Nelfinavir and Its Active Metabolite M8 Are Partial Agonists and Competitive Antagonists of the Human Pregnane X Receptor

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ABSTRACT

The HIV protease inhibitor nelfinavir is currently being analyzed for repurposing as an anticancer drug for many different cancers because it exerts manifold off-target protein interactions, finally resulting in cancer cell death. Xenosensing pregnane X receptor (PXR), which also participates in the control of cancer cell proliferation and apoptosis, was previously shown to be activated by nelfinavir; however, the exact molecular mechanism is still unknown. The present study addresses the effects of nelfinavir and its major and pharmacologically active metabolite nelfinavir hydroxy-tert-butylamide (M8) on PXR to elucidate the underlying molecular mechanism. Molecular docking suggested direct binding to the PXR ligand-binding domain, which was confirmed experimentally by limited proteolytic digestion and competitive ligand-binding assays. Concentration-response analyses using cellular transactivation assays identified nelfinavir and M8 as partial agonists with EC50 values of 0.9 and 7.3 μM and competitive antagonists of rifampin-dependent induction with IC50 values of 7.5 and 25.3 μM, respectively. Antagonism exclusively resulted from binding into the PXR ligand-binding pocket. Impaired coactivator recruitment by nelfinavir as compared with the full agonist rifampin proved to be the underlying mechanism of both effects on PXR. Physiologic relevance of nelfinavir-dependent modulation of PXR activity was investigated in respectively treated primary human hepatocytes, which showed differential induction of PXR target genes and antagonism of rifampin-induced ABCB1 and CYP3A4 gene expression. In conclusion, we elucidate here the molecular mechanism of nelfinavir interaction with PXR. It is hypothesized that modulation of PXR activity may impact the anticancer effects of nelfinavir.

SIGNIFICANCE STATEMENT

Nelfinavir, which is being investigated for repurposing as an anticancer medication, is shown here to directly bind to human pregnane X receptor (PXR) and thereby act as a partial agonist and competitive antagonist. Its major metabolite nelfinavir hydroxy-tert-butylamide exerts the same effects, which are based on impaired coactivator recruitment. Nelfinavir anticancer activity may involve modulation of PXR, which itself is discussed as a therapeutic target in cancer therapy and for the reversal of chemoresistance.

Introduction

The HIV protease inhibitor nelfinavir is no longer commonly used in modern antiretroviral therapy of AIDS because of the introduction of more efficacious medications. However, it is a promising candidate for repurposing as an anticancer drug for many different cancers [for review, see Shim and Liu (2014) and Bhattarai et al. (2016)]. This is because of the manifold off-target interactions of nelfinavir, which not only targets HIV protease but also inhibits, among others, the activities of 20S proteasome (Gupta et al., 2007), cyclin-dependent protein kinase (CDK) 2 (Jiang et al., 2007), site-2-protease (Guan et al., 2011), and heat shock protein 90 (Shim et al., 2012). Proteome-wide in silico analysis of nelfinavir potential off-target interactions suggested the interaction with and possible inhibition of multiple protein kinases (Xie et al., 2011). Because of these interactions, nelfinavir results in diverse effects on cancer cells, such as inhibition of the PI3K-AKT-mTOR pathway and induction of endoplasmic reticulum stress, which finally lead to cell death by cell cycle arrest, triggering of the unfolded protein response, apoptosis, and autophagy (Koltai, 2015).
also designated as M8, demonstrated in vitro antitumor activity comparable with that of the parent compound (Guan et al., 2011). M8 likely contributes to total nelfinavir antiviral and anticancer activity, as its concentrations in human plasma reach up to one-third of those of nelfinavir (Zhang et al., 2001).

The nuclear receptor (NR) pregnane X receptor (PXR) (NR1I2) is one of the many targets of nelfinavir. In response to xenobiotics, PXR transcriptionally regulates the expression of cytochrome P450 drug-metabolizing enzymes and drug transporters, such as CYP3A4 and P-glycoprotein (multidrug resistance protein 1/ABCB1). Since nelfinavir-dependent induction of the respective genes and proteins was demonstrated in rats (Huang et al., 2001), primary human hepatocytes (Dixit et al., 2007; Liu et al., 2012), and the human colorectal adenocarcinoma cell lines LS174T and LS180 (Huang et al., 2001; Gupta et al., 2008), it is assumed that nelfinavir activates PXR. However, inconsistent results were obtained when analyzing nelfinavir-dependent PXR activation using cellular transactivation assays. Although nelfinavir did not activate PXR in a one-hybrid reporter gene assay in monkey kidney fibroblast CV-1 cells (Dussault et al., 2001), it induced PXR-dependent activation of CYP3A4 and CYP2B6 promoter reporter genes in human hepatocellular carcinoma HepG2 cells (Swärd et al., 2010; Lynch et al., 2015). In contrast, knockdown experiments in LS180 cells clearly attributed the nelfinavir-dependent induction of CYP3A4 and ABCB1 gene expression to PXR (Gupta et al., 2008). However, because former work had also demonstrated that nelfinavir neither bound to the PXR ligand-binding domain (LBD) in vitro nor recruited coactivator mediator complex subunit 1 (MED1) to PXR in a mammalian two-hybrid assay (Dussault et al., 2001), the molecular mechanism of nelfinavir-dependent PXR activation remains obscure. As mentioned above, nelfinavir was shown to inhibit CDK2 activity and is further supposed to also inhibit CDK5 and cAMP-dependent protein kinase (Xie et al., 2011), which have all been shown to phosphorylate PXR in vitro and thereby inhibit its transactivation activity [for a review, see Makowiciak and Wang (2016)]. Altogether, published data do not provide evidence for direct binding of nelfinavir to PXR and are consistent with a putative indirect activation mechanism of the drug, which may rely on the inhibition of inhibitory protein kinases.

Given the current efforts to repurpose nelfinavir as an anticancer drug, it is of interest that PXR is discussed as a therapeutic target in anticancer therapy. Depending on the cancer entity, PXR activation may either result in tumor progression and promote chemoresistance or suppress cancer cell growth (Pondugula et al., 2016; Xing et al., 2020). In the latter case, PXR activation may contribute to nelfinavir’s anticancer activity. A thorough understanding of how exactly the drug affects PXR activity will promote our knowledge on the potential of nelfinavir as an anticancer drug.

We aim here to elucidate the molecular mechanism of nelfinavir-dependent activation of PXR and to investigate the PXR interaction properties of the pharmacologically equipotent major metabolite M8. By using a combination of biochemical and cellular assays as well as in silico molecular modeling, it is shown that nelfinavir and M8 bind to human PXR and act both as partial agonists and competitive antagonists. Impaired recruitment of coactivators mainly accounts for these properties. Studies in primary human hepatocytes demonstrate the physiologic relevance of nelfinavir’s agonism and antagonism of PXR; however, nelfinavir exhibits differential induction of PXR target genes. The chemical structure of nelfinavir may thus represent a suitable starting point for the development of PXR-selective modulators.

**Materials and Methods**

**Chemicals and Biological Reagents.** Rifampin was purchased from Merck Chemicals (Darmstadt, Germany); Toxris Bioscience (Bristol, UK) provided T0901317, GW4064, and SR12283; nelfinavir and M8 were purchased from Toronto Research Chemicals (North York, Canada); DMSO, GW3965, triiodo-L-thyronine, and 1α,25-dihydroxyvitamin D3 were provided by Sigma-Aldrich (Munich, Germany); and resiglatzone and CITCO were purchased from Sellkchem (Houston, TX) and ENZO Life Sciences (Lörrach, Germany), respectively. SPA70 was provided by Axon Medchem (Groningen, The Netherlands). Cell culture medium and supplements were obtained from Life Technologies (Darmstadt, Germany).

**Plasmids.** Eukaryotic expression plasmids encoding full-length human nuclear receptors PXR (Geick et al., 2001), constitutive androstane receptor (CAR) 1 (Burk et al., 2002), CAR3 (Arnold et al., 2004), thyroid hormone receptor α1, thyroid hormone receptor β1, vitamin D receptor (VDR) (Burk et al., 2005), liver X receptor (LXR) α, and LXRβ (Piedade et al., 2015) have all been described. Expression plasmids encoding the human small PXR variant, which consists of the ligand-binding domain (amino acids 113–434) only (Jeske et al., 2017), and PXR mutant S296W/S247W/C284W (Burk et al., 2018) have already been described. T. Tanaka kindly provided the human peroxisome proliferator-activated receptor (PPAR) γ1 expression plasmid (Tachibana et al., 2005). Farnesoid X receptor (FXR) ω2 expression plasmid was constructed by cloning the respective open reading frame amplified by polymerase chain reaction (PCR) from the cDNA of primary human hepatocytes using appropriate primers into expression vector pcDNA3 (Thermo Fisher Scientific Invitrogen, Carlsbad, CA). The 5′ upstream primer additionally introduced an optimized Kozak consensus sequence. Sequencing identified an FXR clone lacking the 12-bp insert which encodes amino acids MYTG (i.e., FXRω2).

Expression plasmids encoding fusion proteins of the GAL4–DNA-binding domain (DBD) and the receptor interaction domains (RIDs) of nuclear receptor coactivator (NCOA) 1 (residues 583–783), NCOA2 (residues 583–779), MED1 (residues 527–774) (Arnold et al., 2004), and nuclear receptor corepressor 2 (NCOIR2), (residues 1109–1330) or helix 1 part of the LBD of PXR (residues 132–188) as well as expression plasmids encoding fusion proteins of the VP16 activation domain (AD) and the whole or part of the PXR-LBD (residues 108–434).

**ABBREVIATIONS:** ABCB1, ATP-binding cassette B1; AD, activation domain; AF-2, activation function 2; AKR1B10, aldo-keto reductase 1B10; CAR, constitutive androstane receptor; CDK, cyclin-dependent protein kinase; CI, confidence interval; DBD, DNA-binding domain; EPHX1, epoxide hydrolase 1; FASN, fatty acid synthase; FRET, fluorescence resonance energy transfer; PXR, farnesoid X receptor; HTGR, Human Tissue and Cell Research; ID, identifier; LBD, ligand-binding domain; LBP, ligand-binding pocket; LXR, liver X receptor; M8, nelfinavir hydroxy-tert-butylamide; MDR1, multidrug resistance protein 1; MED1, mediator complex subunit 1; MOE, Molecular Operating Environment; NCOA, nuclear receptor coactivator; NCOIR2, nuclear receptor corepressor 2; NFV, nelfinavir; NR, nuclear receptor; PCR, polymerase chain reaction; PDB, Protein Data Bank; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RID, receptor interaction domain; RIF, rifampin; RMS, root-mean-square; rRNA, ribosomal RNA; SMPDL3A, sphingomyelin phosphodiesterase acid–like 3A; Tk, thymidine kinase; TR-FRET, time-resolved FRET; UGT1A3, UDP-glucuronosyltransferase 1A3; VDR, vitamin D receptor.
and 189–434, respectively) (Burk et al., 2005) have been described. The expression plasmid encoding the fusion protein of GAL4-DBD with PXR-LBD (residues 108–434) was constructed by subcloning the insert of pVP16-AD/PXR-LBD(108–434) into pM (Takara Bio Clontech, Mountain View, CA).

Firefly luciferase reporter gene plasmids have previously been described: CYP3A4 enhancer/promoter reporter gene plasmid pGL4-CYP3A4(7850/Δ7208-304) (Burk et al., 2018); CYP2B6 enhancer/promoter reporter gene plasmid p-1.6kP2B6REM, as provided by H. Wang (Wang et al., 2003); reporter gene plasmid comprising a dimer of the MDR1-DR4(I) motif (Geick et al., 2001); pGL3/DR3,Tk (Hustert et al., 2001); plasmid PPRE X3-Tk-luc1015; Addgene plasmid, as provided by B. Spiegelman (Forman et al., 1995); reporter gene plasmid containing a dimer of the consensus nuclear receptor inverted repeat 1 motif (Piedade et al., 2015); and GAL4-dependent reporter gene plasmid pGL3-G5 (Arnold et al., 2004). With the exception of the CYP3A4 and CYP2B6 reporters and pGL3-G5, the other reporter gene plasmids harbor the thymidine kinase minimal promoter. The Renilla luciferase expression plasmid pGL4.75[hRLuc/CMV] (Promega, Madison, WI) has been used.

**Molecular Docking.** Three-dimensional coordinates for nelfinavir and its metabolite M8 were generated within MOE version 2019.01.02 (Chemical Computing Group Inc., Montreal, Canada). All PXR X-ray crystal structures were downloaded from the Protein Data Bank (Berman et al., 2000). Based on a structural analysis of all available PXR X-ray crystal structures using PROCHECK and ProSa Web (Laskowski et al., 1993; Sippl, 1993), PDB IDs 1M13, 1NRL, and 2O9I were selected for docking. For 1M13, two separate structures with different alternate atoms for Cys284 were generated, and both chains of 1NRL and 2O9I were selected, resulting in an ensemble of six LBD structures. The mutation C284S in 2O9I was mutated back into the wild-type residue using MOE, and solvent atoms were removed. All structures were superposed on Co atoms of amino acids lining the ligand-binding pocket (LBP). Protein structures were prepared using Protonate3D in MOE and energy-minimized (Amber10:EHT force field with R-Field implicit solvent model) using a three-step procedure (Cu atoms, backbone, all atoms) with a tether of 0.5 and a root-mean-square (RMS) gradient of 5. Potential alternative ligand-binding sites on the LBD surface were identified by applying Site Finder in MOE. Compounds were docked using GOLD version 5.8.1 (Cambridge Crystallographic Data Centre, Cambridge, UK). The docking search space for the LBP was defined by a sphere of 13 Å radius centered on the FRET donor of the light-harvesting complex (PDB ID: 1M13), whereas for the AF-2 site, a sphere of 10 Å radius centered on atom CD2 of Leu670 (PDB ID: 1NRL, chain C) was used. The docking search space for the alternative sites was defined by the amino acids lining the pockets. For each compound, 100 docking runs were conducted. The early termination option was switched off.

**Limited Proteolytic Digestion.** Radiolabeled PXR-LBD protein (residues 113–434) was synthesized in vitro using the TNT TT Quick Coupled Transcription/Translation System (Promega) as recommended by the manufacturer. Briefly, 50-μl reactions were set up with 40 μl TNT TT Quick Master Mix, 20 μlCi 35S-methionine (specific activity > 1000 Ci/mmol; Hartmann Analytic, Braunschweig, Germany) and 1 μg of the small PXR expression plasmid. Five-microliter aliquots thereof were preincubated with compounds or solvent DMSO for 30 minutes and then subjected to proteolytic digestion with trypsin for 10 minutes as described (Jeske et al., 2017). Reactions were separated on 12% SDS polyacrylamide gels, which were subsequently stained with Coomassie, dried, and exposed to BAS-IP MS 3225 imaging plates (Fuji, Kanagawa, Japan). Input PXR-LBD protein and protected proteolytic fragments were detected by scanning the imaging plates with the CR35 Bio radioluminography laser scanner (Raytest, Straubenhardt, Germany) and quantified using AIDA software version 4.50.010 (Raytest). Each independent experiment was performed in technical single replicates.

**Competitive Ligand Binding.** Ligand binding to PXR was investigated using the LanthaScreen TR-FRET PXR (SXI) competitive binding assay kit (PV4839, lot 2109118; Thermo Fisher Scientific, Waltham, MA). All assay steps and the detection were carried out according to the protocol and microplate reader-specific guidelines of the manufacturer. In brief, the assay was performed in a volume of 20 μl per well of 384-well, black, nonbinding, low-volume plates (Corning Life Sciences, Amsterdam, The Netherlands) with 2.5 nM glutathione-S-transferase–PXR-LBD, 40 nM Fluoromone PXR Green, and 10 nM terbium-labeled anti–glutathione-S-transferase antibody. Positive control (SR12813) and test compounds nelfinavir and M8 were added at concentrations ranging from 0.5 nM to 30 μM. Reactions were incubated for 60 minutes at room temperature in the dark. The fluorescent emissions were measured on an Infinite M1000 multimode microplate reader (Tecan, Männedorf, Switzerland) at 486 and 515 nm. All measurements were optimized using maximum signal control reactions (100 μl of 100 μl, 200 μs integration, flash mode 2 (100 Hz, ~8 Joule)). The FRET ratio was calculated by dividing the emission signal at 515 nm by that at 486 nm. Two-percent DMSO was used as the negative control (0% relative binding) and 30 μM SR12813 as the positive control (100% relative binding). The data were expressed as relative binding (% relative binding (%) = 100% * (DMSO FRET ratio – compound FRET ratio)/(DMSO FRET ratio – 30 μM SR12813 FRET ratio)), according to Dong et al. (2010).

**Cell Culture.** HepG2 cells (HB-8065, lot number 58341723; American Type Culture Collection, Manassas, VA) and H-P cells, which represent a HepG2 cell clone stably overexpressing human PXR (Bitter et al., 2015), were cultivated in minimal essential medium supplemented with 10% FetalBovineSerum (Gibco), and 0.1 mg/ml streptomycin. In drug treatments, dextran-coated charcoal-stripped FBS replaced regular FBS. Both cell lines were routinely checked for contamination with mycoplasma by PCR (VenerGeM Classic; Minerva Biolabs, Berlin, Germany).

**Concentration-Response Analyses.** Transient transfections of H-P cells were set up according to the batch protocol for jetPEI (Polypus, Illkirch, France). Transfected cells were seeded in 96-well plates with flat-bottom microplates (Sarstedt, Nümbrecht, Germany) using per well 250 μl of a suspension consisting of 200 μl H-P cells (4 × 10^4 cells) and 50 μl transfection mixture containing 0.3 μg CYP3A4 reporter gene plasmid, 0.01 μg pGL4.75[hRLuc/CMV], and 0.6 μg jetPEI transfection reagent in 150 mM NaCl. Twenty-four hours after transfection, cells were treated with varying concentrations of chemicals ranging from 0.03 to 10 μM (nelfinavir and M8) or 30 μM (rifampin) in the presence or absence of fixed concentrations of competing ligands for antagonists nelfinavir and M8 at 10 μM. After a 24-hour incubation, and cells were lysed with 50 μl of 1× passive lysis buffer (Promega). Firefly and Renilla luciferase assays were performed separately with 10 μl lysate each in white opaque 96-well microplates (OptiPlate-96; Perkin-Elmer) by adding 150 or 100 μl of respective assay solutions, which have been described previously (Geick et al., 2001; Piedade et al., 2015). Luminescence was measured after 10-minute incubation at constant shaking with the 2300 EnSpire multimode plate reader (Perkin Elmer, Rodgau, Germany) for 0.1 seconds. Concentration-response experiments were done five to seven times independently, each in technical triplicates.

**Nuclear Receptor Specificity, Mammalian Two-Hybrid, and Mutant PXR Assays.** Transient transfections were performed in 24-well plates with 1.5 × 10^5 HepG2 cells per well seeded the day before, using per well 1 μl jetPRIME transfection reagent (Polyplus, Illkirch, France) and a plasmid DNA mixture consisting of 0.04 μg of the respective NR expression plasmids, 0.3 μg synthesized firefly luciferase reporter gene constructs, and 0.065 μg pGL4.75[hRLuc/CMV] filled up with 1 μg of pUC18 to 0.5 μg. The following NR expression plasmids were used for the NR specificity tests: CYP3A4 reporter (PX) and CYP2B6 reporter (CAR1, CAR2), MDR1-DR4 reporter (LXRα, LXRβ, TRα1, TRβ1), DR3 reporter (VDR), PPRE –3·3 Tg-luc (PPARγ), and consensus inverted repeat 1 reporter (FXR2). In the antagonist mode, the following prototypical agonists were further used: 10 μM rifampin (PXR), 10 μM CICTE (CAR3), 2 μM GW3965 (LXRα, LXRβ), 1 μM triodo-L-thyronine (TRα1, TRβ1), 0.1 μM 1α,25-dihydroxyvitamin D3 (VDR),
1 μM rosiglitazone (PPARγ1), and 1 μM GW4064 (FXRα2). Transfections for mammalian two-hybrid PXR corepressor, coactivator, and LBD assembly assays were set up similarly, using the luciferase plasmids specified in the legend of Fig. 6. In mutant PXR assays, respective PXR expression plasmids were combined with the CYP3A4 reporter as described above. Twenty hours after transfection, cells were treated with chemicals for another 24 hours. Cell lysis and reporter gene assays were performed as described above. All transfections were done four to five times independently, each in technical triplicates and with at least two different preparations of plasmids.

Human Liver Tissue Samples and Primary Human Hepatocytes. Double-coded human liver samples and corresponding data used in this study were provided by the Biobank of the Department of General, Visceral, and Transplantation Surgery in Ludwig-Maximilians University. This Biobank operates under the administration of the Human Tissue and Cell Research (HTCR) Foundation. The framework of HTCR Foundation (Thasler et al., 2003), which includes obtaining written informed consent from all donors, has been approved by the ethics commission of the Faculty of Medicine at the Ludwig-Maximilians University (approval number 025-12) as well as the Bavarian State Medical Association (approval number 11142) in Germany. Experimental procedures were performed in accordance with the Declaration of Helsinki. Donor data are shown in Supplemental Table 1. Primary human hepatocytes were isolated by the Biobank using a two-step collagenase perfusion technique with modifications (Lee et al., 2013). The cells were cultivated and treated with chemicals as described before (Jeske et al., 2017).

RNA Isolation and Reverse-Transcription Quantitative Real-Time PCR Analysis. Total RNA was isolated using the Nucleospin RNA kit (Machery-Nagel, Düren, Germany), including on-column DNase I digest. RNA integrity was analyzed by formaldehyde-agarose gel electrophoresis. cDNA was synthesized as described previously (Jeske et al., 2017).

Relative quantification analyses (ΔΔCt) were performed in technical triplicates by TaqMan RT-qPCR using the BioMark HD system and 48.48 Dynamic Array or Flex Six Gene Expression Integrated Fluidic Circuits (Fluidigm, South San Francisco, CA), as described previously (Jeske et al., 2017). The following TaqMan gene expression assays (Life Technologies) were used: Hs00184500_m1 (ABC1B), Hs01546975_gH (AKR1B10), Hs00946140_g1 (CYP2C8), Hs00640506_m1 (CYP3A4), Hs01005622_m1 (FASN), Hs00378308_m1 (SMPDL3A), and Hs99999902_m1 (RPLP0). The CYP2B6, EPHX1, and Hs00604506_m1 (CYP3A4), Hs01005622_m1 (FASN), Hs00378308_m1 (SMPDL3A), and Hs99999902_m1 (RPLP0). The CYP2B6, EPHX1, and Hs00604506_m1 (CYP3A4), Hs01005622_m1 (FASN), Hs00378308_m1 (SMPDL3A), and Hs99999902_m1 (RPLP0). The CYP2B6, EPHX1, and Hs00604506_m1 (CYP3A4), Hs01005622_m1 (FASN), Hs00378308_m1 (SMPDL3A), and Hs99999902_m1 (RPLP0).

Experimental Design and Data Analyses. All experiments were done in an exploratory manner. Thus, P values have to be interpreted as descriptive only. The decision to perform five independent experiments (except competitive PXR binding and hepatocyte experiments) was made prior to their execution based on the level of variation observed in previous work. In Fig. 4, A, C, and D, the number of independent experiments for controls (RIF, without NFV, or without M8) exceeds five because of technical reasons. To avoid systematic errors due to timing and/or positioning in limited protolytic digestion and transfection experiments, the sequence of chemical treatment of cells and measurement of samples was randomized for independent experiments. Experimental data are shown in scatter plots with mean ± S.D. or medians with interquartile ranges of at least five independent experiments. Numbers of independent experiments are presented in the respective figure legends. Medians were used only with primary human hepatocytes, as independent experiments were performed here with cultures from different donors. Statistical comparisons were performed with repeated-measures one-way or two-way ANOVA or Friedman test using respective post hoc tests for multiple comparisons against specified controls as recommended by the analysis software and described in the figure legends. Comparisons to a hypothetical value were performed with one sample t test or Wilcoxon signed rank test for means or medians, respectively. Pairwise comparisons of medians were performed with Wilcoxon matched-pairs signed rank test. EC50 and IC50 values were determined by nonlinear fit of concentration response using the equation for sigmoidal dose response (log agonist or log inhibitor vs. response (three parameters)). All calculations were done with GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA).

Results
Nelfinavir and Its Metabolite M8 Modulate the Activities of PXR and Further Nuclear Receptors. Expression of drug-metabolizing enzymes and transporters is not exclusively induced via PXR but also by further ligand-activated nuclear receptors [for a review, see Pascucci et al. (2003); Prakash et al. (2015)]. Thus, we investigated whether nelfinavir and its metabolite M8 (Fig. 1A) modulate the activities of respectively selected nuclear receptors using the corresponding promoter reporter gene assays in agonist (Fig. 1B) and antagonist (Fig. 1C) modes. To determine the applicable concentration, the effects of increasing concentrations of the compounds on viability of HepG2 cells were analyzed. Supplemental Fig. 1 shows for both compounds that concentrations exceeding 10 μM resulted in HepG2 viability of less than 80%. Thus, single concentration of 10 μM of nelfinavir and M8 was selected for starting analyses. In accordance with published results (Svärd et al., 2010; Lynch et al., 2015), nelfinavir induced PXR activity by 3.7-fold (95% CI 3.1–4.3-fold) while inhibiting the constitutive activity of CAR1 by 60% (95% CI 51%–72%). In addition, FXRα2 was also activated 2.0-fold (95% CI 1.7–2.3-fold) by nelfinavir. M8 selectively induced PXR activity by 5.1-fold (95% CI 4.5–5.7-fold) (Fig. 1B). The 20% reduction of basal PPARγ1 activity may indicate antagonism of M8. Figure 1C shows that nelfinavir antagonized rifampin-dependent PXR activity by 35% (95% CI 19%–51%), whereas M8 demonstrated 50% (95% CI 24%–77%) inhibition. Ligand-activated VDR was inhibited by 15% or 45% in the presence of nelfinavir or M8, respectively, whereas the activity of GW4064-induced FXRα2 was reduced by 17% or 40%, respectively. In addition, the activities of ligand-activated CAR3, LXRβ, and PPARγ1 were all reduced by approximately 30% in the presence of M8.

To corroborate the above-described effects of nelfinavir and M8 on nuclear receptors besides PXR, respective concentration-response analyses were conducted. Nelfinavir-dependent inhibition of CAR1 and activation of FXRα2 were confirmed unequivocally (Supplemental Fig. 2, A and E). However, the effects on the other nuclear receptors, which mainly consisted of slight inhibition of agonist-induced receptor activities by M8, were exclusively obtained with 10 μM. Concentration-dependent effects, as with CAR1 and FXRα2, were not observed (Supplemental Fig. 2, B–D, F–H). Given the sharp decline in viability of HepG2 between 10 and 30 μM of M8 (Supplemental Fig. 1), it cannot be excluded that the effects observed with 10 μM M8 stemmed from partial cytotoxicity. In conclusion, nelfinavir and its metabolite M8 not only activated PXR but also antagonized the ligand-activated receptor. Additionally,
nelfinavir inhibited CAR1 and activated FXRα2, whereas effects on some other nuclear receptors, especially by M8, have to be regarded as uncertain because of the possibility of partial cytotoxicity.

**Direct Binding of Nelfinavir and M8 to PXR.** As mentioned above, activation of PXR by nelfinavir and M8 may be explained theoretically by the inhibition of inhibitory protein kinases. However, the antagonistic effects on rifampin-induced PXR activity identified here call for a different explanation. Thus, direct binding of the compounds to PXR was reinvestigated. First, we addressed binding by in silico molecular docking of nelfinavir and M8 into different potential binding sites. An ensemble docking approach was chosen by which nelfinavir and M8 were flexibly docked into the LBP and AF-2 groove of six different PXR X-ray crystal structures. Docking scores of the top-ranked poses of both compounds were similar for the LBP (107.4 for nelfinavir vs. 110.4 for M8) and thus do not indicate a preferred binding of either nelfinavir or M8 over the other. Docking scores for the AF-2 groove were substantially lower (nelfinavir: 84.2; M8: 85.4), indicating a preference of both compounds for the LBP (Supplemental Table 2). To evaluate binding of nelfinavir and M8 to alternative sites on the LBD, an in silico approach for pocket detection was employed (Fig. 2A). Only pockets with either a size of >50 alpha spheres or a calculated propensity for ligand binding >1 were considered, resulting in a total of eight potential alternative binding sites (Fig. 2A; Supplemental Table 2) into which nelfinavir and M8 were docked. Docking scores for all alternative sites were substantially lower compared with the scores obtained for the
EC50 value of percentage relative binding to PXR, a competitive PXR binding assay was generated by binding of T0901317. To further confirm direct result in an LBD conformational change different from that suggesting that PXR binding of nelfinavir and M8 may have although to varying extents. Although nelfinavir and M8 only weakly protected the main proteolytic fragments, although to varying extents. Although nelfinavir and M8 only weakly protected the 26-kDa fragment, T0901317 efficiently protected all three, suggesting that PXR binding of nelfinavir and M8 may have resulted in an LBD conformational change different from that generated by binding of T0901317. To further confirm direct binding to PXR, a competitive PXR binding assay was deployed (Fig. 3B). EC50 value of percentage relative binding of positive control SR12813 was determined as 45.6 nM (95% CI 3.7–7.0-fold), whereas M8 showed 5.4-fold increase (95% CI 4.5–6.2-fold). Similarly, the high-affinity PXR ligand T0901317 demonstrated 5.0-fold increase (95% CI 4.3–5.7-fold). T0901317 protected the same main proteolytic fragments, although to varying extents. Although nelfinavir and M8 only weakly protected the 26-kDa fragment, T0901317 efficiently protected all three, suggesting that PXR binding of nelfinavir and M8 may have resulted in an LBD conformational change different from that generated by binding of T0901317. To further confirm direct binding to PXR, a competitive PXR binding assay was deployed (Fig. 3B). EC50 value of percentage relative binding of positive control SR12813 was determined as 45.6 nM (95% CI 22.7–94.1 nM), which is in line with published data (Dong et al., 2010). Nelfinavir and M8 also demonstrated PXR binding in this assay with EC50 values of 89.0 nM (95% CI 48.2–167 nM) and 51.1 nM (95% CI 18.7–148 nM), respectively. In summary, these data provide evidence for direct binding of nelfinavir and M8 to the PXR-LBD.

Nelfinavir and M8 Are Partial Agonists of PXR that Also Act as Competitive Antagonists. Having established ligand binding to PXR, further characterization of the respective activation and inhibition properties of nelfinavir and M8 called for cellular concentration-response analyses. Respective analyses were performed with H-P cells, a HepG2 clone stably expressing PXR (Bitter et al., 2015). Similar to parental HepG2 cells, H-P cells showed decreased viability at compound concentrations exceeding 10 μM; however, they were not as sensitive toward M8 as HepG2 (Supplemental Fig 1). Figure 4A shows that the agonist efficacies of nelfinavir and M8 were reduced if compared with rifampin. At 10 μM, the two compounds exhibited only 30%–40% of the activity seen by the same concentration of rifampin, thereby indicating partial agonism. Keeping in mind that the M8 concentration-response curve did not yet approximate plateau, nelfinavir demonstrated EC50 of 0.9 μM (95% CI 0.58–1.3 μM), whereas M8 showed reduced potency with EC50 of 7.5 μM (95% CI 4.4–14.9 μM). Using the same assay, rifampin EC50 of 2.4 μM (95% CI 1.6–3.6 μM) proved to be elevated compared with previously reported EC50 values of 1.3 and 1.8 μM (Lin et al., 2008; Hoffart et al., 2012). Partial agonism was also observed for nelfinavir-dependent activation of a GAL4-DBD/PXR-LBD fusion protein (Supplemental Fig. 3). Cotreatment with the specific PXR antagonist SPA70 (Lin et al., 2017) almost completely suppressed the reporter activation by nelfinavir and M8 (Fig 4B), thereby testifying to PXR dependency. Competitive antagonism of the compounds was observed when rifampin concentration-response curves were established in the presence of increasing concentrations of nelfinavir (Fig 4C) or M8 (Fig 4D). In both cases, rifampin concentration-response curves were shifted to the right, and respective EC50 values increased with elevated concentrations of the antagonistic compounds (Supplemental Table 4). Analysis of the increase in rifampin EC50 values revealed statistical significance only for 10 μM nelfinavir. However, a respective trend was observed for 3 μM nelfinavir and 10 μM M8 (Fig. 4E). IC50 values of nelfinavir and M8 antagonism were determined for the inhibition of PXR activation by 10 μM rifampin (Fig. 4F). With IC50 of 7.3 μM (95% CI 5.0–11.0 μM), nelfinavir proved to be the more potent antagonist when compared with M8 with its IC50 of 25.3 μM (95% CI 16.1–47.2 μM). Nelfinavir further proved to antagonize PXR activation by the high-affinity ligand T0901317 (Supplemental Fig. 4). Because nelfinavir and M8 comparably affected PXR, and cytotoxicity of M8 was more pronounced, further investigations were performed only with the parent drug.

Competitive Antagonism of Nelfinavir Exclusively Depends on Binding into the PXR Ligand-Binding Pocket. Since the concentration of nelfinavir had to be restricted to 10 μM, additional allosteric antagonism at higher concentrations, as observed for the PXR mixed competitive/noncompetitive allosteric antagonist pimecrolimus (Burk et al., 2018), may have remained undetected. Thus, we investigated the ability of nelfinavir to inhibit the constitutive activity of a PXR mutant with the LBP filled with bulky amino acid residues. Such a mutant is no longer activated by agonists because these cannot bind into the LBP (Wang et al., 2008), but its constitutive activity can be antagonized by compounds exclusively or additionally binding outside of the LBP (e.g., coumestrol, pazopanib, or pimecrolimus) (Wang et al., 2008;
Burk et al., 2018). Accordingly, nelfinavir showed activation of wild-type PXR but not of the LBP-filled triple mutant S208W/S247W/C284W, which is similar to the prototypical PXR agonist rifampin. Nelfinavir, which inhibited the rifampin-dependent activation of wild-type PXR by 54% (95% CI 29%–80%), did not antagonize the high constitutive activity of the mutant (Fig. 5), thereby indicating that it has to bind to the LBP to exhibit its inhibitory effect.

**Impaired Coactivator Recruitment Accounts for the Effects of Nelfinavir on PXR Activation.** To characterize the functional consequences of nelfinavir interaction with PXR, cellular mammalian two-hybrid assays were performed, which address ligand-dependent LBD assembly, corepressor release, and coactivator recruitment. In clear contrast with the partial agonism of nelfinavir in the CYP3A4 enhancer/promoter reporter gene assay (as shown in Fig. 4A), the drug induced the PXR-LBD assembly more strongly (mean increase by 70%, 95% CI 19%–120%) than rifampin (Fig. 6A). The constitutive interaction of PXR with corepressor NCoR2 in the absence of ligand was diminished by nelfinavir to a similar extent as by rifampin (Fig. 6B). In the established sequence of events during nuclear receptor activation, recruitment of coactivators follows the ligand-dependent release of corepressors. Whereas rifampin induced the interaction of PXR with coactivators NCoA1, NCoA2, and MED1 by 8-fold (95% CI 4.6–12.2-fold), 9.3-fold (95% CI 7.3–11.3-fold), and 4.6-fold (95% CI 3.9–5.4-fold), respectively, nelfinavir demonstrated impaired recruitment for all coactivators, which have been tested (Fig. 6, C–E). Furthermore, nelfinavir reduced the rifampin-dependent recruitment of NCoA1 to 3.2-fold (95% CI 1.8–4.6-fold) in cotreatment (Fig. 6C). Similar effects were observed with NCoA2 (4.1-fold, 95% CI 3.5–4.7-fold) (Fig. 6D) and MED1 (2.2-fold; 95% CI 2.0–2.4-fold) (Fig. 6E). Taken together, these data indicate that the partial agonism and competitive antagonism of nelfinavir in PXR activation depend on the impaired recruitment of coactivators. Corepressor release and intramolecular conformational changes in response to ligand binding, the latter as described by the LBD assembly assay, are not compromised.

**Nelfinavir Differentially Induces PXR Target Genes in Primary Human Hepatocytes and Antagonizes Rifampin Induction.** Given the weak activation of PXR by nelfinavir in reporter gene assays, the question arises: To what extent may the compound be able to induce the expression of endogenous PXR target genes? Primary human hepatocytes, which were treated with nelfinavir, demonstrated induction of only a subset of selected PXR-regulated genes as compared with treatment with rifampin. Cytochrome P450 genes, such as CYP2B6, CYP2C8, and CYP3A4, which proved to be highly inducible by rifampin, also demonstrated induction by nelfinavir. Nelfinavir further induced the expression of EPHX1 and UGT1A3. However, genes, such as ABCB1, AKR1B10, FASN, or SMPDL3A, which showed weak induction by rifampin, were not induced by nelfinavir (Fig. 7A). Among the genes induced by both compounds, UGT1A3 was the only one that showed no difference in induction by rifampin and nelfinavir. Neither rifampin nor nelfinavir induced the expression of RPLP0, which is not a target gene of PXR.

Figure 7B shows that cotreatment with increasing concentrations of nelfinavir antagonized the induction of ABCB1 and CYP3A4 by rifampin, whereas the respective effect was not...
statistically significant for CYP2B6, most likely because of the high variability of CYP2B6 induction by rifampin in this experimental set of hepatocytes. Interestingly, treating the cells with 30 μM nelfinavir only did not result in stronger induction of the genes as compared with 10 μM. In the case of CYP3A4, induction even appeared to be suppressed.

Fig. 4. Concentration-dependent response of PXR activation by nelfinavir and M8 and antagonistic effects on activation by rifampin. H-P cells were transfected with CYP3A4 reporter and (A) treated for 24 hours with increasing concentrations of RIF, NFV, or M8; (B) treated with increasing concentrations of nelfinavir or M8 in the presence or absence of 3 μM SPA70; (C and D) cotreated with increasing concentrations of rifampin in the presence of the indicated fixed concentrations of (C) nelfinavir or (D) M8; and (F) cotreated with increasing concentrations of nelfinavir or M8 in the presence of 10 μM rifampin. Mean fold induction ± S.D. (n = 5) by the respective treatment is shown with respect to the normalized reporter activity of cells treated with vehicle DMSO only, which was designated as 1. Nonlinear fit of concentration-dependent response was performed with bottom values fixed to 1 in (A and F). (E) shows the mean ± S.E. of rifampin log10 EC50 values derived from experiments shown in (C and D) (n = 5), which have been calculated as described in Materials and Methods. Differences to rifampin log10 EC50 values in the absence of nelfinavir or M8 were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test according to GraphPad Prism knowledgebase article 144 (GraphPad Software, accessed August 17, 2020, https://www.graphpad.com/support/faq/how-can-i-test-for-differences-among-three-or-more-curves-fitted-to-three-or-more-data-sets/). w/o, without.
Discussion

We have characterized here in detail the interaction of the HIV protease inhibitor nelfinavir and its major metabolite M8 with PXR. Both compounds act as partial agonists and competitive antagonists of human PXR by binding into the LBP of the receptor. The partial agonist/antagonist properties mainly result from impaired coactivator recruitment.

Nelfinavir and its major metabolite M8, which accounts for 80%–90% of primary metabolites detected in human plasma (Zhang et al., 2001), exert the same functional effects on PXR, and thus it is not expected that hepatic metabolism of the drug may limit modulation of the receptor. M8 selectively activated PXR, whereas nelfinavir also induced the activity of FXR and, as already reported (Lynch et al., 2015), inhibited CAR. In the antagonist mode, slight inhibition of the ligand-dependent activation of several other nuclear receptors by 10 μM M8 was observed (see Fig. 1C and Supplemental Fig. 2), which, however, cannot be excluded to result from partial cytotoxicity. Even if the inhibition by 10 μM M8 would reflect true physiologic relevance of such small effects is still questionable.

Direct binding of nelfinavir and M8 to PXR was suggested by docking into the LBP of the receptor’s X-ray crystal structures. Neither the AF-2 groove nor any of the identified potential alternative binding sites seem to be suited to favorably bind any of both compounds. Binding of nelfinavir to the LBP appeared to be more favorable compared with M8 because nelfinavir showed a clear preference for a specific binding mode. This may explain the lower EC50 and IC50 values determined for the parent drug. Because nelfinavir is a more hydrophobic compound (logP: 4.7) compared with M8 (logP: 3.7), nelfinavir binding to the largely hydrophobic PXR-LBP might be more favorable.

The limited proteolytic digestion and TR-FRET–based competitive ligand-binding assays performed here showed for the first time experimental evidence that nelfinavir and its metabolite M8 bind to the LBD of PXR. Using a radio-ligand-binding competition assay, Dussault et al. (2001) failed to demonstrate binding of nelfinavir to PXR. Nevertheless, mean inhibition by 20%–25% is visible in the respective data, which the authors regarded as irrelevant. It may be argued that protection from digestion by trypsin is simply reflecting direct protease inhibition by nelfinavir and its metabolite. However, omitting the ligand preincubation step in the limited proteolytic digestion experiment and only adding the compounds together with trypsin resulted in a strong decrease in protease from digestion by the protease. The residual protection is most likely due to ligand binding during the coinubcation with trypsin, as it was likewise observed with T0901317 (unpublished data). Accordingly, it has been reported that nelfinavir does not inhibit trypsin activity (Wignot et al., 2004; Singh et al., 2009). The result of the limited proteolytic digestion assay was confirmed by the TR-FRET–based PXR ligand-binding competition assay, which further demonstrated nelfinavir and M8 EC50 values for binding to the PXR-LBD comparable with respective EC50 of the potent PXR agonist SR12813. Ligand binding of nelfinavir and M8 does not necessarily disqualify an indirect activation mechanism by the inhibition of protein kinases. This mechanism may additionally contribute to activation of PXR by nelfinavir and may further limit nelfinavir-dependent antagonism. Final proof that kinase inhibition contributed to nelfinavir-dependent PXR modulation awaits the analysis of PXR protein phosphorylation after treatment with the drug.

EC50 value in the range of 0.1 μM for in vitro binding to PXR indicates physiologic relevance of the modulation of PXR activity by nelfinavir. The drug shows extremely high intracellular accumulation (Jones et al., 2001; Mateus et al., 2017). Treatment of lymphoblastoid CEM cells with 10 μM nelfinavir for 18 hours resulted in intracellular concentration of approximately 700 μM (Jones et al., 2001). Since the fraction of unbound intracellular nelfinavir was determined in different cell lines to range from 0.0001 to 0.0004 (Mateus et al., 2017), unbound intracellular concentration can be roughly estimated to range from 0.1 to 0.3 μM, which can be expected as sufficient for PXR activity modulation. Furthermore, dosing mice with nelfinavir in a way that resulted in similar blood concentrations as the therapeutic dosing of patients with HIV subsequently produced amounts in liver, which corresponded to intracellular amounts in HeLa cells treated with 5–10 μM nelfinavir for 6 hours (de Gassart et al., 2016). These data provide indirect evidence that the intracellular concentrations of nelfinavir, which were achieved by
Fig. 6. Effects of nelfinavir on PXR-LBD assembly and transcriptional cofactor interaction. (A) HepG2 cells were cotransfected with combinations of expression plasmids encoding GAL4-DBD/PXR-LBD (132–188) and VP16-AD/PXR-LBD (189–434) or empty vector pVP16-AD. (B–E) HepG2 cells were cotransfected with expression plasmids encoding VP16-AD/PXR-LBD (108–434) fusion protein or empty vector pVP16-AD and expression plasmids encoding fusion proteins of (B) GAL4-DBD/NCOR2-RID, (C) GAL4-DBD/NCOA1-RID, (D) GAL4-DBD/NCOA2-RID, or (E) GAL4-DBD/MED1-RID. Transfected cells were treated with 0.1% DMSO or 10 μM NFV with or without 10 μM RIF for 24 hours. Data are presented as scatter plots with mean (columns) ± S.D. (n = 5) of normalized luciferase activity of the cotransfected pGL3-G5 relative to the activity of cells transfected with pVP16-AD and treated with DMSO only. Differences to respective treatments with DMSO (asterisks, exclusively for single-compound treatments) or with rifampin only (daggars, exclusively for nelfinavir + rifampin cotreatment) were analyzed by repeated-measures two-way ANOVA with Dunnett’s or Sidak’s multiple comparisons tests, respectively. *P < 0.05; **P < 0.01; ***P < 0.001.
treatment of cells with 10 μM of the compound in the culture medium, thereby exerting PXR-dependent effects, can be expected in vivo in the liver by therapeutic dosing. Physiologic relevance is further supported by induction of endogenous gene expression in primary human hepatocytes by 10 μM nelfinavir (see Fig. 7A). Nelfinavir may have demonstrated gene-specific induction, as only part of the genes, which have been analyzed, were induced by the drug. These include CYP2B6, CYP2C8, CYP3A4, EPHX1, and UGT1A3, whereas ABCB1, AKR1B10, FASN, and SMPDL3A were not induced. Missing induction of ABCB1 is in contrast with previous reports, which demonstrated induction of the gene (Dixit et al., 2007, Liu et al., 2012). The difference may be explained by donor-dependent gene expression variability of primary human hepatocytes.

Nelfinavir was proven here to act as both a partial agonist and a competitive antagonist of PXR, inhibiting activation by pure agonists, such as rifampin and T0901317, in hepatic

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**Fig. 7.** Effects of nelfinavir on the expression of endogenous PXR target genes in hepatocytes. (A) Cultures of primary human hepatocytes of six donors were each treated with 0.1% DMSO, 10 μM RIF, or 10 μM NFV for 24 hours. mRNA expression of the indicated genes was determined by TaqMan RT-qPCR and normalized to the expression of 18S rRNA. Data are presented in scatter plots with medians and interquartile ranges. Expression was calculated as fold induction caused by the respective chemical treatment as compared with the expression in cells treated with vehicle DMSO only, which was designated as 1. Differences to this value were analyzed by one sample Wilcoxon signed rank tests (asterisks). Differences between rifampin and nelfinavir treatments were analyzed by Wilcoxon matched-pairs signed rank tests for each gene individually (dagggers). (B) Cultured primary human hepatocytes of four donors were treated for 24 hours with 0.1% DMSO or 10 or 30 μM NFV in the absence or presence of 10 μM RIF. mRNA expression of the indicated genes was calculated and is presented as described above. Differences to treatment with rifampin only were analyzed by Friedman test with Dunn’s multiple comparisons test (asterisks, exclusively for cotreatments of rifampin and nelfinavir). *, †P < 0.05; **P < 0.01.
cells. These characteristics are mainly due to the impaired recruitment of coactivators, whereas corepressor release is not compromised as compared with a full agonist. In this respect, nelfinavir equals partial agonists of PPARγ, such as GW0072, FK614, or PA-082 (Oberfield et al., 1999; Fujimura et al., 2005; Burgermeister et al., 2006), which were proven to be selective receptor modulators. In CV-1 cells, nelfinavir neither activated a fusion protein of the GAL4-DBD with the PXR-LBD nor resulted in recruitment of coactivator MED1 (Dussault et al., 2001). This may indicate cell-type specificity and thus selective receptor modulation. Failure of PXR activation in CV-1 cells was not simply due to the use of a GAL4-DBD/PXR-LBD fusion protein. Performing an equivalent experiment in HepG2 cells resulted in the same partial agonism of nelfinavir (Supplemental Fig. 3) as observed with full-length PXR in CYP3A4 reporter assays. Nelfinavir may thus be regarded as a basic structure for the development of specific PXR-selective receptor modulators. However, this would require detailed information on the binding mode of nelfinavir in the PXR-LBP, which clearly requires the elucidation of the nelfinavir-bound PXR crystal structure.

We have shown here that nelfinavir competitively antagonized PXR activity, which may be of physiologic relevance because the drug also antagonized PXR-dependent induction of ABCB1 and CYP3A4 in hepatocytes (see Fig. 7B). PXR antagonism by nelfinavir may contribute further to the nelfinavir-dependent resensitization of cancer cells to cytotoxic drug treatment (Kim et al., 2019). This applies particularly in the case that the development of chemoresistance involved PXR activation. Several anticancer drugs, such as paclitaxel and cisplatin (Masuyama et al., 2005), vincristine and vinblastine (Harmsen et al., 2010), SN-38, the active metabolite of irinotecan (Basseville et al., 2011), and sorafenib (Feng et al., 2018), were shown to bind to and/or activate PXR. Chemoresistance generated by treatment with these drugs may thus have been caused by PXR activation (Planchque et al., 2016; Feng et al., 2018). Consequently, PXR antagonists may re sensitize chemoresistant cancer cells. This concept was recently validated by the resensitization of cisplatin-resistant HepG2 cells by treatment with the PXR antagonist leflunomide (Yasuda et al., 2019). However, the PXR-dependent effects of nelfinavir in cancer cells and especially in chemoresistant cancer cells require further study.

Modulation of PXR by nelfinavir may not necessarily result in anticancer activity. In models of breast, prostate, or colorectal cancer, PXR induces tumor progression and chemoresistance (Pondugula et al., 2016). If PXR has not yet been inactivated in such a context, treatment with nelfinavir may activate the receptor and counteract nelfinavir-dependent anticancer pathways. However, if PXR has already been activated, nelfinavir may antagonize this activation and result in tumor suppression or counteract PXR-induced chemoresistance. Here, PXR antagonism might synergize with other anticancer pathways, which are triggered by nelfinavir. It is the other way around in cancers, in which PXR acts as a tumor suppressor. Consequently, the here-described partial agonism and competitive antagonism of nelfinavir in the modulation of PXR activity have to be considered if nelfinavir is planned to be used as an anticancer drug.

In conclusion, we have elucidated the molecular mechanism of PXR modulation by nelfinavir and its major metabolite M8. Both compounds bind directly to the receptor and act as partial agonists but also competitively antagonize ligand-mediated PXR activation. Given the current efforts to repurpose nelfinavir as an anticancer drug and considering that PXR plays a role in cancer cell survival pathways and is thought to be involved in the development of chemoresistance, it is important to know how the drug influences PXR activity. The data presented here may thus help to achieve a better understanding of the manifold effects of nelfinavir on cancer cells.

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