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ORIGINAL ARTICLE

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

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Abstract
Purpose 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 (PXR). We therefore screened 18 widely used anticancer drugs for their ability to activate PXR-mediated CYP3A4 induction.
Methods 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 pre-treatment with these agents on the 1'-hydroxylation of midazolam (a specific CYP3A4 probe) was determined.
Results Paclitaxel, erlotinib, tamoxifen, ifosfamide, flutamide and docetaxel are able to activate PXR, while only strong PXR activation leads to significant induction of CYP3A4 activity.
Conclusions The identified PXR agonists may have the propensity to cause clinically relevant drug–drug interactions as a result of CYP3A4 induction.

Keywords PXR · Anticancer drugs · CYP3A4 · Induction · Drug–drug interactions

Abbreviations

BFC	7-Benzyloxy-4-trifluoromethylcoumarin
(h)CAR	(human) Constitutive androstane receptor
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
HFC	7-Hydroxy-4-trifluorocoumarin
1'OH-MDZ	1'-Hydroxymidazolam
MDZ	Midazolam
NADPH	Nicotinamide adenine dinucleotide phosphate
NR	Nuclear receptor
PBS	Phosphate buffered saline
PXR	Pregnane X receptor
RXR	Retinoid X receptor
SD	Standard deviation
VDR	Vitamin D_3 receptor

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

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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 pro-drug 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 (hPXR; 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 (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 18 widely used anticancer drugs for their capacity to modulate nuclear receptor mediated CYP3A4 expression in a cell-based CYP3A4 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 b_5 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 from 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.

CYP3A4 reporter gene assay

LS180 cells were seeded in 96-well plates (5×10^4 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 h, the medium was removed, cells were washed with PBS, and lysed with 20 µl/well passive lysis buffer (Promega, Madison, WI, USA) for 15 min 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 1×10^6 cells/well in 6-well plates in 2 ml 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 (Sigma-Aldrich, 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′-TCAATAACAGTCTTTCCATTCCTCAT-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 3 h. After the incubation period, the medium was aliquoted and centrifuged for 5 min at 10,000 × *g*. The supernatant was further analyzed using HPLC-UV.

HPLC-UV analysis

The HPLC-UV analysis was performed on a Symmetry C18 column (150 mm × 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 min. 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^{-ct} [13]. All statistical calculations were done in SPSS (v14, SPSS Inc., Chicago, IL, USA).

Results

CYP3A4 reporter gene assay

The induction potential of 18 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 Fig. 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 (results not shown).

CYP3A4 mRNA expression

mRNA was isolated from LS180 cells that were exposed for 48 h 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 Fig. 2,

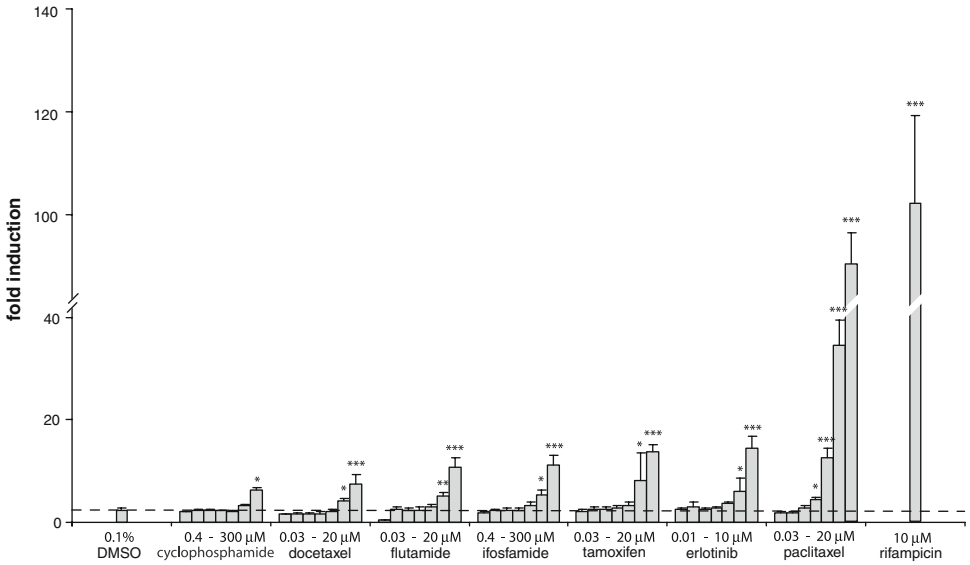

Fig. 1 Pregnane X receptor-mediated induction of CYP3A4 by widely used anticancer drugs. LS180 cells were transfected with the pGL3-CYP3A4-XREM reporter construct, and the nuclear receptor expression vectors pCDG-hPXR, and the pRL-TK control vector. After 24 h of transfection, cells were exposed to the anticancer drugs serially diluted in DMSO with a dilution factor of 3 from 20 to 0.3 µM, with the exception of erlotinib, which was serial diluted from 10 to 0.1 µM and ifosfamide and cyclophosphamide, which were serially diluted from 300 to 0.4 µM. Rifampicin (10 µM) was used as a prototypical CYP3A4 inducer and PXR agonist. After 48 h, luciferase activity was measured. These results are derived from a representative experiment and data are the mean ± 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]. *Dotted line* represents the CYP3A4 level after treatment with the vehicle 0.1% DMSO

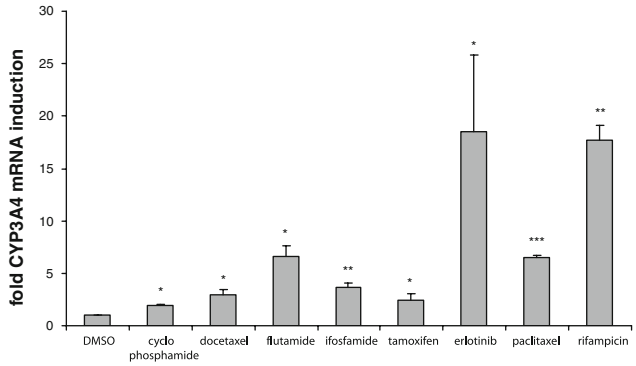

Fig. 2 Anticancer drug induced CYP3A4 mRNA expression levels. The CYP3A4 mRNA levels were determined after 2 × 24 h treatment of the cells with one concentration of the anticancer drugs; cyclophosphamide (300 µM), docetaxel (20 µM), flutamide (20 µM), ifosfamide (300 µM), tamoxifen (20 µM), erlotinib (10 µM) and paclitaxel (20 µM). Rifampicin (10 µM) was used as a positive control. For each anticancer drug, three 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 housekeeping gene [significance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) compared to the vehicle 0.1% DMSO]

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.

CYP3A4 immunoreactive protein expression levels

CYP3A4 protein levels were evaluated using Western immunoblotting and densitometric analysis, and plotted as fold induction. As shown in Fig. 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 induced CYP3A4 protein levels.

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-h 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 (Fig. 4). Flutamide caused a twofold increase in 1'-hydroxymidazolam formation, while erlotinib was shown to decrease the formation of the 1'-hydroxy metabolite. All other compounds did not

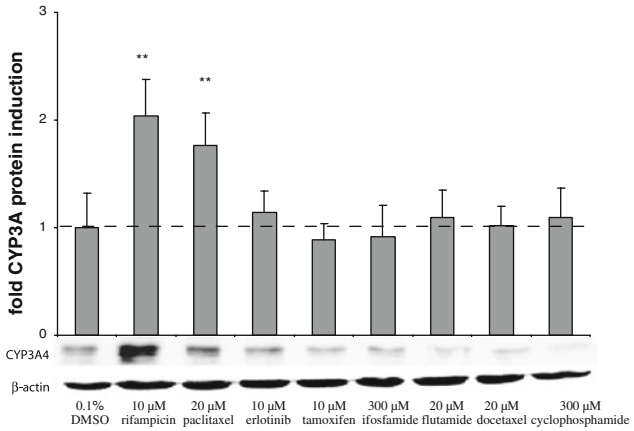

Fig. 3 CYP3A4 protein expression levels. CYP3A4 protein expression was determined with Western blotting after 2 × 24 h treatment with the anticancer drugs; cyclophosphamide (300 μM), docetaxel (20 μM), flutamide (20 μM), ifosfamide (300 μM), tamoxifen (20 μM), erlotinib (10 μM) and paclitaxel (20 μM). 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

significantly affect 1'-hydroxymidazolam formation compared to the vehicle.

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 (20 μM) had no significant effect on the formation of HFC, while erlotinib (10 μM) non-competitively inhibited HFC formation (Fig. 5).

Discussion

Eighteen 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 18 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.

Pregnane X receptor was shown to be involved in the induction of CYP3A4 by 7 of the 18 anticancer drugs (Fig. 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,

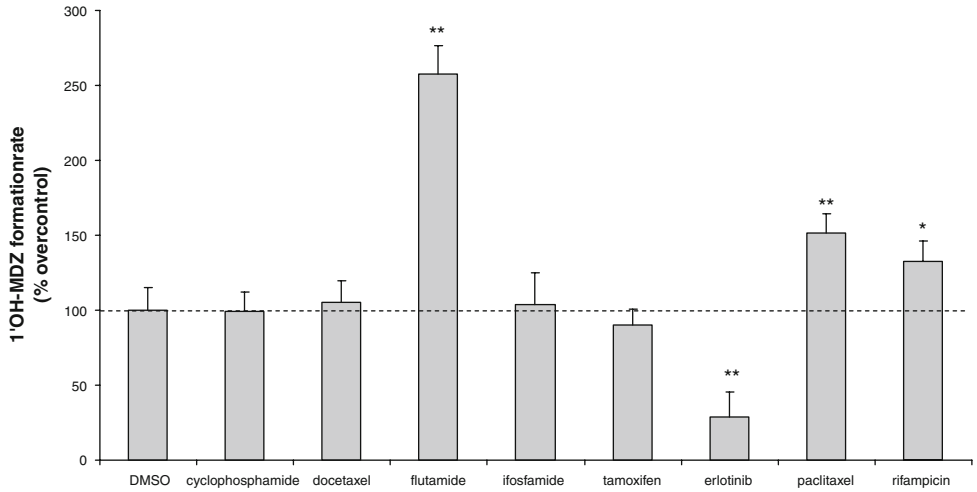

Fig. 4 Cell-based CYP3A4 activity assay. CYP3A4 activity was assessed after 2 × 24 h treatment with the anticancer drugs; cyclophosphamide (300 μM), docetaxel (20 μM), flutamide (20 μM), ifosfamide (300 μM), tamoxifen (20 μM), erlotinib (10 μM) and paclitaxel (20 μM). Rifampicin (10 μM) was used as a positive control. These results are derived from a representative experiment and data are the mean ± 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]

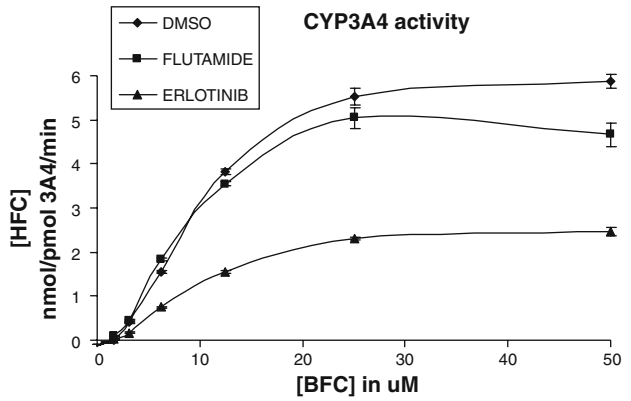

Fig. 5 Effect of flutamide and erlotinib on CYP3A4 activity. Flutamide (20 μM) and erlotinib (10 μM) were co-incubated with the non-fluorescent CYP3A4 substrate BFC. Erlotinib was shown to non-competitively inhibit CYP3A4, while flutamide did not significantly alter rate of substrate conversion compared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

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 h 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 contrast 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 cannot 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 h 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 pre-treatment with flutamide did not correlate with CYP3A4 reporter gene, CYP3A4 mRNA and 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 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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