clinical anticancer drug-drug interactions are scarce, due to the difficulty to recognize drug-drug interactions as such, and the limited number of preclinical drug interaction studies. Clinicians should be aware of drug-drug interactions when combining multiple (anticancer) drugs. Especially (anticancer) drugs that activate PXR-mediated CYP3A4 induction have the potential to cause clinically relevant drug-drug interactions by affecting the pharmacokinetic profile of co-administered agents.

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Chapter 4

PXR-mediated induction of P-glycoprotein by anticancer drugs

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Abstract

The development of multidrug resistance (MDR) is one of the major limitations in the treatment of cancer. Induction of P-glycoprotein (Pgp) has been regarded as one of the main mechanisms underlying drug-induced MDR. Since the induction of Pgp is regulated by the pregnane X receptor (PXR), the ability of a panel of widely used anticancer drugs to activate PXR-mediated Pgp induction was investigated. Our study shows that treatment with 8 of the 10 selected anticancer drugs (vincristine, tamoxifen, vinblastine, docetaxel, cyclophosphamide, flutamide, ifosfamide and paclitaxel) activated PXR-mediated Pgp induction, which was additionally shown to affect the intracellular accumulation of the Pgp probe rhodamine 123. Moreover, PXR activation was also shown to reduce the cytotoxic activity of doxorubicin, which is also a substrate for Pgp. Our results indicate that anticancer drugs can induce MDR in cancer cells by activation of PXR-mediated Pgp induction.

Introduction

A major drawback in the successful treatment of advanced malignancies is the development of multidrug resistance (MDR). One of the main causes by which cancer cells acquire the multidrug resistant phenotype in response to chemotherapy is the induction of ATP-binding cassette (ABC) drug efflux transporter proteins. An important mediator of MDR that has been associated with poor treatment response in various malignancies is the ABC-family member P-glycoprotein (Pgp; ABCB1; MDR1) [1-3]. Pgp is a transmembrane protein that protects the cell from xenobiotic stress by facilitating the extrusion of a broad spectrum of substrates, including anticancer drugs such as the anthracyclines, epipodophyllotoxins, Vinca alkaloids and taxanes. As a consequence, induction of Pgp affects the efficacy of these agents by decreasing their intracellular accumulation in cancer cells.The pregnane X receptor (PXR; NR1I2) has been identified as a major regulator of Pgp induction [4], and, apart from expression in the liver and small intestines, has been shown to be expressed in several cancerous tissues such as breast, colon, bone, prostate and endometrial cancers [5-9]. PXR is a very promiscuous receptor that is activated by a wide variety of structurally unrelated ligands including rifampicin, hyperforin and the anticancer drug paclitaxel. Due to the promiscuity of PXR other widely used anticancer drugs could possibly also activate PXR-mediated Pgp induction, and as a consequence induce MDR in cancer cells.

In the current study a panel of widely used anticancer drugs were evaluated for their ability to activate PXR-mediated Pgp protein induction in the colon carcinoma-derived cell line LS180. In addition, the effect of PXR activation on the intracellular accumulation of Pgp substrates was determined. Our results demonstrate that several widely used anticancer drugs can activate PXR thereby inducing Pgp, and as a consequence, decrease the intracellular accumulation and efficacy of (cytotoxic) Pgp substrates.

Materials and methods

Materials

All cell culture media and supplements were purchased from Invitrogen (Breda, The Netherlands). Carboplatin, ifosfamide, tamoxifen citrate, and etoposide were obtained from Axxora (San Diego, CA, USA). Zosuquidar (LY335979) was obtained through Kanisa Pharmaceuticals Inc. (Irvine, CA, USA). All other chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Plasmids

The pGL3-MDR1 (p-10224) luciferase reporter construct was generously provided by Dr. Oliver Burk (Institute of Clinical Pharmacology, Eberhard-Karls University, Tübingen, Germany). The pCDG-hPXR expression vector was generously provided by Dr. Ron Evans (Salk institute for biological studies, La Jolla, CA, USA). The pRL-TK control plasmid was obtained from Promega (Madison, WI, USA). Plasmids were checked by enzyme restriction and agarose gel electrophoresis and purified using Promega's Pureyield Midi-prep (Madison, WI, USA) according to the instructions of the manufacturer.

Cell culture

The human colon adenocarcinoma-derived cell line, LS180 was purchased from the ATCC (Manassas, VA, USA). The cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (with 25 mM HEPES and L-glutamine, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin), at 37°C under a humidified atmosphere of 5% CO_2.

Pgp reporter gene assay

The Pgp reporter gene assay was performed in a similar manner as described previously [10]. In brief, LS180 cells were transfected with a pCDG-hPXR expression vector, a pGL3-MDR1 luciferase reporter construct, and a pRL-TK control plasmid for 24 hours. After transfection, the transiently transfected LS180 cells were treated with the highest non-toxic concentration of each anticancer drug. The cytotoxicity of the anticancer drugs was assessed with a MTT-assay (results not shown). After 48 hours incubation, the reporter activity was determined.

Cell treatment

LS180 cells were plated at a density of 5 x 10^4 cells/well in 96-well plates (Pgp protein level determination) or 1 x 10^5 cells/well in 1 ml RMPI in 24 well plates (Pgp transport activity). After reaching 80-90% confluency, medium was replaced with medium containing the different anticancer drugs: carboplatin (10 μM), cyclophosphamide (100 μM), ifosfamide (100 μM), docetaxel (10 μM), paclitaxel (10 μM), flutamide (10 μM), and tamoxifen (10 μM). Rifampicin (10 μM) was used as a positive control and DMSO (0.1%) as a negative control. The cells were treated for 48 hours with the drugs and the controls. At the end of each treatment the cells were processed for immunoblotting or rhodamine 123-based Pgp activity assays.

RNA interference of PXR

The siRNA sequence targeting human PXR (sense: cguuuguucgcuuccugagtt; antisense: cucaggaagcgaacaaacgtg) and the negative control, which consisted of a noncomplementary sequence, were purchased from Ambion (Austin, TX, USA). LS180 cells were reversely transfected 48 hours prior to the Pgp induction experiment with 50 nM siRNA PXR or 50 nM negative control siRNA using Lipofectamine RNAi Max (Invitrogen, Breda, The Netherlands). Subsequently, PXR knockdown and control LS180 cells were treated with different anticancer drugs for 48 hours.

Immunoblot analysis

After 48 hours cells were washed with phosphate buffered saline (PBS),

and lysed in 1 mL/well MilliQ water that contained protease inhibitors (Roche, Basel, Switzerland).The lysate was spinned down (5000 rpm, 5 min, 4°C), and the remaining pellet was resuspended in 100 µL RIPA buffer. The lysate was spinned down (10000 rpm, 5 min, 4°C) and the supernatant was transferred to a new collection tube. Protein concentrations were determined by a Pierce BCA protein assay (Pierce, Rockford, IL, USA). 10 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis using NuPage novex 4-12% bis-tris gradient gels (Invitrogen, Breda, The Netherlands). Proteins were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA, USA). After blocking (1 hour at RT) in 3% BSA in tris-buffered saline containing 0.1% Tween-20, the membranes were incubated overnight at 4°C with a murine monoclonal anti-Pgp primary antibody (C219, 1:500; Abcam, Cambridge, UK) followed by incubation with a goat anti-mouse IgG coupled to horse radish peroxidase (HRP) secondary antibody (1:1000; ImmunoPure Peroxidase Conjugated Goat anti-Mouse IgG (H + L); Pierce, Rockford, IL, USA). β-actin (1:10000; AC-15; Abcam, Cambridge, UK) was used as a loading control. The proteins were visualized by a chemiluminescence-based detection reagent (SuperSignal West Femto; Pierce, Rockford, IL, USA) and the intensities of the Pgp bands were determined on a Gel Doc XRS Imaging system with Quantity One analysis software (Bio-Rad, Hercules, CA, USA). The ratio of the Pgp signal and the β-actin signal were indicative for Pgp protein expression.

Rhodamine 123 accumulation assay

The efflux activity of Pgp was determined by measuring the accumulation of the fluorescent Pgp probe rhodamine 123 as described by [11]. In brief, LS180 cells were pretreated with the anticancer drugs as described above. After 48 hours, the cells were washed with Hank's Balanced Salt Solution (HBSS) and incubated at 37°C for 1 hour with rhodamine 123 (10 µM) in the presence or absence of the Pgp-specific inhibitor zosuquidar (LY335979; 5 µM; [12]) in phenolred-free RPMI medium. After washing thrice with ice cold HBSS, the cells were lysed with Triton X-100 (0.1%) in MilliQ water (Millipore, Bedford, MA, USA). The ratio of intracellular rhodamine 123 concentrations in the absence and presence of 5 µM zosuqui dar is indicative for the efflux activity of Pgp.

Doxorubicin accumulation

LS180 cells (0.5 x 10^6 cells/well) were plated in a 24-well plate. After a 48 h incubation with 0.1% DMSO or 10 µM rifampicin, cells were treated with 5 µM doxorubicin for 90 min. Cells were washed thrice with ice cold PBS, and lyzed in 250 µL MilliQ water, and homogenized by ultrasonification Labsonic P (Braun; cycle 0.2 min en Amplitude 60%). 100 µL of the homogenate was processed for HPLC analysis. Daunorubicin was used as an internal standard. The samples and standard solutions were prepared as described by [13].

Doxorubicin HPLC analysis

Doxorubicin was quantified using a method as previously described by [14]. In brief, samples were eluted isocratically with a mobile phase that consisted of 28% acetonitril in 60 mM phosphoric acid. A Waters Symmetry C18 3.5 µM, 4.6mm x 100mm reverse-phase column was used. Fluorescence was detected using a JASCO FP-920 fluorometer (excitation: 455 nm; emission: 550 nm), Shimadzu LC10AT-VP pumps, and a Shimadzu SCL10A-VP controller. The flow rate was set at 1.0 ml/min. Doxorubicin and daunorubicin retention times were 2.38 and 5.01 min, respectively. A standard curve of doxorubicin (0.08, 0.4, 2, 10, and 50 µM) was prepared in milliQ water.

Doxorubicin cytotoxicity assay

LS180 cells were plated (2x10^4 cells/well; 96-well), and treated with 10 µM rifampicin or 0.1% DMSO. Following 72 h incubation, cells were exposed to doxorubicin (serially diluted 0-20 µM; dilution factor 2). Cell viability after 48 h doxorubicin exposure was assessed using a neutral red assay [15].

Statistical analysis

One-way ANOVA with Bonferroni *post hoc* testing was performed for statistical comparison of the obtained Pgp reporter gene and activity results and considered statistically significant when $P<0.05$. All statistical calculations were done in SPSS (v14, SPSS inc. Chicago, IL, USA).

Results

PXR-mediated Pgp reporter activation by anticancer drugs

First, the ability of a panel of widely used anticancer drugs to induce the expression of Pgp via activation of PXR was determined. LS180 cells were transfected with a Pgp reporter construct as well as a PXR expression plasmid. Treatment of the transiently transfected LS180 cells with cyclophosphamide, ifosfamide, docetaxel, paclitaxel, flutamide, and tamoxifen resulted in dose-dependent increases in Pgp reporter activity, while exposure to different concentrations of carboplatin and etoposide did not increase PXR-mediated Pgp reporter activity (figure 1). As a control, the same compounds were tested in LS180 cells that were only transfected with the Pgp reporter gene construct without co-transfection of a PXR expression plasmid. In these cells, none of the compounds was able to activate Pgp reporter activity (results not shown).

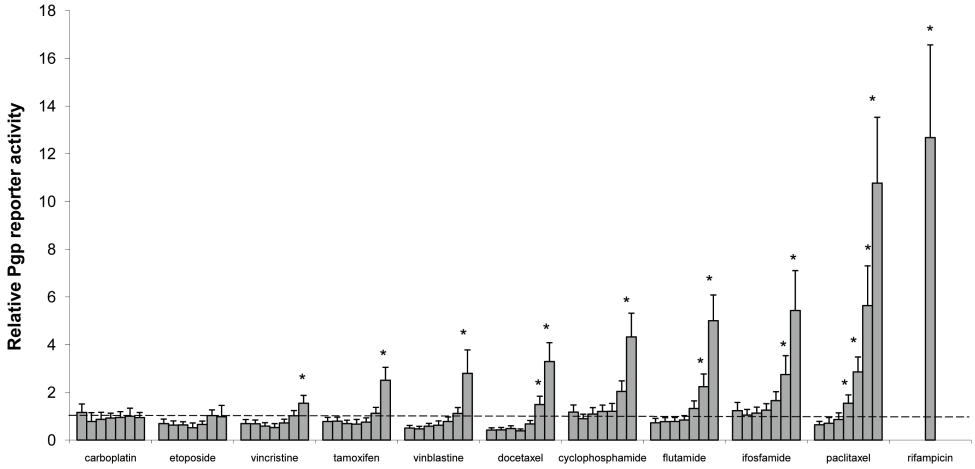

Figure 1 PXR mediated Pgp reporter gene activity following exposure to anticancer drugs
LS180 cells were transfected with the pGL3-MDR1 reporter construct, the nuclear receptor expression vectors pCDG-hPXR, and the pRL-TK control vector. Cells were exposed to anticancer drugs (range: 0.1-10 µM; dilution factor 3) with the exception ifosfamide and cyclophosphamide (0.3 – 100 µM; dilution factor 3). Rifampicin (10 µM) was used as positive control. These results are the means (± SD) of three separate determinations and are expressed as relative fold inductions compared to 0.1% DMSO (*P<0.05).

Induction of Pgp protein expression

To evaluate whether Pgp reporter activation is predictive for Pgp protein induction, the effect of the selected anticancer drugs on the protein expression levels of Pgp after 48 hours treatment in LS180 cells was determined. As shown in figure 2, all compounds that were shown to activate PXR-mediated Pgp reporter activity (vincristine, tamoxifen, vinblastine, docetaxel, cyclophosphamide, flutamide, ifosfamide and paclitaxel) also induced Pgp protein levels in LS180 cells. In addition, both etoposide and carboplatin did not induce Pgp protein expression, which is in accordance with the results of the Pgp reporter assay. To further demonstrate that PXR plays an important role in the upregulation of Pgp by the anticancer drugs, Pgp protein expression after exposure to the same anticancer drugs was determined in PXR knockdown LS180 cells. The knockdown efficiency of PXR was 51% as was determined 48 hours after siRNA transfection by Western blot (results not shown). As shown in figure 2, Pgp protein induction was significantly lower in the PXR knockdown LS180 cells after treatment with the anticancer drugs compared to the control cells (LS180 cells transfected with non-targeting siRNA). However, Pgp induction was not completely abolished, most likely as a result of the knockdown efficiency of 51%.

Rhodamine 123 accumulation assay

To assess whether PXR-mediated Pgp induction affects the accumulation of Pgp substrates, the transport activity of Pgp was analyzed using a rhodamine 123 accumulation assay. Following 48 hours pretreatment with the anticancer drugs, cells were exposed to rhodamine 123 in the presence or absence of the Pgp specific inhibitor zosuquidar (LY335979; IC50=4 nM) [16]. The ratio between the cellular fluorescence of rhodamine 123 in the presence and absence of zosuquidar is indicative for the Pgp transport activity in the treated LS180 cells. As shown in figure 3, rhodamine 123 accumulation corresponds with Pgp protein expression data; low cellular rhodamine 123 accumulation was observed for paclitaxel, docetaxel, and flutamide, which were shown to induce Pgp, while etoposide and carboplatin did not affect rhodamine 123 accumulation. In contrast, the accumulation of rhodamine 123 in the cells that were treated with the Vinca alkaloids, cyclophosphamide, ifosfamide, and tamoxifen did not corre-

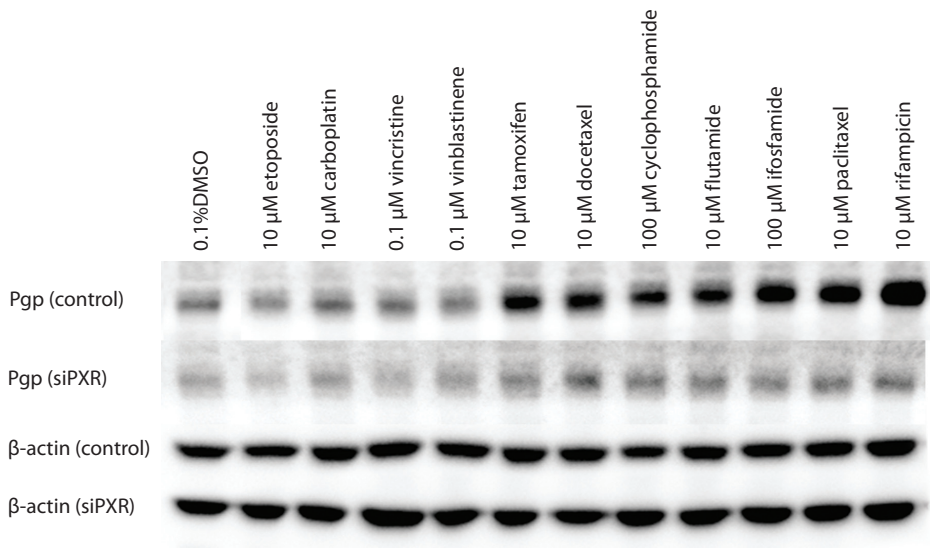

Figure 2 Anticancer drugs affect Pgp protein expression
Pgp protein expression after exposure of the LS180 to the anticancer drugs as was determined with immunoblotting using Pgp- and β-actin specific antibodies. The figure shows a representative immunoblot of Pgp protein expression in LS180 cells (both in control and PXR knock down cells) following 48 hours treatment with the indicated anticancer drugs.

spond with Pgp protein expression, possibly these compounds interact with Pgp, or other transporter proteins such as multidrug resistance associated protein (MRP) 1 [17], which have also been shown to transport rhodamine 123, thereby affecting its accumulation.

Effect of PXR activation on doxorubicin cytotoxicity

To study whether activation of PXR affects the efficacy of a cytotoxic Pgp substrate, the effect of PXR activation on the cytotoxicity of doxorubicin in LS180 was assessed. Therefore, prior to doxorubicin exposure, LS180 cells were pretreated with the prototypical PXR agonist 10 µM rifampicin or 0.1 % DMSO (solvent) for 48 hours. As shown in figure 4a, rifampicin pretreatment affects the intracellular accumulation of doxorubicin. To determine whether reduced accumulation of doxorubicin also affects the efficacy of this agent, rifampicin or solvent pretreated cells were exposed to different doxorubicin concentrations (ranging from 0.3 – 20 µM; dilution factor 2).As shown in figure 4b, following doxorubicin exposure cell viability was significantly ($P<0.05$) higher in the rifampicin pretreated

cells (IC50=2.5 μM) compared to the solvent (0.1% DMSO) pretreated cells (IC50=1.5 μM). This indicates that PXR activation indeed can reduce the activity of cytotoxic Pgp substrates as a result of decreased intracellular accumulation.

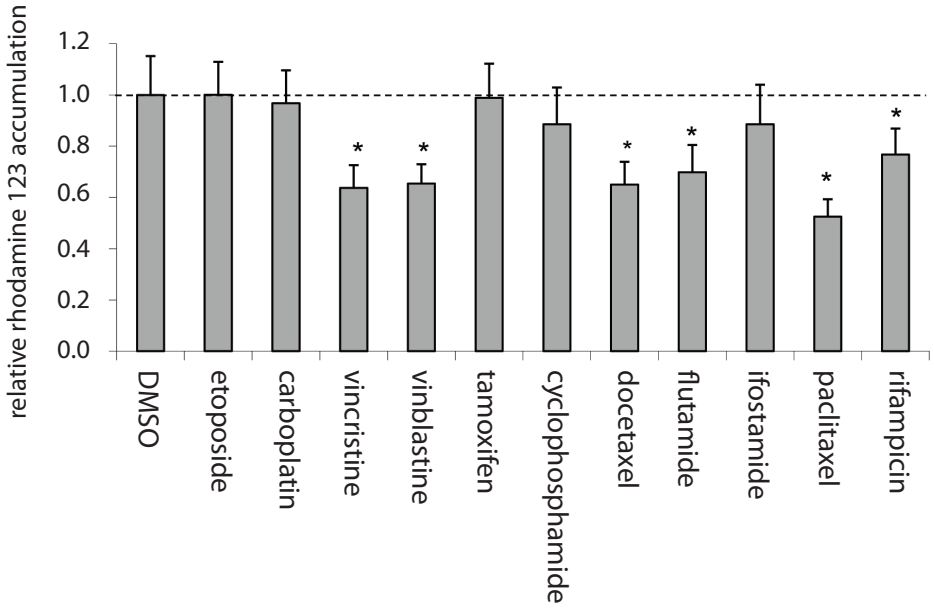

Figure 3 Anticancer drug pretreatment affects Pgp substrate accumulation
LS180 cells were exposed to the indicated anticancer drugs for 48 hours, after which rhodamine 123 accumulation was determined in the absence or presence of the Pgp-specific inhibitor zosuquidar. The ratio of intracellular rhodamine 123 fluorescence in the absence or presence of zosuquidar is indicative for the functionality of Pgp. The data are normalized to the DMSO controls and are presented as mean data (± SD) of three different experiments (*P<0.05).

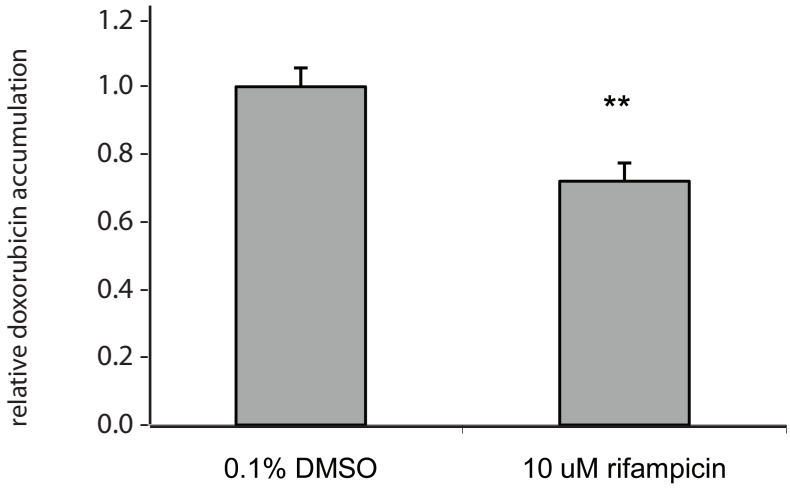

Figure 4a Rifampicin pretreatment decreases doxorubicin accumulation in LS180 cells
Doxorubicin accumulation was assessed in LS180 cells after 48 h pretreatment with rifampicin (10 μM). These results are derived from a representative experiment and data are the means (± SD) from three separate determinations and is expressed as fold decrease in doxorubicin accumulation compared to 0.1% DMSO (**P<0.01).

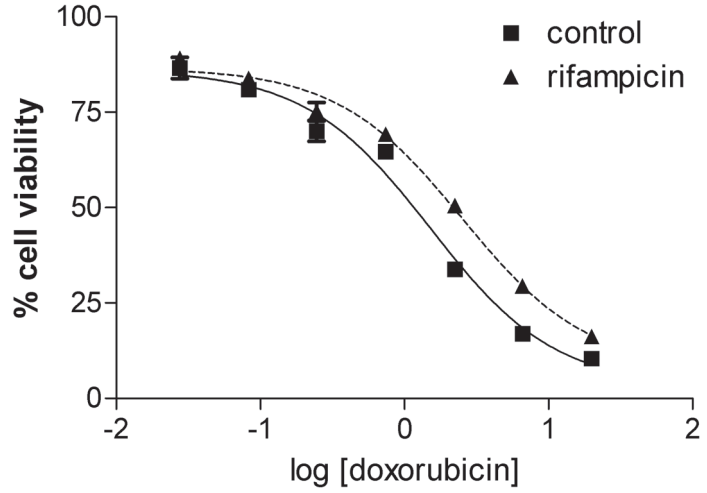

Figure 4b Rifampicin pretreatment reduces the cytotoxicity of doxorubicin in LS180 cells
The effect of 96 h rifampicin pretreatment on the cytotoxicity of doxorubicin in LS180 was determined with a neutral red assay. The results are presented as mean viabilities (± SD) and are derived from four separate determinations (*P<0.05).

Discussion

The development of MDR in response to anticancer treatment is a major problem in the clinic. Since induction of Pgp is one of the main mechanisms underlying the development of MDR in response to chemotherapy, the ability of 10 anticancer drugs to activate PXR-mediated Pgp induction was evaluated.

In a Pgp-reporter gene assay, 8 of the selected anticancer drugs were shown to induce Pgp reporter activity via activation of PXR. In accordance, vincristine, vinblastine, cyclophosphamide, ifosfamide, docetaxel, flutamide, and tamoxifen, which activated PXR-mediated Pgp reporter gene activity also induced Pgp protein expression in LS180 cells. Knockdown of PXR resulted in an attenuation of Pgp induction after treatment with these anticancer drugs. These results clearly show that PXR plays an important role in the induction of Pgp protein expression following treatment with anticancer drugs. Additionally, the effect of PXR activation by anticancer drugs on the intracellular accumulation of Pgp substrates was assessed. As expected, PXR-mediated Pgp induction decreased the accumulation of the Pgp probe rhodamine 123 in the case of docetaxel, flutamide, paclitaxel and rifampicin, while both carboplatin and etoposide, which did not induced Pgp, also did not affect rhodamine 123 accumulation. In contrast, vincristine and vinblastine, which hardly increased Pgp protein expression, significantly decreased the accumulation of rhodamine 123. Since other drug transporters such as multidrug resistance associated protein (MRP; ABCC) 1, have also been shown to contribute to the efflux of rhodamine 123, possibly MRP1 mediated efflux, and not Pgp mediated efflux, is affected by vincristine and vinblastine. Huang *et al*. (2006) indeed have shown that vincristine treatment induced the expression of ABCC1-3 rather than Pgp in LS180 cells [18]. Therefore, it seems that vincristine and vinblastine affect rhodamine 123 accumulation by inducing MRP1 and not Pgp.

On the other hand, tamoxifen, which was shown to induce Pgp protein expression, did not affect rhodamine 123 accumulation. Most likely this is due to inhibition of Pgp by tamoxifen [19]. Together, these results show that some anticancer drugs not only interfere with drug transporter regulation, but also with drug transporter activity.

Since activation of PXR-mediated induction of Pgp by anticancer drugs can lead to decreased accumulation of Pgp substrates, the effect of PXR

activation on the activity of the cytotoxic Pgp substrate doxorubicin was evaluated. To avoid pharmacodynamical interactions between the selected anticancer drugs and doxorubicin, the prototypical PXR agonist rifampicin was used to assess these effects. Rifampicin pretreatment of LS180 cells resulted in decreased intracellular accumulation of doxorubicin, and in addition, also significantly reduced the cytotoxic efficacy of this drug. PXR activation thus was shown to induce resistance towards doxorubicin in these cells.

In addition to showing that several widely used anticancer drugs such as vincristine, vinblastine, cyclophosphamide, ifosfamide, docetaxel, flutamide, and tamoxifen activate PXR, we also assessed the potential role of PXR in the development of drug-induced MDR. Our results confirm the studies of Chen *et al*. (2007) and Gupta *et al*. (2008), who showed that activation of PXR promotes the multidrug resistant phenotype of prostate and ovarian cancer cell lines, respectively [9, 20]. In addition to their results, this study showed that activation of PXR could also induce the MDR phenotype in colon cancer cells, which further confirms the role of PXR as a major regulator of MDR.

Apart from the role in inducing the MDR phenotype of cancer cells, PXR mediated-Pgp induction might also be involved in drug-drug interactions. Since Pgp is co-expressed with PXR in important barrier tissues, such as the intestines and the liver, activation of PXR-mediated Pgp induction could affect the pharmacokinetic profile of (oral) anticancer drugs. Indeed, drug-drug interactions as a result of PXR-mediated Pgp induction have been reported. For instance, intake of St.John's wort, which contains the PXR agonist hyperforin, by healthy volunteers resulted in a 1.9-fold increase in the oral clearance of the Pgp substrate fexofenadine [21], while rifampicin pretreatment resulted in a 1.3- to 5.3-fold increase in the oral clearance of fexofenadine [22]. In the same manner, anticancer drugs that activate PXR might affect their own pharmacokinetics, but also that of other concomitantly administered (anticancer) drugs.

In conclusion, our study has identified several widely used anticancer drugs as activators of PXR-mediated Pgp induction. In addition, activation of PXR-mediated Pgp induction was shown to provide survival-benefits to cancer cells that were exposed to doxorubicin. Together, these results might indicate that PXR activation by anticancer drugs initiates the development of MDR in certain malignancies.

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Chapter 5

PXR-mediated induction of P-glycoprotein by small molecule tyrosine kinase inhibitors

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Abstract

The rapid development of drug resistance as a result of exposure to small molecule tyrosine kinase inhibitors (TKIs) is an important drawback to the successful use of these agents in the clinic. Although one of the most established mechanisms by which cells acquire drug resistance to anticancer drugs is the upregulation of drug efflux transporters such as P-glycoprotein (Pgp), it is currently still unknown whether TKIs have the propensity to induce Pgp. Therefore, to determine whether TKIs affect Pgp protein expression *in vitro*, LS180 cells were exposed to several clinically administered TKIs. At least 5 out of the 9 tested TKIs (erlotinib, gefitinib, nilotinib, sorafenib, vandetanib) were able to induce the expression of Pgp within 48 hours in these cells. Accordingly, these TKIs were also shown to affect the accumulation of a P-glycoprotein specific probe substrate. Furthermore, we showed that the pregnane X receptor (PXR), which is an important regulator of Pgp induction, is involved in the upregulation of Pgp protein expression following exposure to these TKIs. In conclusion, our data show that PXR-mediated upregulation of Pgp expression by TKIs might be a possible mechanism underlying acquired drug resistance in cancer cells.

Introduction

Small molecule tyrosine kinase inhibitors (TKIs) are an important new class of anticancer drugs. Their targets, protein tyrosine kinases, are crucial mediators in signaling pathways controlling cell proliferation, differentiation and apoptosis. In cancer, protein tyrosine kinases such as the fusion protein BCR-ABL, several growth factor receptors, RAF, SRC and c-kit kinases, are often deregulated and potentiate the malignant phenotype [1]. Small molecule TKIs compete for the ATP-binding site of the intracellular catalytic domains of the oncogenic protein tyrosine kinases, thereby preventing autophosphorylation and subsequent propagation of downstream signals.

The clinical efficacy of TKIs, however, is compromised by acquired drug resistance of cancer cells [2]. Mechanisms that underlie the development of anticancer drug-induced resistance include target protein mutations, altered target protein expression, alternative pathway activation, or constitutive activation of downstream signaling effectors [2, 3]. However, one of the most established mechanisms behind acquired drug resistance in response to anticancer drugs is the upregulation of ATP-binding cassette (ABC) drug efflux transporters [4]. An important member of this family that has been implicated in (multi)drug resistance is P-glycoprotein (Pgp, ABCB1, MDR1; [5, 6]). P-glycoprotein has a pivotal role in the uptake and extrusion of a wide variety of substrates including clinically relevant anticancer drugs such as the taxanes, anthracyclines, epipodophyllotoxins, and campthothecins. Recently, it was also shown that several TKIs such as dasatinib, erlotinib, gefitinib, imatinib, and lapatinib are also transported by Pgp [7-13]. Since the clinical efficacy of TKIs is highly dependent on the accessibility of these agents to their intracellular targets [2], increased Pgp-mediated extrusion of TKIs will result in decreased intracellular accumulation, which in turn reduces the clinical efficacy of TKIs.

However, although several widely used anticancer drugs were already shown to induce Pgp expression such as paclitaxel [14], it is still unknown whether also TKIs have the propensity to upregulate Pgp expression. Therefore, we determined whether TKIs are able to induce Pgp in cancer cells and if TKI-mediated upregulation of Pgp affects the accumulation of a Pgp probe-substrate. In addition, since the pregnane X receptor (PXR; NR1I2) is an important regulator of xenobiotic-mediated upregulation of Pgp [14], we examined if this ligand-activated nuclear receptor is involved in the regulation of Pgp induction by TKIs.

Material and Methods

Materials

The human colon carcinoma-derived cell line LS180 was obtained from American Type Culture Collection. Madin-Darby canine kidney (MDCK)II-MDR1 cells were provided by the Netherlands Cancer Institute. All human cell culture media and reagents were purchased from PAA Laboratories (Colbe, Germany) unless otherwise indicated. Fetal bovine serum (FBS) was obtained from Invitrogen (Breda, The Netherlands). Dasatinib, erlotinib, gefitinib, imatinib mesylate, lapatinib tosylate, nilotinib, sorafenib tosylate, sunitinib maleate and vandetanib (Sequoia Research Products Ltd, Pangbourne, UK) were dissolved in DMSO. Zosuquidar (LY335979) was provided by the Netherlands Cancer Institute (Amsterdam, The Netherlands). All other chemicals were of the highest available grade and purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).

Cell culture

LS180 cells were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine and 25 mM HEPES, supplemented with antibiotics. MDCKII-MDR1 were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, supplemented with antibiotics. All cells were maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37°C.

siRNA transfection and TKI treatment

LS180 cells were subjected to transfections with siRNA targeting PXR (sense: cguuuguucgcuuccugagtt; antisense: cucaggaagcgaacaaacgtg), and nontargeting siRNA, with Lipofectamine RNAiMAX in parallel in 96-well plates. Briefly, LS180 cells (2.5 x 10^4 cells/well) were reversely transfected with 10 nM siRNA Silencer duplexes (Ambion, Foster City, USA) according to the manufacturer's protocol for Lipofectamine RNAiMAX (Invitrogen). Following 48 hours transfection, the TKI treatments were initiated. Cells were treated with dasatinib (0.1 μM), erlotinib (10 μM), gefitinib (10 μM), imatinib (10 μM), lapatinib (10 μM), nilotinib (10 μM), sorafenib (1 μM), sunitinib (10 μM), and vandetanib (10 μM). At the end of the 48 hours TKI exposure, cells were processed for immunoblotting assays.

Immunodetection of Pgp and PXR

Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodiumdeoxycholate, 5 mM EDTA and protease inhibitors (Roche, Basal, Switzerland)). Protein content was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Proteins were reduced in Nupage LDS sample buffer (Invitrogen) containing a final concentration of 30 mM dithiothreitol (DTT). Proteins (10µg) were separated by SDS-polyacrylamide gel electrophoresis on NuPage Novex Bis-Tris precast 4-12% gradient gels (Invitrogen) and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 3% bovine serum albumine (BSA) in Tween-20 (0.5%)/tris buffered saline (TBS-T), pH 7.4 for 1 hour at RT and then incubated overnight with primary anti-Pgp (C219; 1:1000) or anti-PXR (H4417; 1:1000) antibody (Abcam, Cambridge, UK) followed by a 1 hour incubation with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:1000; Perbio Science, Erembodegem, Belgium). The protein bands were visualized using an enhanced chemiluminescence-based detection reagent (West Femto; Pierce) and the intensities of the Pgp and PXR bands were determined on a ChemiDoc XRS Imaging system and analyzed with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Rhodamine 123 accumulation assay

The efflux activity of Pgp was determined by measuring the accumulation of the fluorescent Pgp probe rhodamine 123 as described by Collett *et al*. [15]. In brief, LS180 cells were pretreated with the TKIs. After 48 hours, the cells were washed with Hank's Balanced Salt Solution (HBSS) and incubated at 37^{o}C for 1 hour with rhodamine 123 (10 µM) in the presence or absence of the Pgp-specific inhibitor zosuquidar (LY335979; 10 µM; [16] in phenolred-free RPMI medium. After washing thrice with ice cold HBSS, the cells were lysed with Triton X-100 (0.1%) in MilliQ water (Millipore, Bedford, MA, USA). The ratio of intracellular rhodamine 123 concentrations in the absence and presence of 10 µM zosuquidar is indicative for the efflux activity of Pgp.

P-glycoprotein inhibition assay

MDCKII-MDR1 cells were plated at a concentration of 2.5 x 10^5 cells/well in 24-well plates. After 24 hours, cells were pre-incubated with 0.1% DMSO, 10 μM erlotinib, 10 μM nilotinib and 10 μM zosuquidar in phenolred-free DMEM medium. After 30 minutes, the medium was replaced with phenolred-free DMEM containing 10 μM rhodamine 123 supplemented with 0.1% DMSO, 10 μM erlotinib, 10 μM nilotinib and 10 μM zosuquidar for 60 minutes. Rhodamine 123 accumulation was determined in cell lysates that were prepared by lyzing the cells in 0.1% triton X-100 in MilliQ water.

Statistical Analysis

The Student's t test (two tails) was used to analyze the difference between two groups. Multiple group comparisons were analyzed using ANOVA with Bonferroni post-hoc testing and were considered significant when $P<0.05$. The statistical tests were done using SPSS version 14 (SPSS Inc., Chicago, IL,USA).

Results

Induction of P-glycoprotein by small molecule tyrosine kinase inhibitors

The effect of TKIs on the protein expression of Pgp was determined in LS180 cells that were treated for 48 hours with 9 registered TKIs; dasatinib (0.1 μM), erlotinib (10 μM), gefitinib (10 μM), imatinib (10 μM), lapatinib (10 μM), nilotinib (10 μM), sorafenib (1 μM), sunitinib (10 μM), and vandetanib (10 μM). The concentrations used were the highest non-toxic concentrations as determined by an Alamar Blue-based cell viability assay (results not shown). As shown in figure 1a, erlotinib, gefitinib, nilotinib, sorafenib, and vandetanib significantly induced the expression of Pgp compared to the vehicle (0.1% DMSO), while treatment with dasatinib, imatinib, lapatinib and sunitinib did not have a significant effect on the Pgp protein levels.

Knockdown of PXR decreases TKI induced protein expression of P-glycoprotein

To examine the involvement of PXR in the TKI-mediated induction of Pgp, PXR was knocked down in the LS180 cells by transfecting the cells with siRNA targeting PXR. The knockdown efficiency of PXR protein was 50.9

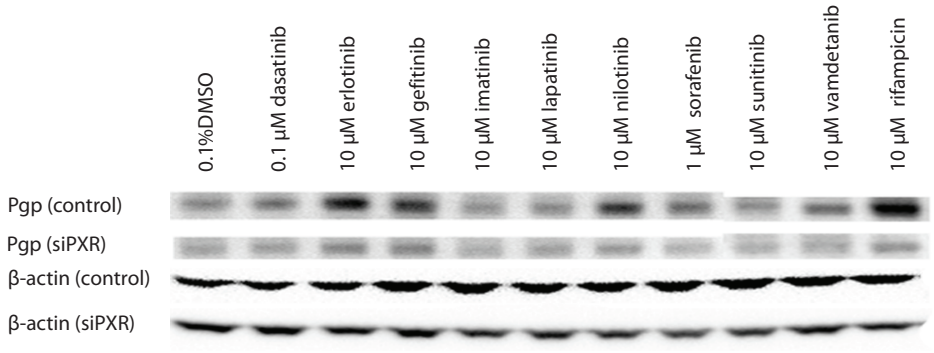

Figure 1a Increased Pgp protein expression following TKI treatment
Pgp is upregulated by TKIs in LS180 cells as was determined with immunoblotting using Pgp- and β-actin specific antibodies. The figure shows a representative immunoblot of Pgp protein expression in LS180 cells (both in control and PXR knockdown cells) following 48 hours treatment with the indicated TKIs.

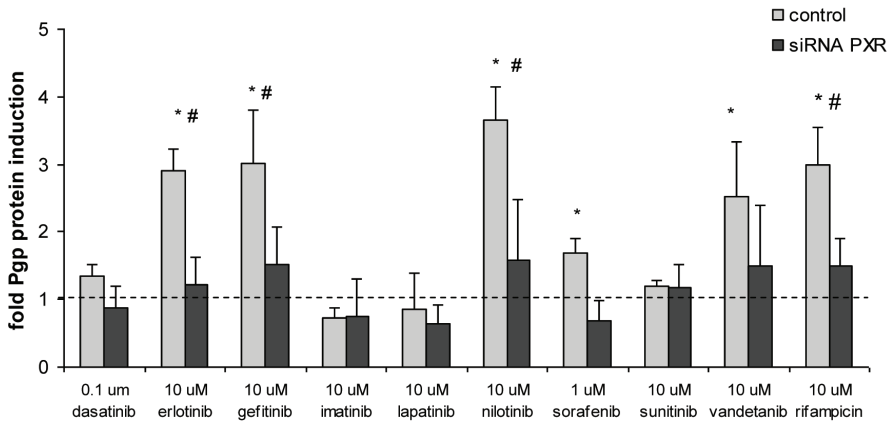

Figure 1b Inhibition of Pgp protein expression following TKI treatment by co-incubation of PXR-targeting siRNA
Pgp protein expression in PXR knockdown LS180 cells and control LS180 cells was assessed after treatment with the indicated TKIs. The bar graph shows the mean data (± SD) of the Pgp protein expression of three independent experiments. These data are normalized to the DMSO controls (**P*<0.05 fold Pgp induction following TKI treatment compared to the DMSO treated cells; #*P*<0.05 fold Pgp induction following TKI treatment in the control cells (control) compared to the PXR knockdown cells (siRNA PXR)).

± 16.0 % as was determined by western blot 48 hours after transfection (results not shown). As shown in figure 1b, Pgp protein expression levels were significantly lower in the PXR knockdown LS180 cells after treatment with erlotinib, gefitinib, nilotinib, sorafenib and vandetanib compared to the levels of Pgp in the control cells (LS180 cells transfected with non-targeting siRNA) indicating that PXR plays a role in the TKI-mediated induction of Pgp.

Rhodamine 123 accumulation assay

To examine whether TKI-mediated Pgp protein upregulation affects the accumulation of Pgp substrates, the accumulation of the Pgp probe rhodamine 123 was measured in the presence or absence of the Pgp specific inhibitor zosuquidar (LY335979; [16, 17]) in LS180 cells that were pretreated for 48 hours with the different TKIs. The ratio of intracellular rhodamine 123 concentrations in the absence and presence of 10 μM zosuquidar is indicative of the efflux activity of Pgp. With the exception of nilotinib, the TKIs that induced Pgp protein expression also significantly decreased the rhodamine 123 accumulation in these cells (figure 2).

Pgp inhibition assay

Additionally, to explain the discrepancy between the high Pgp protein expression levels and the minor effect of nilotinib pretreatment on rhodamine 123 accumulation, the potential of nilotinib to inhibit Pgp was determined by pre-incubating MDCKII-MDR1 cells. These cells express high levels of Pgp, with erlotinib (10 μM), nilotinib (10 μM), zosuquidar (10 μM; positive control) or 0.1% DMSO (vehicle) prior to rhodamine 123 exposure. As shown in figure 3, pre-incubation of MDCKII-MDR1 cells with nilotinib and zosuquidar significantly affected the accumulation of rhodamine 123 in these cells, which indicates that nilotinib inhibited Pgp activity.

Discussion

Small molecule tyrosine kinase inhibitors are an important new class of anticancer drugs. However, drug resistance induced by treatment with TKIs is a major drawback that compromises the clinical efficacy of these

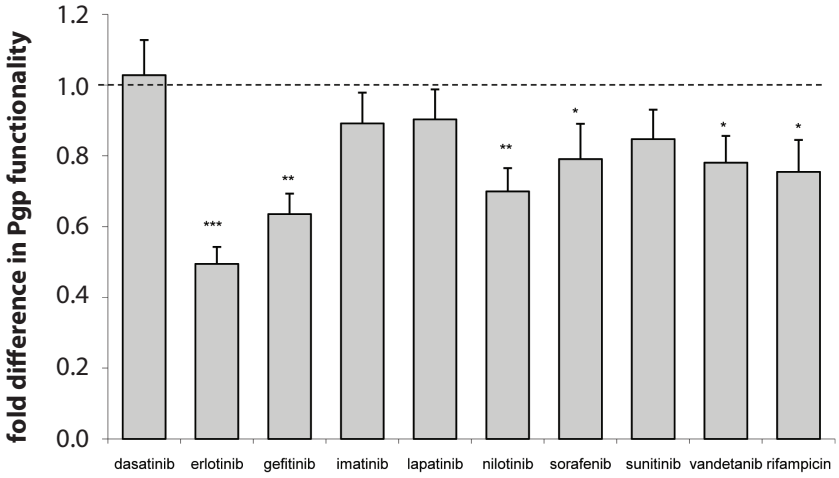

Figure 2 The effect of TKI pretreatment on Pgp functionality
LS180 cells were exposed to the indicated TKIs for 48 hours, after which rhodamine 123 accumulation was determined in the absence or presence of the Pgp-specific inhibitor zosuquidar. The ratio of intracellular rhodamine 123 fluorescence in the absence or presence of zosuquidar is indicative for the functionality of Pgp. The data are normalized to the DMSO controls and are presented as mean data (± SD) of three different experiments. *P<0.05, ** P<0.01, ***P<0.001.

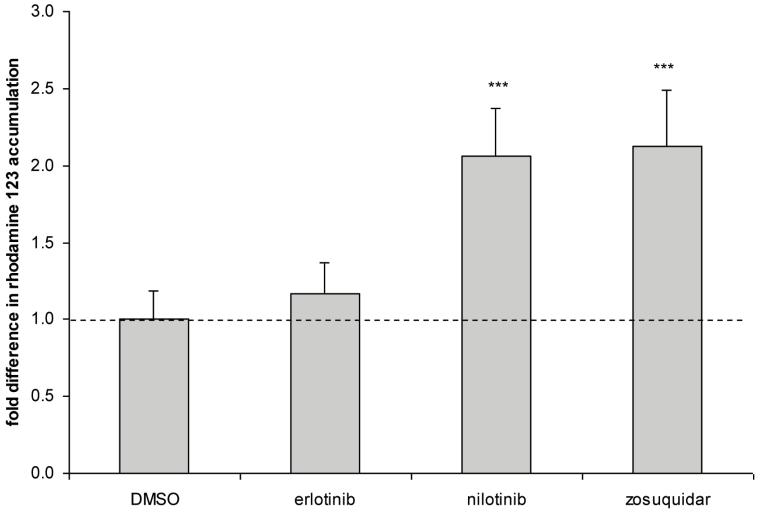

Figure 3 Inhibitory effect of nilotinib on Pgp in Pgp-overexpressing MDCKII-MDR1 cells.
Prior to determining the accumulation of the Pgp probe rhodamine 123 in MDCKII-MDR1 (as described in the Material and Methods section), these cells were incubated with erlotinib, nilotinib, the specific Pgp inhibitor zosuquidar (positive control) or with the negative control DMSO. The results are presented as mean data (± SD) of three different experiments (*P<0.05, ** P<0.01, ***P<0.001).

agents [2, 18]. Although several mechanisms have been identified by which cancer cells acquire resistance to these agents, not much is known about the effects of TKI-treatment on the expression of Pgp, while over-expression of Pgp is often associated with a drug resistant phenotype in cancer cells. In this study we demonstrated that TKIs have the propensity to induce Pgp, and we further showed that this TKI-mediated induction of Pgp requires PXR.

Exposure of LS180 cells, a proven model to study PXR functionality [19], to TKIs resulted in upregulation of Pgp within 48 hours. Especially, erlotinib, gefitinib, nilotinib, sorafenib and vandetanib significantly induced Pgp in wild-type LS180 cells. Knockdown of PXR in these cells resulted in a markedly lower, but not completely abolished, upregulation of Pgp in the case of erlotinib, gefitinib, nilotinib, sorafenib and vandetanib. This indicates that PXR plays a significant role in the TKI-mediated upregulation of Pgp. However, Pgp upregulation was not completely abolished in PXR knockdown LS180 cells, which may (in part) be explained by the knockdown efficiency of ~51%. It could also indicate that the TKIs interact with other (nuclear) transcription factors or signaling pathways that are involved in regulation of Pgp expression such as NF-κB [20], protein kinase C [21] and the PI3-kinase/Akt pathway [22]. Recently, Smith *et al*. (2008) demonstrated that, in contrast to our findings, gefitinib downregulated the expression of Pgp in ovarian cancer cells [23]. Most likely, this cell line does not express PXR, since treatment with paclitaxel [14], a known activator of PXR-mediated induction of Pgp, also did not induce Pgp in this cell line. The underlying mechanism behind the downregulation of Pgp by gefitinib in that cell line is still unknown.

Since upregulation of Pgp is an important cause of (multi)drug resistance, we evaluated whether TKI-mediated upregulation of Pgp could affect the accumulation of an established Pgp substrate; rhodamine 123. Indeed, the TKIs that caused upregulation of Pgp protein expression also decreased the accumulation of rhodamine 123, with the exception of nilotinib. The discrepancy between the strong increase in Pgp protein expression (~4 fold) and the moderate effect on rhodamine 123 accumulation (which was ~25% lower compared to the control) following treatment with nilotinib could be ascribed to Pgp protein inhibition as was shown in the rhodamine 123 accumulation assay in the Pgp over-expressing MDCKII-MDR1 cells. Together, these results directly link PXR activation to reduced accumulation of Pgp substrates in response to TKI exposure and thus

confirms the role of PXR in the regulation of drug resistance, as was also suggested by Chen *et al*. [24].

Apart from (multi)drug resistance, Pgp also plays an important role in the pharmacokinetics (e.g. absorption and excretion) of (anticancer) drugs. Pgp is expressed in important barrier tissues like the lining of the small intestine and in the liver, and therefore PXR-mediated upregulation of Pgp in these tissues may result in reduced absorption of (anticancer) drugs or increased biliary excretion of (anticancer) drugs. Indeed, two separate studies in which volunteers were pretreated with either rifampicin or hyperforin (active constituent of St.John's wort), which are both PXR agonists, show a significant effect on the pharmacokinetic profile and bioavailability of the metabolically inert Pgp probe substrate fexofenadine. Pretreatment with St.John's wort resulted in a 1.9-fold increase in fexofenadine's oral clearance [25], while rifampicin pretreatment resulted in a 1.3- to 5.3-fold increase in the oral clearance of fexofenadine [26]. These two studies may illustrate that PXR-mediated upregulation of Pgp also plays a role in clinically relevant drug-drug interactions. Since TKIs were shown in this study to have the ability to cause PXR-mediated upregulation of Pgp as well, these agents may also be involved in clinically relevant drug-drug interactions.

The clinical implications of our study remain to be explored. Currently, not much is known about Pgp-mediated drug resistance or drug-drug interactions following TKI treatment. This may be due to the novelty of these agents, but can also be caused by the complex interaction that many of these agents have with Pgp. Since several TKIs including erlotinib [27] are substrates of Pgp, these TKIs can auto-induce their own clearance by inducing intestinal and hepatic Pgp protein expression levels. Furthermore, with respect to the predictability of drug resistance or drug-drug interactions, another interesting question is raised by this study. Since the 4-anilinoquinazoline derived TKIs (erlotinib, gefitinib and vandetanib) were shown to activate PXR-mediated Pgp induction, possibly the 4-anilinoquinazoline backbone may serve as a pharmacophore for PXR activation. Structure-activity relationship and PXR-binding studies may help to predict clinically relevant drug-drug interactions and the propensity of compounds to induce cellular drug resistance.

In conclusion, we showed that TKIs are able to significantly upregulate Pgp expression and consequently decrease the accumulation of a Pgp-probe substrate. In addition, we showed that PXR is an important me-

diator of Pgp upregulation by TKIs. Although our results suggests that PXR-mediated Pgp upregulation in cancer cells following TKI treatment could be a relevant mechanism by which cancer cells protect themselves against these agents, the clinical implications of our study remain to be explored and further research is warranted.

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Chapter 6

Nuclear receptor-mediated induction of multidrug resistance associated proteins in four widely used cell lines

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Abstract

Multidrug resistance associated proteins (MRP) 1-9 are increasingly associated with multidrug resistance (MDR) in cancer. Several MRP proteins, especially MRP1, 2, 5, and 7, have been found to confer resistance to a wide variety of anticancer drugs. At the moment it is unclear whether the expression of MRP proteins can be induced by anticancer drugs. While MRP regulation studies in animals have provided evidence that nuclear receptors are involved in MRP induction, due to major interspecies differences, it is unclear whether the results of these studies are predictive for MRP regulation in humans. Therefore, we evaluated whether human cell lines (e.g. A549, HepG2, LS180, and T84) could be used to study nuclear receptor-mediated MRP(1-9) protein induction. We demonstrate that although most of the nuclear receptors and MRP proteins are expressed in the cell lines, activation of nuclear receptors did not result in induction of MRP1-7 protein expression levels. Only MRP8 protein expression was shown to be induced upon activation of FXR, PPARα and γ, AhR, and Nrf2. Overall, we conclude that although MRP protein expression is not affected by nuclear receptor activation in the T84 cell line, due to its versatile and ubiquitous expression of MRP proteins, it could be a very suitable model to study MRP substrate transport instead.

Introduction

The development of multidrug resistance (MDR) is a major limitation to the successful treatment of cancer patients. One of the main mechanisms underlying the development of MDR in response to anticancer drugs is induction of members of the ATP-binding cassette (ABC) superfamily of drug efflux transporters. These transporters efficiently mediate the efflux of many widely used anticancer drugs out of the cell. Well-known members of this family that already have been shown to be upregulated by anticancer drugs are P-glycoprotein (Pgp; ABCB1; MDR1) [1], and breast cancer resistance protein (BCRP; ABCG2) [2]. In addition, there is increasing evidence that also MRPs (MRP1-6, 7-9; ABCC1-6, ABCC10-12) can confer resistance to anticancer drugs [3, 4]. However, it is still unclear whether anticancer drugs can induce the expression of the different MRP isoforms. Therefore, there is a need for good models to study MRP induction by anticancer drugs.

It has been suggested that nuclear receptors may be involved in the induction of MRPs in humans. Nuclear receptors are ligand-activated transcription factors that, upon ligand binding, activate the upregulation of important drug metabolizing enzymes and drug transporters. Activation of the pregnane X receptor (PXR) by anticancer drugs has been shown to induce the expression of cytochrome P450 (CYP) 3A4 and Pgp proteins in cancer cells. In addition, this was shown to affect the pharmacokinetic profile of CYP3A4 and Pgp substrates [1]. Furthermore, induction studies in mice have revealed that nuclear receptors such as PXR, the constitutive androstane receptor (CAR) and the arylhydrocarbon receptor (AhR) could be involved in the regulation of several MRP isoforms [5]. However, due to major interspecies differences in ligand affinity and target gene regulation [6-9] , it is uncertain whether the conclusions drawn from these studies are predictive for the regulation of MRPs in human (malignant) tissues.

To study the involvement of nuclear receptors in the potential induction of MRPs by anticancer drugs in humans, it is important to have good *in vitro* models. Primary human hepatocytes or enterocytes are of special interest, because they mostly resemble the *in vivo* situation. However, the interindividual donor variability, rapid loss of drug metabolizing enzyme expression, poor availability, and the costs are major drawbacks of primary cells. Human cell lines are interesting alternatives, because they

are relatively cheap, easy to maintain and allow for high throughput screening approaches. In addition, some human cell lines, such as HepG2 [10-12] and LS180 [13], have already been used to study the effect of nuclear receptor activation on MRP mRNA expression. However, since Hagmann *et al*. (2008) recently showed that there is a poor correlation between basal mRNA and protein expression of different MRP isoforms in a panel of pancreatic cell lines [14], it is considered more clinically relevant to determine the effect of nuclear receptor activation on MRP protein expression levels. Therefore, in the present study the effect of nuclear receptor activation on the protein expression of different MRP isoforms (1-9) was evaluated in four different human cell lines (HepG2, LS180, T84, and A549) to determine which cell lines could serve as a good model to study (nuclear receptor-mediated) MRP induction by anticancer drugs.

Materials and methods

Materials

All human cell culture media and reagents were purchased from PAA Laboratories (Colbe, Germany) unless otherwise indicated. Fetal bovine serum (FBS) was obtained from Invitrogen (Breda, The Netherlands). 6-(4-Chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO) was purchased at Enzo Life Sciences BVBA (Biomol; Zandhoven, Belgium). All other chemicals were supplied by Sigma Aldrich (Zwijndrecht, The Netherlands) and were of the highest available grade.

Cell culturing

Lung carcinoma-derived A549 cells, hepatocarcinoma-derived HepG2 cells, colorectal carcinoma-derived LS180 and T84 cells, which are derived from a lung metastasis of a colorectal carcinoma, were obtained from LGC standards (ATCC; Middleton, UK). A549 and T84 were cultured in the supplier propagated media, while LS180 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 25 mM HEPES, and HepG2 in high glucose (2.5 g/L) Dulbecco's Minimal Essential Medium (DMEM). All media contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. Cells were maintained at 37^{o}C

under a humidified atmosphere of 5% CO_2.

Cell treatment

The basal expression of nuclear receptors and MRP isoforms was determined in cell lysates, which were prepared from confluent monolayers. The RIPA lysis buffer contained protease inhibitors (Hoffman La Roche, Basel, Switzerland). The inducible expression of the nuclear receptor marker proteins and MRP isoforms was assessed by treating the different cells with 10 µM rifampicin (PXR), 1 µM CITCO (CAR), 1 µM GW4064 (FXR), 1 µM troglitazone (PPARγ), 50 µM clofibrate (PPARα), 10 nM TCDD (AhR), or 1 µM tBHQ (nrf2) for 48 hours.

Immunoblot analysis

After 48 hours treatement, cells were washed with phosphate buffered saline (PBS), and lysed in 1 mL/well MilliQ water that contained protease inhibitors (Roche, Basal, Switzerland). The lysate was spinned down (5000 rpm, 5 min, 4°C), and the remaining pellet was resuspended in 100 µL RIPA buffer. The lysate was spinned down (10000 rpm, 5 min, 4°C) and the supernatant was transferred to a new collection tube. Protein concentrations were determined by a Pierce BCA protein assay (Pierce, Rockford, IL, USA). 10 µg of total protein was separated by SDS-polyacrylamide gel electrophoresis using NuPage novex 4-12% bis-tris gradient gels (Invitrogen, Breda, The Netherlands). Proteins were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA, USA). After blocking 1 h at RT in 3% BSA in tris-buffered saline containing 0.1% Tween-20, the membranes were incubated overnight at 4°C with primary antibody. The antibody clones that were used to detect the different MRP isoforms, nuclear receptors and marker proteins are found in table 1. Primary antibody incubation was followed by incubation with an appropriate secondary antibody coupled to horse radish peroxidase (HRP). β-actin (1:10000; AC-15; Abcam, Cambridge, UK) was used as a loading control. The proteins were visualized with a chemiluminescence-based detection reagent (SuperSignal West Femto; Pierce, Rockford, IL, USA) and the intensities of the protein bands were determined on a Gel Doc XRS Imaging system with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

TABLE 1 Antibody clones

Transporter	Antibody	Nuclear receptor	Antibody	Marker protein	Antibody
MRP1	MRPr1	PXR	H-11	Pgp*	C219
MRP2	M2III-6	CAR	M-127	CYP7A1	H-58
MRP3	M3II-9	FXR	H-130	CYP4A11	M25-P2A10
MRP4	M4I-10	PPARα	H-98	CYP1A1	H-70
MRP5	M5I-1	PPARγ	E-8	NQO1*	A180
MRP6	M6II-31	AhR	C-4		
MRP7	H-300	Nrf2	H-300		
MRP8	H-190				
MRP9	H-215				

*obtained from Abcam (Cambridge, UK). All others from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany)

Results and discussion

Since MRPs are increasingly shown to confer resistance to anticancer drugs, induction of MRPs might contribute to the development of MDR in cancer [15]. Therefore, it is important to have good models to study anticancer drug-mediated induction of MRP protein expression. In the current study, the lung carcinoma (A549), hepatocarcinoma (HepG2), colorectal carcinoma (LS180) and colorectal lung metastasis derived (T84) cell line, were evaluated as models to study nuclear receptor-mediated MRP protein induction.

Nuclear receptor expression and functionality

To show that the cell lines express the nuclear receptors that could potentially be involved in the regulation of MRP induction, the basal protein expression of PXR, CAR, farnesoid X receptor (FXR), nuclear factor – erythroid 2 – related factor 2 (nrf2), AhR, and peroxisome proliferator-activated receptor (PPAR) α/γ, was characterized. As shown in figure 1, the protein expression of the nuclear receptors varied highly between the different cell lines. PXR had a high protein expression in A549 and T84, while it was lower in LS180 and HepG2 cell, which was a remarkable finding since LS180 and HepG2 are regarded as suitable models to study PXR mediated enzyme induction [16-18]. Unlike the bile acid sensor FXR, which was only expressed in T84, CAR was found in all cell lines. PPARα

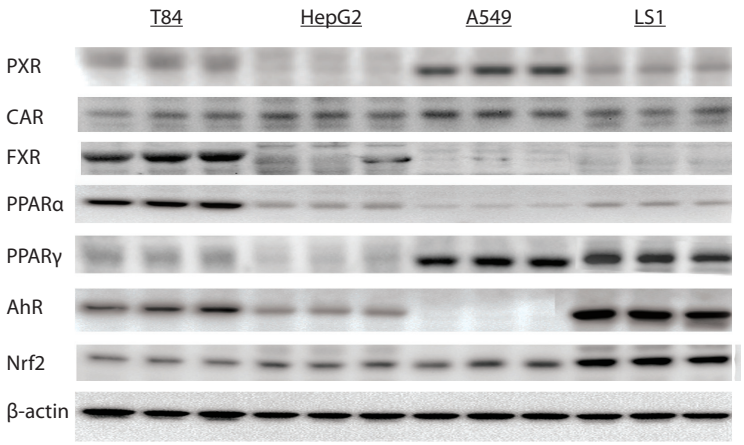

Figure 1 Basal nuclear receptor protein expression in different cell lines
The basal protein levels of the nuclear receptors were assessed in 4 cell lines. Proteins (10 µg) were separated using gradient 4-12% SDS-PAGE, and transferred to PVDF membrane. Immunoblotting was used to detect the proteins. Equal loading was verified with β-actin as a loading control. Blots are representative of three different determinations.

had a high expression in T84, but could not be detected in A549, which seems remarkable because it was shown that A549 expresses high PPARα mRNA levels compared to the mRNA expression of other nuclear receptor such as PXR, FXR, CAR [19]. PPARγ on the other hand had a higher expression in A549 and LS180 cells compared to T84 and HepG2 cells. With the exception of A549 cells, AhR could be detected in the other cell lines, with a high expression in LS180. In addition, nrf2 also showed the highest expression in LS180 compared to the other cell lines. In summary, HepG2, LS180 and T84 express all nuclear receptor proteins and therefore could potentially be interesting models to study nuclear receptor mediated induction of MRP protein expression. The suitability of A549 cells might be restricted to study PXR, CAR, PPARγ and nrf2 mediated enzyme induction.

Although it was shown that the cell lines express most nuclear receptors, it is unclear whether these receptors are functional. Therefore, to show that the expressed nuclear receptors are functional, the cells were treated with prototypical agonists of each nuclear receptor. As shown in figure 2, PXR-mediated induction of Pgp was functional in both T84 and

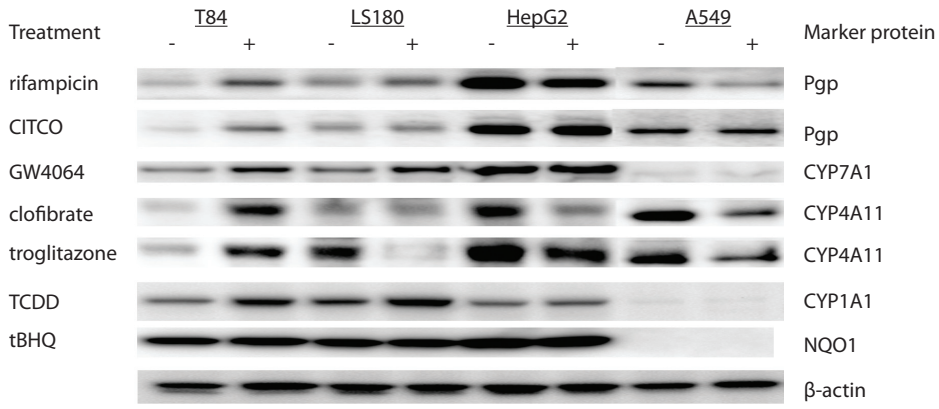

Figure 2 Nuclear receptor functionality
The functionality of the nuclear receptors was assessed by determining the induction of the respective marker protein of each nuclear receptor upon treatment with (+) or without (-) an agonist. Marker protein levels were determined with immunoblot analysis. Proteins (10 µg) were separated using gradient 4-12% SDS-PAGE. Equal loading was verified with β-actin as a loading control. Blots are representative of three different determinations.

LS180, but was not active in HepG2 and A549. CAR on the other hand was only found to mediate the upregulation of Pgp in T84 cells and did not mediate the induction of Pgp in LS180 and HepG2. These results confirm the findings of Gupta *et al*. (2008) [20] and Kobayashi *et al*. (2003) [21], who showed that CAR is not functional in LS180 and HepG2 cell lines. FXR activation resulted in induction of CYP7A1 in T84 and LS180, but in downregulation of CYP7A1 in HepG2 cells. In addition, treatment with the PPARα agonist clofibrate and PPARγ agonist troglitazone resulted in induction of their marker protein CYP4A11 [8] in T84 cells, while in the other cell lines a downregulation was observed. An explanation for the conflicting findings regarding FXR, PPARα and PPARγ activation could not be found in literature, and warrants further research. However, it does show that FXR-, PPARα- and PPARγ-mediated marker protein induction is cell type dependent. The induction of the AhR marker protein CYP1A1 resembled the expression pattern of AhR; a high CYP1A1 induction after treatment with the AhR agonist TCDD in T84 and LS180 cells, while CYP1A1 could hardly be induced in HepG2, and was not detectable in A549 cells. In addition, the nrf2 marker protein NAD(P)H-quinone oxi-

doreductase 1 (NQO1) was also not detectable in A549 cells after exposure to the nrf2 activator tBHQ. In the other cells NQO1 was detected, but was not induced after treatment with tBHQ. This indicates that nrf2 might not be active in these cells.
Based on the nuclear receptor functionality results, the T84 and LS180 cell lines are the most promising models to study nuclear receptor-mediated enzyme induction. Furthermore, the results show that although nuclear receptors are expressed, this not necessarily means that these receptors are functional and can contribute to enzyme induction. In addition, some nuclear receptors (e.g. FXR, PPARα/γ) were shown to regulate their marker genes in a cell type dependent manner.

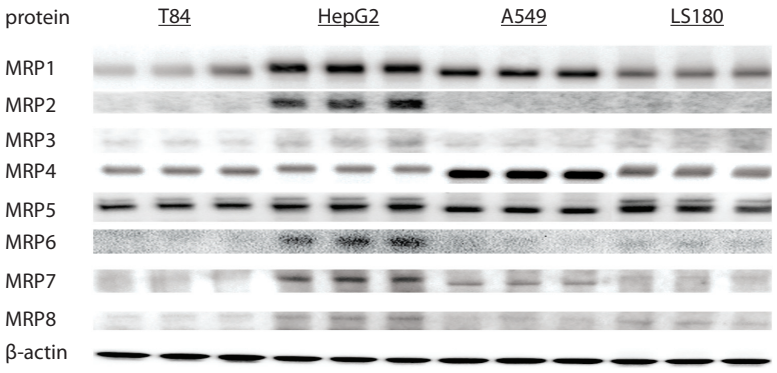

Figure 3 Basal MRP1-9 protein expression in different cell lines
The basal protein levels of MRP isoforms 1-9 were assessed in 4 cell lines. Proteins (10 µg) were separated using gradient 4-12% SDS-PAGE, and transferred to PVDF membrane. Immunoblotting was used to detect the proteins. Equal loading was verified with β-actin as a loading control. Blots are representative of three different determinations.

MRP isoform expression and regulation

The basal MRP1-9 protein expression patterns in the different cell lines were characterized. As shown in figure 3, all cell lines expressed MRP1, 4 and MRP5 protein. Interestingly, this paralleled the observation of Hagmann *et al*. (2008), who found that a panel of pancreatic cell lines also ubiquitously expressed the same three MRP proteins [14]. MRP2 protein was highly detectable in HepG2 and to a lesser extent in T84. MRP3 was hardly detectable in any of the cell lines. In addition, protein expression of MRP6 was only detected in LS180 and HepG2, while MRP7 and MRP8 were expressed in all cell lines, with the highest expression in HepG2.

In contrast, protein expression of MRP9 was not detectable in any of the cell lines, which is supported by the findings of Bera *et al*. (2001), who have shown that MRP9 protein expression is restricted to breast cancer tissue [22]. Overall, HepG2 cells showed the highest and most versatile expression of the different MRP isoforms, followed by T84. However, in contrast to HepG2, T84 cells are able to form polarized monolayers [23], which allow apical-to-basolateral or apical-to-basolateral MRP substrate transport studies.

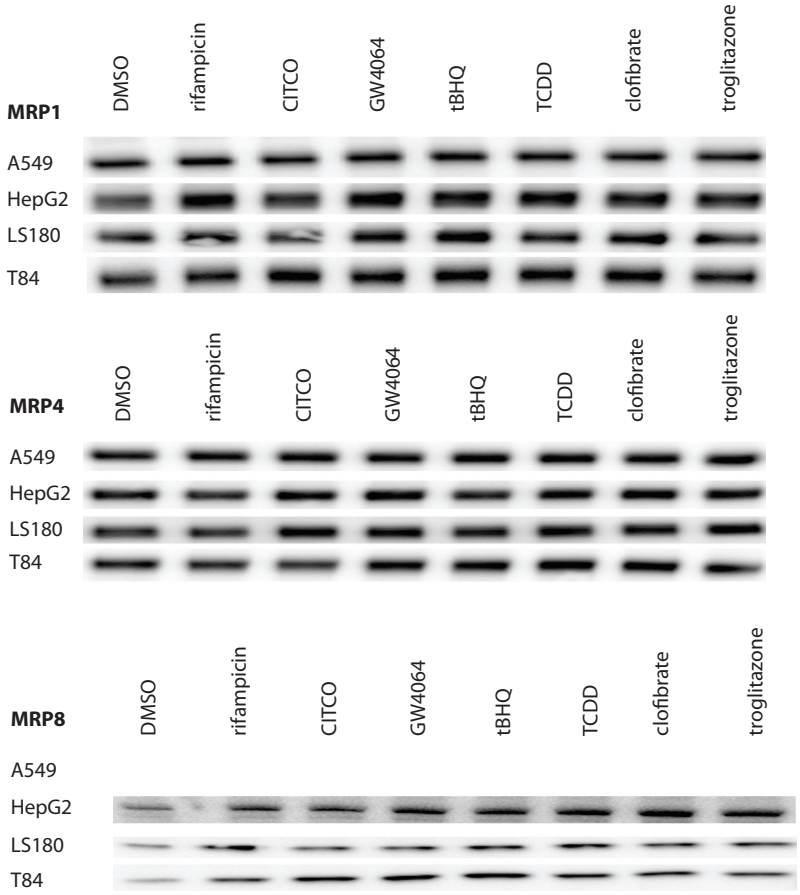

Figure 4 Regulation of MRP protein expression by nuclear receptors in different cell lines
Inducible protein expressions of MRP1-8 were determined upon exposure to the agonists of the nuclear receptors (PXR, CAR, FXR, Nrf2, AhR, PPARα, and PPARγ, respectively). Proteins (10µg) were separated using gradient 4-12% SDS-PAGE, and transferred to PVDF membrane. Immunoblotting was used to detect the proteins. Equal loading was verified with β-actin as a loading control. Protein expressions of MRP1-7 were not induced upon treatment. Two representative blots of MRP1 (A) and MRP4 (B) are shown. MRP8 protein was inducible in HepG2, LS180 and T84, but not in A549 cells (C).

To assess whether nuclear receptors are involved in the upregulation of the different MRP isoforms, the protein expression levels of MRP1-9 were determined after 48 h treatment with prototypical nuclear receptor agonists. MRP8 protein levels were shown to be upregulated by FXR, AhR, nrf2 and PPARα/γ in HepG2, LS180, and T84 (figure 4). MRP8 has been shown to confer resistance to 5-fluorouracil (5FU) [24] and, therefore, drugs that are agonists of the indicated nuclear receptors might interfere with the pharmacological activity of 5FU by inducing MRP8 in tumors that functionally express these nuclear receptors. In contrast to MRP8, none of the other MRP protein expression levels were induced upon treatment with nuclear receptor agonists (figure 4; immunoblots of two representative MRPs are shown). While MRP regulation studies in human cell lines [10-12] and in rodents [5, 25] have shown that nuclear receptor agonists can induce MRP mRNA expression, our results clearly show that nuclear receptor agonists do not affect the expression of MRP1-7 proteins in four human cell lines. These findings are in concordance with a study by Hagmann *et al*. (2008), who showed that MRP mRNA expression was not predictive for MRP protein expression in pancreatic cell lines [14]. Why mRNA expression is not predictive for MRP protein expression is not understood and remains to be explored.

Conclusion

Although several nuclear receptors that have been implicated in the transcriptional regulation of MRP isoforms are functionally expressed in the four cell lines, only MRP8 protein expression was induced upon activation of FXR, AhR, nrf2 and PPARα/γ in HepG2, LS180 and T84 cells. Overall, our findings suggest that the protein expression of MRPs is poorly regulated by nuclear receptors in the four cell lines. However, this does not necessarily mean that MRP protein expression can not be induced. Since also kinase signalling (e.g. JNK and ERK) was shown to be involved in the upregulation of MRPs [26, 27], it might be relevant to study the effect of JNK/ERK activation on MRP protein expression.

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Chapter 7

Daunorubicin induced-breast cancer resistance protein upregulation is regulated by stress activated protein kinases in the acute leukemia cell line KG1a

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J.H.Beijnen and J.H.M. Schellens

(preliminary report)

Abstract

Clinical resistance to anticancer drugs hinders the successful treatment of leukemia. Resistance of hematological malignancies to anticancer drugs often involves the upregulation of breast cancer resistance protein (BCRP), a member of the ATP-binding cassette (ABC) drug efflux transporter family. To be able to predict or prevent MDR in acute myeloid leukemia (AML), the involvement of nuclear receptors and stress activated protein kinases in BCRP induction was investigated. The present study demonstrates that in contrast to estrogen receptor (ER) and arylhydrocarbon receptor (AhR), activation of the other nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D_3 receptor (VDR), nuclear factor-erythroid 2 related factor 2 (nrf2), and peroxisome proliferator-activated receptor α/γ (PPARα/β) by prototypical agonists did not result in upregulation of BCRP. Combination treatment of daunorubicin with specific inhibitors against the protein kinases PI3K, JNK, p38/MAPK and PKC showed an attenuation of the daunorubicin induced BCRP upregulation by all of these protein kinase inhibitors. Protein kinase inhibitors may therefore be effective in preventing or reversing BCRP-mediated clinical resistance in AML.

Introduction

The development of clinical resistance to anticancer drugs is one of the major causes of therapeutic failure in leukemia. Clinical resistance can arise from exposure to a single drug and render a tumor resistant to multiple structurally unrelated drugs, a phenomenon known as multidrug resistance (MDR). MDR often involves the overexpression of members of the ATP-binding cassette (ABC) efflux transporter family, in particular P-glycoprotein (Pgp; MDR1; ABCB1), multidrug resistance associated protein-1 (MRP1; ABCC1) and breast cancer resistance protein (BCRP; ABCG2; CD338). Especially BCRP expression has been related with resistance to anticancer drugs that are extensively used in leukemia treatment regimens such as mitoxantrone, daunorubicin, doxorubicin, topotecan and imatinib [1-3]. BCRP was shown to be overexpressed in relapsed AML patients and has also been associated with secondary AML in geriatric patients [4, 5]. Furthermore, BCRP expression has been identified as a prognostic factor for poor treatment outcome in AML [4, 6, 7].

The expression of BCRP was shown to be upregulated in response to exposure to anticancer drugs such as doxorubicin and topotecan [8]. Several mechanisms have been implicated in the regulation of BCRP such as nuclear receptor activation (e.g. estrogen receptor α (ERα), arylhydrocarbon receptor (AhR), peroxisome proliferator- activated receptor γ (PPARγ) and AKT signaling [9-12]. Other nuclear receptors such as the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have been shown to be involved in the regulation of other ABC-efflux transporters such as Pgp and MRP2 [13-15]. However, so far they have not been implicated in BCRP regulation. Understanding the mechanisms underlying anticancer drug induced MDR could improve clinical drug efficacy in AML. Therefore, in the present study the regulation of BCRP expression by nuclear receptors and stress-activated protein kinases was investigated. This study provides evidence for the involvement of the stress-activated protein kinases JNK, p38/MAPK, PKCγ, and PI3K, in the upregulation of BCRP in the AML cell line KG1a. Our results demonstrate that combined administration of daunorubicin and protein kinase inhibitors may create opportunities for the development of new strategies for the reversal of MDR in AML.

Material and methods

Materials

The human acute myeloid leukemia cell line KG1a was a kind gift from Dr.R.A. Raymakers (Radboud UMC, Nijmegen, The Netherlands). All human cell culture media and reagents were purchased from PAA Laboratories (Colbe, Germany) unless otherwise indicated. Fetal bovine serum (FBS) was obtained from Invitrogen (Breda, The Netherlands). SP600125, Bisindolylmaleimide IX (Bis-IX; Ro-31-8220), SB203580 and wortmannin were obtained from Biaffin (Kassel, Germany). All other chemicals were of the highest available grade and purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Anti-BCRP (BXP-21) and β-actin coupled to horseradish peroxidase (HRP) antibody (AC-15) were obtained from Abcam (Cambridge, UK).

Cell culture

KG1a cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM), which contained 10% FBS, 2 mM L-glutamine, and supplemented with penicilline and streptomycin. Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 at 37°C.

Cell treatment

Nuclear receptors

KG1a (2.5×10^5 cells/ml) cells were treated with 10 μM rifampicin, 1 μM 6-(4-Chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO), 100 nM calcitriol, 50 μM clofibrate, 1 μM troglitazone, 10 μM tert-butylquinone (tBHQ), 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 1 μM 17β-estradiol, 10 μM menadione or 0.1% DMSO. After 24 h the cells were processed for immunoblotting assays.

Kinase inhibitors

KG1a (2.5 x 10^5 cells/ml) cells were pre-incubated for 30 min with 50 nM Bis-IX , 500 nM SB203580, 1 μM SP600125, 50 nM wortmannin, or 0.1% DMSO. Following pre-incubation, the kinase inhibitors were combined with 0.1 μM daunorubicin or 0.1% DMSO. After 24 h the cells were processed for immunoblotting assays.

Immunodetection of BCRP

Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodiumdeoxycholate, 5 mM EDTA, protease and phosphatase inhibitors (Roche, Basal, Switzerland)). Proteins in the cell lysates were reduced by addition of sample buffer (Invitrogen) containing 30 mM dithiothreitol (DTT) was added, and heated at 95°C for 5 minutes. Proteins (10μg) were separated on NuPage Novex Bis-Tris precast 4-12% gradient gels (Invitrogen) and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 3% bovine serum albumine (BSA) in Tween-20 (0.5%)/tris buffered saline (TBS-T), pH 7.4 for 1 h at RT and then incubated overnight with primary antibody followed by a 1 h incubation with the appropriate secondary HRP conjugated antibody. The BCRP and β-actin protein bands were visualized using an enhanced chemiluminescence-based detection reagent (West Femto; Pierce) and the intensities of the BCRP and β-actin protein bands were determined on a ChemiDoc XRS Imaging system and analyzed with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

A student's *t* test was used to analyze the difference between two groups. Multiple group comparisons were analyzed using ANOVA with Bonferroni post-hoc testing and were considered significant when $P<0.05$. The statistical tests were performed using SPSS version 14 (SPSS Inc., Chicago, IL,USA).

Results and discussion

Upregulation of BCRP is an important cause of MDR in hematological malignancies. Since the inducible protein expression of important members of the ABC-subfamilies (e.g. ABCB and ABCC) that are already implicated in MDR is regulated by nuclear receptors, the involvement of these nuclear receptors in the regulation of BCRP is investigated. KG1a cells were treated with several established nuclear receptor agonists or activators such as rifampicin (PXR), CITCO (CAR), calcitriol (vitamin D_3 receptor; VDR), clofibrate (PPARα), troglitazone (PPARγ), TCDD (AhR), 17β-estradiol (ERα) and tBHQ (Nuclear factor-erythroid 2 related factor 2; Nrf2). Only 17β-estradiol and TCDD were able to significantly alter the expression of BCRP in KG1a cells (figure 1).

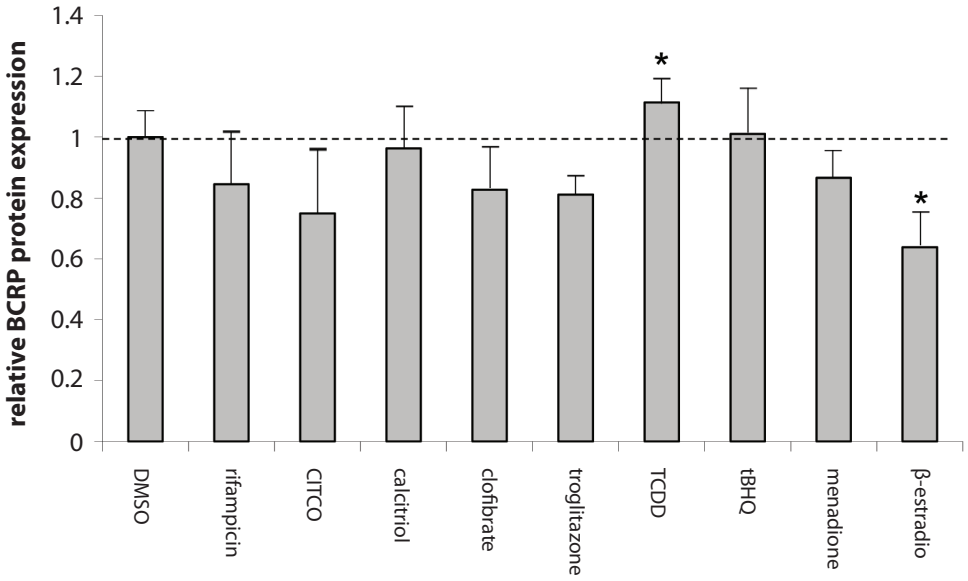

Figure 1 Effect of nuclear receptor activation on BCRP expression in KG1a cells

BCRP expression in KG1a cells following treatment with 10 μM rifampicin (PXR), 1 μM 6-(4-Chlorophenyl) imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO; CAR), 100 nM calcitriol (VDR), 50 μM clofibrate (PPARα), 1 μM troglitazone (PPARγ), 10 μM tert-butylquinone (tBHQ; nrf2), 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; AhR), 1 μM 17β-estradiol (ERα), 10 μM menadione (nrf2) or 0.1% DMSO. The bargraph shows the mean data (± SD) of three independent experiments. These data are normalized to the DMSO controls.

TCDD has already been shown to induce the expression of BCRP in Caco-2 cells [10], but this was never shown in leukemia cells. In addition, in our study 17β-estradiol downregulated BCRP protein expression in KG1a cells. Downregulation of BCRP was also observed in the placental BeWO cell line upon 17β-estradiol treatment [16], while in primary trophoblasts 17β-estradiol induced the protein expression of BCRP [17]. This suggests a cell type dependent regulation of BCRP by ERα. The same holds for PPARγ, which has been shown to induce BCRP expression in dendritic cells [11]. However, in KG1a no induction was observed after treatment with the PPARγ agonist troglitzone in KG1a cells. Possibly, PPARγ mediated induction of BCRP is also cell type dependent.

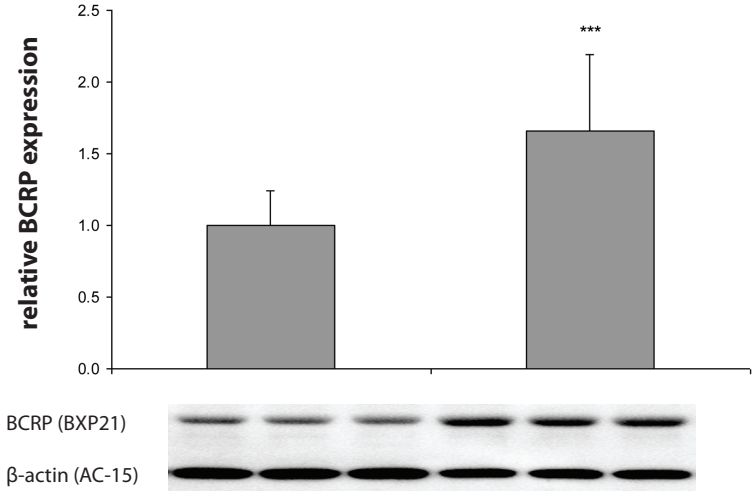

Figure 2 Daunorubicin induced upregulation of BCRP
BCRP expression in KG1a cells as was determined after treatment with 0.1 μM daunorubicin for 24 h The bargraph shows the mean data (± SD) of three independent experiments. These data are normalized to the DMSO controls. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

In addition to nuclear receptors, the involvement of stress-activated protein kinases in the regulation of BCRP induction was assessed. Since daunorubicin is known to activate several stress-activated kinase signaling pathways (as reviewed by Laurent *et al*. (2001) [18]), KG1a cells were treated with daunorubicin. As shown in figure 2, daunorubicin indeed induced the expression of BCRP in KG1a cells.

Therefore, the involvement of the stress-activated protiein kinases PI3K, p38/MAPK, JNK, and PKCγ in daunorubicin-mediated induction of BCRP was evaluated. As shown in figure 3(a/b), cotreatment of daunorubicin with wortmannin, SB203580, SP600125, or Bis-IX (inhibitors of the respective proteins) significantly abolished daunorubicin-mediated induction of BCRP, while the inhibitors alone did not affect BCRP expression levels in the KG1a cells. A cytotoxicity assay was performed to assess the cytotoxicity of the individual compounds and the compound combinations. No cytotoxicity was observed for the concentrations used (results not shown). These results indicate that PI3K, p38/MAPK, JNK, and PKC signaling cascades are involved in BCRP upregulation. However, it is unsure if these kinase inhibitors have off-target effects, therefore knock-down of the specific kinases with siRNA should produce more convincing results that support the finding that these kinases are involved in daunorubicin-induced BCRP upregulation.

Overall, the preliminary results presented here indicate that BCRP induction is mainly regulated by stress-activated protein kinases, namely PI3K, JNK, p38/MAPK, and PKC. Inhibition of these stress-activated protein kinases resulted in attenuation of the daunorubicin-induced BCRP protein upregulation. Further experiments should provide information whether inhibition of kinase mediated-BCRP induction indeed results in increased BCRP substrate accumulation. FACS determination of differences between the accumulation of a fluorescent BCRP substrate such as mitoxantrone, topotecan, or HOECHST33342 in control cells, daunorubicin treated cells or cells that are treated with a combination of daunorubicin and a SAPK inhibitor, should further produce results that support the concept of modulating BCRP induction to enhance the effectivity of anticancer drugs.

This study demonstrates that cotreatment of daunorubicin with inhibitors of stress-activated protein kinases attenuates anticancer-drug induced BCRP expression, and thus might be an interesting new approach in preventing or reversing daunorubicin-induced BCRP upregulation in AML patients.

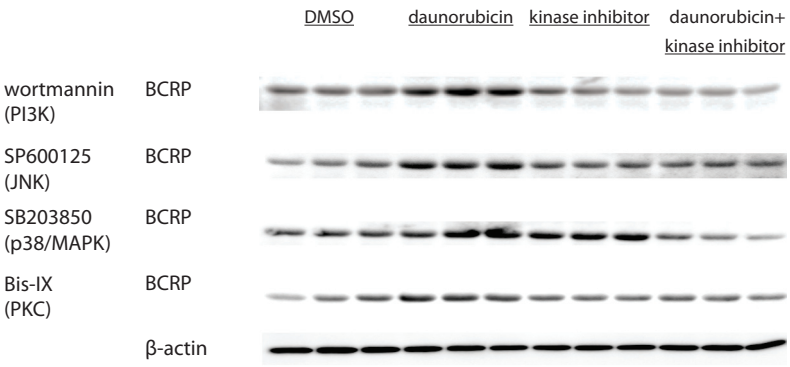

Fig.3a BCRP induction by daunorubicin is attenuated by combined treatment with protein kinase inhibitors

BCRP expression following combination treatment of 0.1 μM daunorubicin with 50 nM wortmannin, 500 nM SB203580, 1 μM SP600125, or 50 nM Bis-IX or 0.1% DMSO for 24 h in KG1a cells. The BCRP expression on the immunoblots is representative for the BCRP expression levels of three independent experiments (n=3).

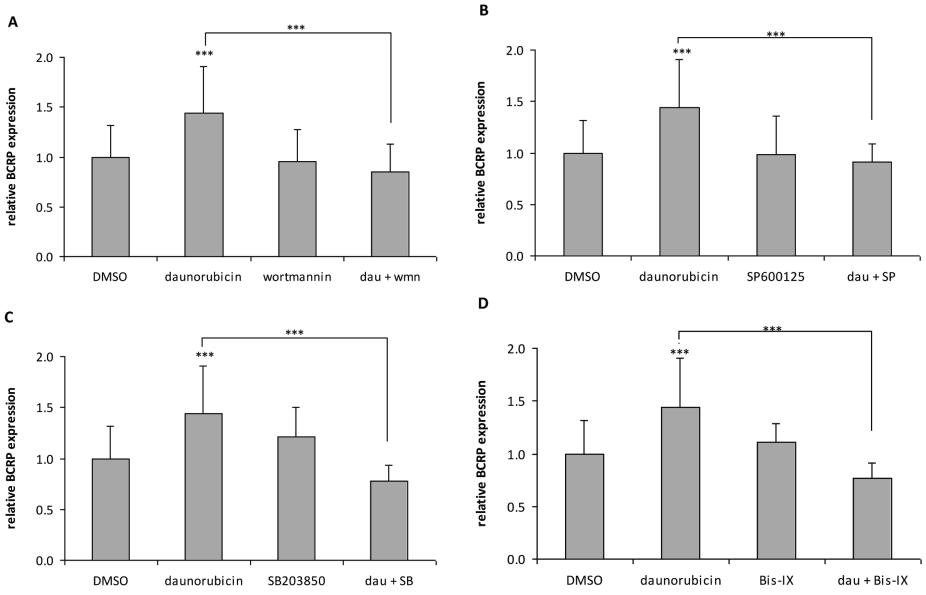

Fig.3b BCRP induction by daunorubicin is attenuated by combined treatment with protein kinase inhibitors

BCRP expression following combination treatment of 0.1 μM daunorubicin with 50 nM wortmannin (A), 1 μM SP600125 (B), 500 nM SB203580 (C), or 50 nM Bis-IX (D) for 24 h in KG1a cells. The bargraph shows the mean data (± SD) of three independent experiments. The data are normalized to DMSO controls. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

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Chapter 8

Conclusion and perspectives

Conclusions and Perspectives

Drug metabolizing enzymes and drug efflux transporters of the ABC-family are key determinants of the pharmacokinetic profile of anticancer drugs and drug sensitivity of tumors. Changes in the expression or activity of drug metabolizing enzymes and transporters, as a result of inhibition or induction, can affect the pharmacokinetic profile of anticancer drugs. Due to the specific properties of anticancer drugs, such as steep dose-toxicity curves and narrow therapeutic indices, already small pharmacokinetic changes can have major clinical implications such as reduced or even loss of therapeutic efficacy, but also lethal intoxications. Direct inhibition of anticancer drug metabolism or transport will rapidly lead to increased plasma levels of cytotoxic agents resulting in acute intoxications. These are often easily recognized and associated to a specific drug. In contrast, enzyme induction in general will lead to reduced plasma levels of anticancer drugs resulting in therapeutic failure or drug resistance. Enzyme induction is hardly recognized as the underlying mechanism, due to the slow onset of enzyme induction and because therapeutic failure and drug resistance are common in cancer treatment. Therefore, often the clinical consequences of enzyme induction are mistaken for being treatment related, and as a result, the clinical effects of enzyme induction are underestimated. However, from literature it is known that several widely used drugs, that include the antibiotic rifampicin, the anticonvulsants phenobarbital and phenytoin, and the over-the-counter herbal antidepressant St.John's wort, can induce drug metabolizing enzymes and transporters. These inducers adversely affected the pharmacokinetic profile of anticancer drugs, which was shown to have far reaching clinical implications. For instance, rifampicin was shown to drastically reduce the plasma levels of the anticancer drug irinotecan. However, so far anticancer drugs themselves have only scarcely been associated with enzyme induction. Therefore, the rationale behind this thesis was to investigate whether anticancer drugs can induce drug metabolizing enzymes and transporters and thereby adversely affect their own pharmacokinetic profile, efficacy or toxicity, or that of co-administered drugs such as other anticancer drugs or drugs to treat regimen-related side effects.

After conducting extensive *in vitro* studies, we conclude that many widely used anticancer drugs can induce the expression of cytochrome P450 (CYP) 3A4 and P-glycoprotein (Pgp) (**Chapter 3 - 5**). Consequently,

these agents were shown to be able to adversely affect the pharmacokinetics of CYP3A4 and Pgp substrates. Since CYP3A4 and Pgp are involved in the metabolism and disposition of many widely used anticancer drugs, the anticancer drugs identified as enzyme inducers thus might alter their own pharmacokinetic profile or that of other agents that are substrate for these enzymes. In addition to the more conventional anticancer drugs, also some of the very promising recently approved tyrosine kinase inhibitors were shown to induce Pgp and adversely affect the accumulation of Pgp substrates (**Chapter 5**). Three of these tyrosine kinase inhibitors (TKIs), erlotinib, gefitinib, and nilotinib were even shown to be as potent as the infamous inducer rifampicin that has been implicated in many clinically relevant pharmacokinetic drug-drug interactions. This implies that it is very likely that these TKIs cause clinically significant interactions. Based on our results, physicians should be aware of pharmacokinetic drug interactions as an underlying mechanism of unexpected therapeutic failure or toxicities when administering these agents.

Apart from drug-drug interactions, drug induced-multidrug resistance (MDR) is another important drawback of the treatment of cancer. One of the most established mechanisms behind acquired drug resistance in response to anticancer drugs is the induction of ATP-binding cassette (ABC) drug efflux transporters by cancer cells. Drug efflux transporters play an important role in the uptake and extrusion of a wide variety of anticancer drugs. Since the clinical efficacy of anticancer drugs is highly dependent on the accessibility of these agents to their intracellular targets, increased transporter-mediated extrusion will result in decreased intracellular accumulation, rendering the tumor more resistant. Pgp has already been associated with MDR, and was identified as a marker for treatment response as high Pgp expression is correlated with poor treatment outcome. In view of the fact that Pgp also plays a very important role in MDR, the data presented in **Chapter 4** and **5**, might also indicate that the anticancer drugs that were shown to induce Pgp expression, could induce the development of MDR in cancer.

Together, our results show that induction of drug metabolizing enzymes and transporters by anticancer drugs could be an important underlying mechanism in clinically relevant drug-drug interactions and the development of multidrug resistance in oncology. Modulating the process of enzyme induction, therefore, might be an interesting approach to prevent or circumvent pharmacokinetic drug interactions or the development of

MDR. However, therefore first the mechanisms underlying enzyme induction have to be clarified. In this thesis we demonstrate that the nuclear pregnane X receptor (PXR) is one of the main regulators of anticancer drug-induced upregulation of CYP3A4 and Pgp. Furthermore, activation of PXR was shown to affect the pharmacokinetics of respective enzyme substrates. In this view, PXR activation might contribute to pharmacokinetic drug interactions and the development of MDR in cancer (**Chapter 3, 4** and **5**). Moreover, apart from nuclear receptors, stress-activated kinases (SAPKs) were shown to be involved in anticancer drug-induced upregulation of the breast cancer resistance protein, a protein that has also been frequently implicated in MDR, especially in leukemias.

Thus, many potential targets were identified that could be exploited to inhibit the process of anticancer drug-induced enzyme induction. Indeed, targeting the expression of PXR with siRNA, resulted in attenuation of drug-induced Pgp expression, and as a result enhanced the cytotoxic activity of the Pgp substrate doxorubicin (**Chapter 4**). In addition, inhibition of the SAPKs was shown to attenuate daunorubicin-induced upregulation of BCRP (**Chapter 7**). These results clearly support the idea of exploiting nuclear receptors and SAPK as potential drug targets to enhance the accumulation of anticancer drugs, and thus prevent the development of MDR.

However, therefore it is first important to map the complex network underlying drug metabolizing enzyme and transporter induction. Although we have shown that nuclear receptors and some kinases are involved in the regulation of enzyme induction, several papers have also shown that nuclear receptors themselves such as hepatocyte nuclear factor 4α (HNF4α) and the glucocorticoid receptor (GR) regulate the expression of PXR and CAR (**Chapter 1**). In addition, kinase signaling cascades such as NF-κB have also been shown to regulate the expression of nuclear receptors such as PXR and CAR. Thus, by transcriptionally controlling the regulation of nuclear receptors, NF-κB is able to indirectly regulate enzyme induction. On top of that, nuclear receptors require complexation with co-regulators to be able to activate target gene transcription (**Chapter 1**). Conformational changes induced by ligand binding either allow the recruitment of a co-activator, which subsequently activates gene transcription, or a co-repressor, which prevents gene transcription.

The seeming complexity of this whole regulatory network involved in enzyme induction warrants the use of high throughput based approaches

such as *in silico* screening, microarrays and protein arrays. Currently, *in silico* screenings allow the screening of promoter sequences for specific response elements within the human genome, and thus can be used to identify which genes are regulated by specific nuclear receptors or kinases. Chromatin immunoprecipitation (ChIP) on chip (ChIP-on-chip) can be used to actually study the interaction of nuclear receptors with their response elements in the gene-promoters on a genome wide scale, both *in vitro* and *in vivo*. Furthermore, microarrays can be assessed to study the changes in the mRNA expression profiles of genes induced by nuclear receptor activation, and provide information on which genes are transcriptionally activated by nuclear receptors. However, we have shown (**Chapter 6**) that the mRNA expression of certain transporters is not predictive. In addition, because proteins are the actors of the cell, changes in protein levels may be more relevant. Therefore, protein array approaches such as reverse-phase immunoassays, that allow the determination of the expression of hundreds of proteins following nuclear receptor activation, are preferred. Moreover, since nuclear receptors require complexation to co-regulators to initiate gene-transcription, it is important to identify which co-regulators can bind to nuclear receptors, and because ligand binding-induced conformational changes highly impact the affinity of nuclear receptors for co-regulators, also the effects of ligands on the co-regulator binding profile should be evaluated. An interesting technique that can be used to determine such protein-protein interactions is ligand fishing. Basically, immobilized nuclear receptors (either in their apo or ligand bound state) are used to "fish" out proteins from a cell lysate. Proteomic approaches such as 2D-gel separation and subsequent MS/MS are used to identify the proteins (but also DNA) that are bound to a specific nuclear receptor. This provides information on how ligands mediate gene transcription by changing the co-regulator profile. In summary, the above described approaches can help to elucidate the complex network involved in enzyme induction, and may additionally identify interesting new drug targets.

Identification of new drug targets, such as PXR and SAPKs, provides opportunities to develop drugs that exploit these targets. Since PXR activation has been shown to play an important role in inducing pharmacokinetic drug-drug interactions and the development of multidrug resistance (**Chapter 4**), inhibiting the action of this receptor may provide interesting therapeutic options to battle these adverse events. At the

moment, many PXR agonists and only a few antagonists have been identified. Computational approaches, such as modeling, may provide new drug candidates by identifying (new) pharmacophores in known nuclear receptor ligands. Additional affinity assays, such as ligand fishing, can then be applied to identify whether the newly designed drugs have affinity for a specific nuclear receptor. Ligand fishing can also be used to determine PXR (ant)agonists in complex mixtures such as herbal extracts, which are often used by patients.

Because many high throughput approaches heavily rely on cell lysates, it is very important to choose the right cell model. The FDA guidelines prescribe primary cultures of human hepatocytes as the preferred model to evaluate the DME induction potential of new drug candidates. However, low availability, high cost, high interindividual donor variation, rapid decline of metabolic enzyme expression and the additives required to culture hepatocytes such as the very potent PXR agonist dexamethasone, are major drawbacks of this model. Cell lines seem better alternatives, and have been demonstrated to be useful in assessing nuclear receptor mediated induction of drug metabolizing enzymes and transporters by anticancer drugs (**Chapter 2** and **Chapter 6**). However, when using cell lines it is very important to evaluate whether the specific cell line suits the requirements. We have demonstrated that the widely used HepG2 cell line, which due to its hepatic origin is regarded as the most suitable model to study drug metabolizing enzyme induction, does not express CYP3A4, but only fetal CYP3A7. Because CYP3A7 is regulated by the glucocorticoid receptor and not like CYP3A4 by PXR, using the HepG2 cell line to study CYP3A4 induction could lead to false conclusions. The colon cancer-derived LS180 cell line was shown to be a far more suitable model to study CYP3A4 induction, and thus was intensively used throughout the other studies described in this thesis. In addition, the expression of nuclear receptors was shown to vary highly between cell lines (**Chapter 6**), and therefore it is advised to evaluate cell lines for functional expression of the nuclear receptor of interest before implementing the cell line in an enzyme induction study.

In conclusion, in this thesis it is described that several widely used anticancer drugs can induce the expression of important drug metabolizing enzymes and transporters by activating nuclear receptors. Thereby, these agents can adversely affect their own pharmacokinetics, but also that of

co-administered anticancer drugs, which increases the risk for pharmacokinetic interactions and the development of multidrug resistance.
This increased understanding of how the complex network of nuclear receptors regulates DMEs and DTs may eventually contribute to circumvent or even prevent pharmacokinetic drug interactions and multidrug resistance. This will enhance the efficacy of anticancer drugs, and as a result may produce safer treatment options in oncology.

Appendices

Summary

Unwanted drug-drug interactions can seriously complicate the successful treatment of cancer patients. These patients are at high risk of such interactions, because they are regularly treated with combinations of multiple cytotoxic anticancer drugs or hormonal agents that are often co-administered with prophylactic antiemetics and analgesics to provide palliation. Interactions between drugs occur when one drug alter the pharmacokinetics of another drug. This is especially relevant in oncology, because anticancer drugs have a very narrow therapeutic window, and therefore small changes in their pharmacokinetic profile can have serious clinical consequences. For instance, enhanced clearance of anticancer drugs as a result of the induction of enzymes involved in the metabolism or disposition can lead to subtherapeutic dosing. On the other hand, enhanced bioactivation of anticancer prodrugs can lead to life-threatening toxicities especially when the active metabolites are more toxic than the parent compound. Induction of cytochrome P450 (CYP) 3A4, an enzyme involved in the metabolism of >50% of all drugs, by the antibiotic rifampicin or the over-the-counter herbal antidepressant hyperforin (constituent of St.John's wort) has frequently been shown to considerably decrease the plasma levels of many widely used anticancer drugs. In addition, induction of the drug efflux transporter P-glycoprotein (Pgp) by rifampicin and hyperforin in the small intestine has been reported to significantly affect the absorption of orally administered drugs. The underlying mechanism by which rifampicin and hyperforin induce enzymes such as CYP3A4 and Pgp is the activation of a nuclear receptor that acts as a xenobiotic sensor: the pregnane X receptor (PXR). Since also anticancer drugs such as paclitaxel, cyclophosphamide and tamoxifen were shown to activate PXR, we have evaluated whether other anticancer drugs could also activate this receptor and if activation of PXR by these agents could potentially cause drug-drug interactions. In addition, we also evaluated whether anticancer drugs could induce the expression of other important drug transporters such as the multidrug resistance associated proteins (MRPs) and the breast cancer resistance protein (BCRP) and determined which mechanisms underlied the induction of these transporters.

In **Chapter 1**, a literature review is presented that addresses the clinical implications of pharmacokinetic drug-drug interactions in oncology and discusses the molecular mechanisms involved in these interactions, with a special focus on nuclear receptors. In addition, an overview of widely used techniques to study nuclear receptor-mediated enzyme induction is given.

To be able to study the effect of anticancer drugs on the induction of CYP3A4 a good cell model was required. Since primary human hepatocytes and enterocytes have major drawbacks such as poor availability and poor reproducibility, we focused on human cell lines. In **Chapter 2**, the human colon cancer derived LS180 cell line is compared with the human hepatocellular carcinoma HepG2 cell line in terms of CYP3A expression, CYP3A4 inducibility, and CYP3A4 reporter activity.

In contrast to HepG2, the LS180 cell line was shown to express CYP3A4 protein that could be induced upon activation of nuclear receptors such as PXR. The LS180 cell line therefore proved to be a more suitable model to study nuclear receptor mediated CYP3A4 induction than the HepG2 cell line.

In **Chapter 3**, the effect of a panel of widely used anticancer drugs on PXR-mediated CYP3A4 induction is evaluated in LS180 cells. The panel consisted of a wide variety of structurally unrelated anticancer drugs with different antineoplastic mechanisms and included topoisomerase I/II inhibitors, microtubule-(de)stabilizing agents, antimetabolites and alkylating agents. Seven of these anticancer drugs (cyclophosphamide, ifosfamide, flutamide, tamoxifen, erlotinib, docetaxel and paclitaxel) were shown to activate PXR. However, only the strong PXR activator paclitaxel was shown to increase CYP3A4 protein expression and as a result affect the metabolism of the CYP3A4 probe substrate midazolam. Thus paclitaxel might have the propensity to cause clinically relevant pharmacokinetic drug-drug interactions when combined with (anticancer) drugs that are metabolized by CYP3A4.

Another important gene that has been shown to be regulated by PXR is multidrug resistance 1 (MDR1) which encodes for a drug efflux transporter protein called P-glycoprotein (Pgp). **Chapter 4** describes the ability of conventional anticancer drugs to induce the expression of Pgp, while in **Chapter 5** the focus is on Pgp induction by a novel class of anticancer drugs: the small molecule tyrosine kinase inhibitors. Several widely used conventional anticancer drugs (vincristine, vinblastine, cyclophos-

phamide, ifosfamide, flutamide, tamoxifen, docetaxel and paclitaxel), but also some novel tyrosine kinase inhibitors (erlotinib, gefitinib, nilotinib, sorafenib and vandetanib) were shown to induce the protein expression of Pgp as a result of PXR activation. Moreover, many of the anticancer drugs that induced Pgp protein expression were also shown to affect the accumulation of a Pgp probe substrate indicating that these agents could cause clinically relevant drug interactions by influencing the absorption and excretion of concomitantly administered (anticancer) drugs. However, not all anticancer drugs (e.g. tamoxifen and nilotinib) that activated PXR-mediated Pgp induction affected the accumulation of the Pgp probe substrate. This could be explained by the fact that both tamoxifen and nilotinib were shown to inhibit the efflux functionality of Pgp.

Pgp is not only expressed by healthy tissues, but is also an important defense mechanism that is exploited by cancer cells to become resistant to multiple anticancer drugs a phenomenon known as multidrug resistance (MDR). Overexpression of Pgp by cancer cells is often associated with a poor prognosis in terms of treatment outcome. Since PXR has been shown to regulate Pgp expression, in **Chapter 4** the role of PXR activation with respect to anticancer drug resistance is evaluated. Indeed, activation of PXR by the prototypical PXR agonist rifampicin resulted in a cancer cell that was more resistant to the anticancer drug doxorubicin indicating that PXR activation also plays an important role in the development of MDR. On the other hand, knockdown of PXR resulted in attenuation of rifampicin-induced Pgp upregulation.

Apart from Pgp, also other members of the ABC-family have been implicated in pharmacokinetic drug-drug interactions and MDR: the multidrug resistance associated proteins (MRPs) and the breast cancer resistance protein (BCRP). In **Chapter 6** the regulation of MRPs is evaluated. Currently, not much is known about the regulation of the MRPs in humans. However, studies in animals have provided evidence that nuclear receptors are involved in the regulation of MRPs, but due to major interspecies differences it is unknown whether these data are predictive for the regulation of human MRPs. We demonstrated that although most of the nuclear receptors were functionally expressed in the selected cell lines, only MRP8 was induced when HepG2, LS180 and T84 were treated with nuclear receptor agonists. This might indicate that MRPs are poorly regulated by nuclear receptors in humans, and that possibly

other mechanisms such as kinase signaling or post-translational regulation are involved in the regulation of these proteins.
Upregulation of BCRP is often implicated in hematological malignancies such as acute myeloid leukemia, and has been associated with resistance to anticancer drugs that are extensively used in leukemia treatment regimens. **Chapter 7** describes a preliminary study of the regulation of BCRP. Apart from regulation by two nuclear receptors, the arylhydrocarbon receptor and the estrogen receptor, BCRP was also shown to be regulated by protein kinases, namely PI3K, JNK, p38/MAPK and PKC. Activation of these kinases by daunorubicin resulted in upregulation of BCRP protein, which could be attenuated by concomitant treatment with inhibitors of these protein kinases. Although more research is warranted, possibly co-administration of anticancer drugs with inhibitors of PI3K, JNK, p38/MAPK and PKC may be promising in preventing or reversing BCRP-mediated resistance to anticancer drugs in acute myeloid leukemia.

In conclusion, this thesis describes that activation of nuclear receptor-mediated induction of drug metabolizing enzymes and drug transporters by anticancer drugs could be an important underlying mechanism of drug-drug interactions and the development of multidrug resistance. In addition, not only nuclear receptors, but also stress activated protein kinases are involved in the process of enzyme induction. Furthermore, we have shown that we could prevent enzyme induction by knocking down nuclear receptors or by inhibiting the stress activated protein kinases. Therefore, combining nuclear receptor/kinase antagonists/inhibitors or gene therapy aimed at knocking down specific nuclear receptors/kinases with anticancer drugs could be promising in preventing or reversing anticancer drug-induced drug interactions or multidrug resistance, and eventually may even provide more potent and safer cancer treatment regimens

Nederlandse Samenvatting

Ongewenste geneesmiddelinteracties kunnen een succesvolle behandeling van kankerpatiënten ernstig in de weg staan. Kankerpatiënten hebben een verhoogd risico op het ontwikkelen van geneesmiddelinteracties gezien het feit dat ze vaak gelijktijdig behandeld worden met meerdere cytostatica en daarnaast ook nog medicijnen krijgen toegediend om de bijwerkingen van chemotherapeutica te verlichten en symptomen van hun ziekte te bestrijden. Interacties tussen geneesmiddelen kunnen ontstaan wanneer het ene geneesmiddel de farmacokinetiek van een ander geneesmiddel beïnvloed. Simpelweg beschrijft de farmacokinetiek wat het lichaam met een geneesmiddel doet nadat het is toegediend. Processen die hierbij een rol spelen zijn opname, verdeling, afbraak en uitscheiding van het geneesmiddel. Omdat cytostatica zeer zware medicijnen zijn en er een kleine grens bestaat tussen de effectiviteit en de giftigheid kan een kleine verandering in het farmacokinetisch profiel van dit soort geneesmiddelen ernstige klinische gevolgen hebben. Zo zou een verhoogde klaring (snelheid waarmee het lichaam zich ontdoet van geneesmiddelen) van cytostatica kunnen leiden tot onderdosering. Aan de andere kant, wanneer zogenaamde prodrugs (geneesmiddelen die door het lichaam geactiveerd moeten worden voordat ze werkzaam zijn) versneld worden omgezet in hun actieve vorm, kan dit leiden tot ernstige intoxicaties, zeker wanneer het gevormde actieve product toxischer is dan de prodrug zelf. Een proces dat ten grondslag kan liggen aan verhoogde klaring van cytostatica is enzyminductie. Inductie is het proces waarbij het lichaam meer van een bepaald enzym aanmaakt. Aangezien enzymen betrokken zijn bij de opname, omzetting/afbraak en uitscheiding van cytostatica, kan enzym inductie dus leiden tot een verhoogde klaring van cytostatica. Het enzym cytochroom P450 (CYP) 3A4 bevindt zich met name in de darm en de lever (organen die betrokken zijn bij de opname/uitscheiding en omzetting/afbraak van geneesmiddelen) en is betrokken bij de omzetting van ruim de helft van alle voorgeschreven geneesmiddelen (inclusief cytostatica). Van een aantal medicijnen is bekend dat zij de inductie van CYP3A4 stimuleren en op de manier dus invloed hebben op de farmacokinetiek van de helft van alle voorgeschreven geneesmiddelen. Het antibioticum rifampicine en het vrij verkrijgbare antidepressivum St.Janskruid staan er om bekend CYP3A4 te induceren en als

gevolg hiervan de klaring van andere gelijktoegediende cytostatica te verhogen. Dezelfde stoffen kunnen ook een enzym induceren dat betrokken is bij de uitscheiding van geneesmiddelen in de darm. Dit enzym heet P-glycoproteine (Pgp). Inductie van Pgp zorgt ervoor dat cytostatica versneld worden uitgescheiden in de darm, maar kan ook de opname van orale cytostatica beïnvloeden. Doordat rifampicine en St.Janskruid beide zowel CYP3A4 en Pgp induceren lijkt het erop alsof er een gemeenschappelijk mechanisme ten grondslag ligt aan de inductie van dit soort enzymen. Dit is inderdaad het geval. In het lichaam bevinden zich receptoren die mogelijk schadelijke lichaamsvreemde stoffen kunnen opmerken. Een van deze receptoren is de pregnaan X receptor (PXR). PXR is een soort rookmelder die, wanneer hij bepaalde lichaamsvreemde stoffen (rifampicine, St.Janskruid) waarneemt, ervoor zorgt dat CYP3A4 en Pgp worden geïnduceerd. Beide enzymen zullen er dan vervolgens voor zorgen dat de mogelijk schadelijke stoffen zo snel mogelijk worden uitgescheiden. Naast rifampicine en St.Janskruid is gebleken dat sommige cytostatica (paclitaxel, cyclophosphamide en tamoxifen) PXR kunnen activeren en op deze manier CYP3A4 en Pgp induceren. Omdat het vooralsnog onbekend is of ook andere cytostatica PXR kunnen activeren, hebben wij dit onderzocht. Ook is er gekeken of cytostatica naast CYP3A4 en Pgp, ook andere enzymen kunnen induceren die betrokken zijn bij de farmacokinetiek van cytostatica. Tevens hebben we gekeken of er naast PXR ook andere receptoren betrokken kunnen zijn bij het activeren van inductie van de verschillende enzymen.

Om het inducerend vermogen van cytostatica op enzymen zoals CYP3A4 te kunnen bestuderen is het van belang om goede celmodellen te hebben. Primaire cellen die direct afkomstig zijn uit de lever of de darm lijken het meest ideaal, maar door de beperkte beschikbaarheid en de hoge variatie tussen menselijk donormateriaal is er voor gekozen om in plaats van primaire cellen gebruik te maken van humane cellijnen. Cellijnen vertonen over het algemeen veel minder variatie en kunnen voor langere periodes in kweek genomen worden. In **Hoofdstuk 2** worden twee cellijnen met elkaar vergeleken om te bepalen welke cellijn als standaardmodel gebruikt kan worden om CYP3A4 inductie te bestuderen. In tegenstelling tot de HepG2 leverkanker cellijn, bleek CYP3A4 wel geinduceerd te worden in de LS180 dikkedarmkanker cellijn na activatie van nucleaire receptoren. Daarom werd besloten de LS180 cellijn

als standaardmodel te gebruiken om CYP3A4 inductie te bestuderen.
Om vervolgens te bepalen of cytostatica ook in staat zijn om CYP3A4 te induceren via activatie van nucleaire receptoren zoals PXR werd de LS180 cellijn blootgesteld aan verschillende klassen van cytostatica. **Hoofdstuk 3** beschrijft dat een aantal veelgebruikte cytostatica inderdaad PXR-gemedieerde CYP3A4 inductie kunnen activeren. Bovendien bleek behandeling met paclitaxel ook de omzetting van een ander CYP3A4 substraat te beïnvloeden. Dit geeft aan dat paclitaxel mogelijk ook in patiënten interacties kan aangaan met andere cytostatica of andere medicijnen die door CYP3A4 gemetaboliseerd worden en op dat zelfde moment door de patient worden gebruikt.
Een ander belangrijk gen dat gereguleerd wordt door PXR is het Multidrug resistance 1 (MDR1) gen. Dit gen codeert voor een eiwit genaamd P-glycoproteine (Pgp) dat de cel beschermt tegen schadelijke lichaamsvreemde stoffen. Aangezien cytostatica en andere geneesmiddelen ook lichaamsvreemd zijn, kan dit eiwit de opname van deze medicijnen bemoeilijken, zeker als er door inductie veel van dit eiwit aanwezig is in de cel. Het is daarom van belang om te kijken of cytostatica hun eigen of de opname van andere stoffen beïnvloeden als gevolg van Pgp inductie. In **Hoofdstuk 4** wordt beschreven in hoeverre reguliere cytostatica in staat zijn PXR-gemedieerde Pgp inductie te activeren, terwijl in **Hoofdstuk 5** Pgp inductie door een geheel nieuwe klasse van cytostatica wordt bestudeerd: de tyrosine kinase remmers. Naast verschillende reguliere cytostatica, bleken ook de tyrosine kinase remmers Pgp te kunnen induceren als gevolg van PXR activatie. Bovendien bleken een aantal van deze cytostatica de opname van een modelstof dat specifiek door Pgp getransporteerd wordt te beïnvloeden. Het zou dus best zo kunnen zijn dat door het gebruik van de beschreven cytostatica de werking van andere gelijktijdig gebruikte cytostatica of medicijnen verminderd wordt (geneesmiddelinteractie) als gevolg van de verhoogde hoeveelheid Pgp.
Niet alleen gezonde cellen maken gebruik van Pgp om zichzelf te beschermen tegen schadelijke lichaamsvreemde stoffen, ook kankercellen doen dit. Inductie van Pgp kan er zelfs toe leiden dat kankercellen zichzelf zo goed beschermen dat ze ongevoelig worden voor veel gebruikte cytostatica; dit fenomeen staat bekend als “multidrug resistentie”. Aangezien PXR activatie tot de inductie van Pgp kan leiden, hebben wij in **Hoofdstuk 4** gekeken of activatie van PXR ten grondslag kan liggen aan een verminderde gevoeligheid van de cel voor cytostatica als gevolg van

Pgp inductie. Dit bleek inderdaad het geval. Nadat een cel behandeld was met de PXR activator rifampicine, bleek de cel minder gevoelig geworden voor het veelgebruikte cytostaticum doxorubicine. Dit zou dus mogelijk ook kunnen betekenen dat cytostatica die PXR activeren multidrug resistentie in de hand werken.
Naast Pgp zijn er ook nog andere geneesmiddeltransporterende eiwitten: de multidrug resistentie geassocieerde eiwitten (MRPs) en het borstkanker resistentie eiwit (BCRP). Op het moment is er weinig bekend over hoe de inductie van MRPs en BCRP gereguleerd wordt. Wel is bekend dat verhoogde expressie van dit soort eiwitten invloed heeft op de gevoeligheid van een kankercel voor bepaalde cytostatica. Het is dus van belang om te begrijpen hoe dit soort eiwitten gereguleerd worden. Uit dierstudies is inmiddels gebleken dat nucleaire receptoren een rol spelen bij de regulatie van MRP inductie. Er bestaan echter grote species verschillen tussen mens en muis met betrekking tot de regulatie van dit soort eiwitten en het is daarom onduidelijk of deze studies iets zeggen over de situatie in de mens. In **Hoofdstuk 6** hebben we daarom onderzocht in hoeverre nucleaire receptoren betrokken zijn bij de inductie van humane MRPs. Hoewel we hebben laten zien dat in verscheidene cellijnen de verschillende nucleaire receptoren functioneel zijn, hebben we alleen voor MRP8 kunnen aantonen dat nucleaire receptoren betrokken zijn bij de inductie van deze transporter. Vervolgstudies zullen moeten aantonen of MRP8 inductie invloed kan hebben op de werkzaamheid van therapeutica, en hoe de inductie van de overige MRPs gereguleerd wordt.
Ondanks dat de naam anders doet vermoeden, blijkt een verhoging van het borstkanker resistentie eiwit (BCRP) met name in hematologische kankers zoals acute myeloide leukemie een belangrijke rol te spelen in de resistentie tegen chemotherapie. In **Hoofdstuk 7** laten we zien dat naast twee nucleaire receptoren (estrogeen receptor en de arylhydrocarbon receptor), verschillende signaaleiwitten (kinases) betrokken zijn bij de inductie van BCRP. Activatie van deze kinases door het cytostaticum daunorubicin leidde tot een inductie van BCRP. Wanneer daunorubicin echter gecombineerd werd met specifieke remmers van de betrokken kinases bleef BCRP inductie uit. Dit kan belangrijke therapeutische gevolgen hebben. Mogelijk kan combinatietherapie (daunorubicin en kinase remmers) leiden tot het uitstellen of zelfs voorkomen van resistentie als gevolg van BCRP inductie in acute myeloide leukemie.

Concluderend, in deze thesis staat beschreven dat activatie door cytostatica van nucleaire receptor-gemedieerde inductie van enzymen kan leiden tot een verhoogde klaring van geneesmiddelen en op deze manier geneesmiddel interacties in de hand kunnen werken. Tevens kan hetzelfde proces ten grondslag liggen aan het ontstaan van multidrug resistentie in kankers. We hebben ook laten zien dat naast nucleaire receptoren bepaalde signaaleiwitten een rol kunnen spelen in enzyminductie. Omdat remming van deze signaaleiwitten leidde tot het uitblijven van inductie, zou dit kunnen betekenen dat combinaties van cytostatica met deze signaaleiwitremmers of remmers van nucleaire receptoren het ontstaan van geneesmiddelinteracties en de ontwikkeling van multidrug resistentie kunnen voorkomen. Dit zou vervolgens kunnen leiden tot efficiëntere en veiligere behandelingen van kanker.

Curriculum Vitae

Stefan Harmsen werd geboren op 5 december 1979 te Haarlem. In 1998 behaalde hij het VWO gymnasium diploma aan het Gymnasium Felisenum te Velsen-Zuid. Hetzelfde jaar begon hij met zijn studie Scheikunde aan de Vrije Universiteit te Amsterdam. In de doctoraalfase specialiseerde hij zich in de moleculaire toxicologie. Het hoofdvakonderzoek getiteld "Development and validation of a novel cytochrome P450 bio-affinity and a radical inducer and antioxidant detection system coupled online to gradient reversed-phase HPLC" werd begeleid door Dr.J.Kool, Dr.J.N.M.Commandeur en Prof.Dr.N.P.E.Vermeulen. Zijn literatuurstudie beschreef de rol van nucleaire receptoren in de regulatie van geneesmiddel-metaboliserende enzymen en was getiteld "Implications of nuclear receptor regulated induction of drug metabolizing enzymes and transporter proteins on cancer therapy". In 2005 werd het doctoraal diploma behaald. In datzelfde jaar trad hij aan als Assistent in Opleiding in dienst van de Faculteit Farmaceutische Wetenschappen aan de Universiteit Utrecht. In de disciplinegroep Biomedische Analyse, sectie klinische geneesmiddelentoxicologie, werkte hij onder leiding van Prof.Dr.J.H.M.Schellens, Prof.Dr.J.H.Beinen en Dr.Ir.I.Meijerman aan het in dit proefschrift beschreven promotieonderzoek. Vanaf 1 januari 2009 is hij werkzaam als Postdoc aan de disciplinegroep Biofarmacie aan de Universiteit Utrecht.

List of Publications

Harmsen S, Meijerman I, Beijnen JH and Schellens JH (2007) The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. Cancer Treat Rev **33**(4):369-380.

Kool J, van Liempd SM, **Harmsen S**, Beckman J, van Elswijk D, Commandeur JN, Irth H and Vermeulen NP (2007) Cytochrome P450 bio-affinity detection coupled to gradient HPLC: on-line screening of affinities to cytochrome P4501A2 and 2D6. J Chromatogr B Analyt Technol Biomed Life Sci **858**(1-2):49-58.

Kool J, Van Liempd SM, **Harmsen S**, Schenk T, Irth H, Commandeur JN and Vermeulen NP (2007) An on-line post-column detection system for the detection of reactive-oxygen-species-producing compounds and antioxidants in mixtures. Anal Bioanal Chem **388**(4):871-879.

Harmsen S, Koster AS, Beijnen JH, Schellens JH and Meijerman I (2008) Comparison of two immortalized human cell lines to study nuclear receptor-mediated CYP3A4 induction. Drug Metab Dispos **36**(6):1166-1171.

Harmsen S, Meijerman I, Beijnen JH and Schellens JH (2009) Nuclear receptor mediated induction of cytochrome P450 3A4 by anticancer drugs: a key role for the pregnane X receptor. Cancer Chemother Pharmacol **64**(1): 35-43.

Zimmermann C, van Waterschoot RA, **Harmsen S**, Maier A, Gutmann H and Schinkel AH (2009) PXR-mediated induction of human CYP3A4 and mouse Cyp3a11 by the glucocorticoid budesonide. Eur J Pharm Sci **36**(4-5):565-571.

In preparation

Harmsen S, Meijerman I, Maas-Bakker RF, Beijnen JH and Schellens JHM. PXR-mediated induction of P-glycoprotein by small molecule tyrosine kinase inhibitors

Harmsen S, Meijerman I, Febus CL, Maas-Bakker RF, Beijnen JH and Schellens JHM. PXR-mediated induction of P-glycoprotein by anticancer drugs

Harmsen S, David DM, Maas-Bakker RF, Beijnen JH, Schellens JHM and Meijerman I Nuclear receptor-mediated induction of multidrug resistance associated proteins
in four widely used cell lines

Buda G, **Harmsen S**, Bergevoet S, van der Reijden BA, Meijerman I, Raymakers RAP, and Raaijmakers MHGP. Rapid upregulation of a broad range of ATP-binding cassette transporters upon anthracycline exposure in leukemic progenitor cells.

Selected abstracts

Harmsen S, Meijerman I, Beijnen JH and Schellens JHM Nuclear receptor mediated induction of cytochrome P450 3A4 by anticancer drugs: a key role for the pregnane X receptor (EACPT 2007, Amsterdam)

Harmsen S, Meijerman I, Beijnen JH and Schellens JHM Pregnane X receptor mediated induction of P-glycoprotein by anticancer drugs (EACPT 2007, Amsterdam)

Harmsen S, Meijerman I, Koster AS, Beijnen JH and Schellens JHM Comparison of two immortalized human cell lines to study nuclear receptor-mediated CYP3A4 induction (EACPT 2007, Amsterdam)

Harmsen S, Meijerman I, Beijnen JH and Schellens JHM Induction Of ATP-Binding Cassette Efflux Transporters By Small Molecule Tyrosine Kinase Inhibitors; A Role For Nuclear Receptors? (ISSX 2008, San Diego, USA)

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Stefan

Abbreviations

ABC, ATP-binding cassette

ADME, absorption, distribution, metabolism, excretion

AML, acute myeloid leukemia

APC, 7-ethyl-10-[4-N-(5-aminopentanoicacid)1-piperidino]carbonyloxycamptothecin

AhR, arylhydrocarbon receptor

ATP, adenosine triphosphate

AUC, area under the curve

BCRP, breast cancer resistance protein

BFC, 7-benzyloxy-4-trifluoromethylcoumarin

CAL, calcitriol

CAM, complementary alternative medicines

(h)CAR, (human) constitutive androstane receptor

CCRP, cytoplasmic CAR retention protein

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

CYP, cytochrome P450

DBD, DNA-binding doman

DME, drug metabolizing enzyme

DT, drug efflux transporter

ERα, estrogen receptor α

FBS, foetal bovine serum

FXR, farnesoid X receptor

GRIP, glucocorticoid receptor interacting protein

(h)GR, (human) glucocorticoid receptor

GST, glutathione S transferase

HBSS, Hank's Balanced Salt Solution

HFC, 7-hydroxy-4-trifluorocoumarin

HNF4α, hepatocyte nuclear factor 4α

HRP, horse radish peroxidase

JNK, jun N-terminal kinase

LBD, ligand-binding domain

LORR, loss of righting reflex

LXR, liver X receptor

MDCKII, Madin Darby canine kidney II

MDR, multidrug resistance

MDZ, midazolam

MRP, multidrug resistance associated protein

NADPH, nicotinamide adeninedinucleotide phosphate

NCoR, nuclear receptor co-repressor

NQO1, NAD(P)H Quinone Oxidoreducatse 1

NR, nuclear receptor

Nrf2, nuclear factor – erythroid 2 related factor 2

1′-OH-MDZ, 1′-hydroxymidazolam

PBS, phosphate buffered saline

PCN, pregnenolone 16α carbonitrile

Pgp, P-glycoprotein

PPAR, peroxisome proliferator-activated receptor

PXR, pregnane X receptor

PB, phenobarbital

RIF, rifampicin

RXR, retinoid X receptor

siRNA, short inhibitory rinonucleic acid

SMRT, silencing mediator of retinoid and thyroid receptor

SRC1, steroid receptor coactivator-1

SULT, sulfotransferase

siRNA, small interfering ribinucleic acid

UGT, uridinediphospho(UDP)-glucuronosyltransferase

VDR, vitamin D3 receptor