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Gefitinib Inhibits Rifampicin-Induced CYP3A4 Gene Expression in Human Hepatocytes

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ABSTRACT: During multidrug combination chemotherapy, activation of the nuclear receptor and the transcription factor human pregnane xenobiotic receptor (hPXR) has been shown to play a role in the development of chemoresistance. Mechanistically, this could occur due to the cancer drug activation of hPXR and the subsequent upregulation of hPXR target genes such as the drug metabolism enzyme, cytochrome P450 3A4 (CYP3A4). In the context of hPXR-mediated drug resistance, hPXR antagonists would be useful adjuncts to PXR-activating chemotherapy. However, there are currently no clinically approved hPXR antagonists in the market. Gefitinib (GEF), a tyrosine kinase inhibitor used for the treatment of advanced non-small-cell lung cancer and effectively used in combinational chemotherapy treatments, is a promising candidate owing to its hPXR ligand-like features. We, therefore, investigated whether GEF would act as an hPXR antagonist when combined with a known hPXR agonist, rifampicin (RIF). At therapeutically relevant concentrations, GEF successfully inhibited the RIF-induced upregulation of endogenous CYP3A4 gene expression in human primary hepatocytes and human hepatocells. Additionally, GEF inhibited the RIF induction of hPXR-mediated CYP3A4 promoter activity in HepG2 human liver carcinoma cells. The computational modeling of molecular docking predicted that GEF could bind to multiple sites on hPXR including the ligand-binding pocket, allowing for potential as a direct antagonist as well as an allosteric inhibitor. Indeed, GEF bound to the ligand-binding domain of the hPXR in cell-free assays, suggesting that GEF directly interacts with the hPXR. Taken together, our results suggest that GEF, at its clinically relevant therapeutic concentration, can antagonize the hPXR agonist-induced CYP3A4 gene expression in human hepatocytes. Thus, GEF could be a potential candidate for use in combinational chemotherapies to combat hPXR agonist-induced chemoresistance. Further studies are warranted to determine whether GEF has sufficient hPXR inhibitor abilities to overcome the hPXR agonist-induced chemoresistance.

INTRODUCTION

The human pregnane X receptor (hPXR) is the master xenosensor responsible for xenobiotic detection, metabolism, and elimination. In response to xenobiotic binding and activation, hPXR acts as a transcription factor to upregulate the expression of drug-metabolizing enzymes, such as CYP3A4.[1−3] CYP3A4 contributes to the metabolism of over 50% of clinically active drugs. Therefore, the overexpression of this enzyme can lead to a loss of drug efficacy.[4,5] This is a major concern for cancer patients as chemoresistance can occur when chemotherapeutics act as agonists of hPXR, leading to CYP3A4 overexpression and altered metabolism of chemotherapy drugs. Indeed, combination therapies containing agonists of hPXR can drive increased metabolism of chemotherapy drugs such as cobimetinib,[6] cabozantinib,[7] imatinib,[8] irinoecan,[9] and vemurafenib.[10]

Efforts to overcome the hPXR agonist-induced chemoresistance have resulted in a growing list of compounds that have been studied to determine their capacity to antagonize or inhibit the agonist-activated hPXR and reduce hPXR target gene expression.[11−14] There have been reported many structurally diverse hPXR antagonists such as ET-743, ketoconazole, FLB-12, sulforaphane, coumestrol, camptothecin, A792611, metformin, and SPA70.[12] However, with the exception of SPA70, these compounds have been determined to possess characteristics that render them clinically unusable, such as a lack of *in vivo* efficacy and significant toxicities.[12,15]

Repurposing FDA-approved drugs has been a promising strategy in the treatment of many conditions.[16,17] Interestingly,

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many FDA-approved drugs have been proven to possess hPXR antagonistic and/or inhibitory capabilities that allow for the modulation of hPXR-mediated gene expression. For example, the diabetes medication metformin and the chemotherapeutic camptothecin are capable of reducing hPXR target gene expression.[18,19] However, they do not seem to interact directly with hPXR, and follow-up studies showing their ability to overcome chemoresistance mediated by hPXR have yet to be conducted.[18,19] Nevertheless, the rationale behind testing previously approved drugs for their hPXR antagonistic potential offers several enticing benefits. Safety, toxicity, and pharmacokinetic (PK) information will already be established for approved drugs, which will reduce approval time and associated costs of candidate drugs.[20] Furthermore, if an approved clinical drug was able to antagonize hPXR at therapeutic concentrations, it would successfully overcome the primary limiting trait of previous hPXR antagonists, that is, unacceptable toxicities that exist at the suprapharmacological concentrations required to antagonize hPXR. Additionally, if an hPXR antagonist was an approved chemotherapeutic used in combinational therapies, then we gain the benefits of proven anticancer properties and successful combinatorial usage. An additional emerging function of hPXR relates to its ability to perpetuate stemness (*e.g.*, colon cancer) and cancer re-growth.[21−31] These functions of hPXR in human tumor tissues could be conjointly inhibited by antagonists.

Recently, our lab has demonstrated that the anticancer drug, belinostat, could successfully antagonize hPXR-mediated gene expression and attenuate the hPXR agonist-induced chemotherapy resistance.[13] Based on our previous work, we determined to continue our screening of a selection of clinically approved drugs that could potentially be repurposed to antagonize hPXR at therapeutically relevant concentrations.

Gefitinib (GEF) (Figure 1) is a tyrosine kinase inhibitor approved for the treatment of non-small-cell lung cancer (NSCLC). GEF has many attractive characteristics compared to failed hPXR antagonists, which would make it a promising addition to combination therapy as an hPXR antagonist. These desirable traits include the reported manageability of its common adverse events (mild to moderate (grade 1/2) skin rash, diarrhea, and nausea) and enhancing the benefit of other chemotherapy regimens. *In vitro* and *in vivo* studies revealed that GEF can enhance the anticancer effects of chemotherapy drugs in combination with chemotherapies.[32−34] Patient studies have shown that GEF can effectively be used in combinational treatments with other chemotherapeutics, such as pemetrexed and carboplatin, to improve advanced lung adenocarcinoma patient prognosis.[35] Carboplatin was more efficacious when used with an hPXR antagonist such as ketoconazole in HepG2 hepatocellular carcinoma cells.[36] Additionally, other clinical trials were successful in using GEF in combinational treatments. For example, GEF used in combination with cisplatin and radiotherapy to treat late-stage III/IV nonmetastatic head and neck squamous cell carcinoma also showed some success [NCT00229723]. Previously, it was shown that the antitumor effects of cisplatin can be increased by the hPXR antagonist, leflunomide,[37] and that cisplatin may be a possible agonist of hPXR,[38] which may help explain why coadministration with hPXR antagonists, such as GEF and leflunomide, increased cisplatin antitumor effects.

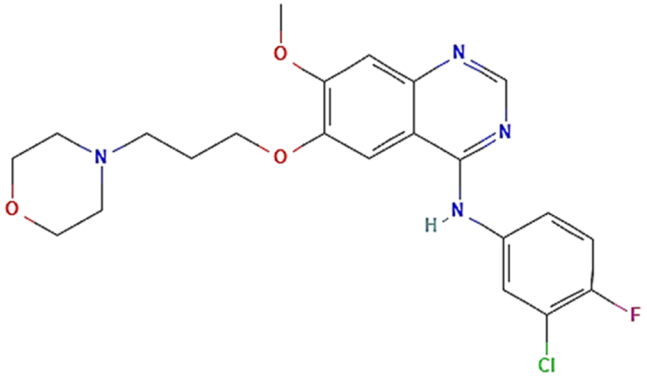

Figure 1. Chemical structure of gefitinib (GEF).

If GEF could serve to antagonize the agonist-induced hPXR activity, then it is possible that hPXR target gene expression, including CYP3A4, could be reduced. However, it is unknown whether GEF antagonizes drug-activated hPXR target gene expression in humans. In the current study, we show that GEF, at its C_{max} achievable under therapeutic dosages, can antagonize the agonist-activated hPXR and reduce the hPXR-mediated expression of CYP3A4 in human hepatocytes. Mechanistically, molecular modeling and the time-resolved fluorescence resonance energy transfer (TR-FRET) assays reveal that GEF binds to hPXR. Based on these characteristics, we propose GEF as a candidate with the potential to abrogate hPXR-mediated chemotherapy resistance in combination chemotherapies.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO; ≥99.9%), rifampicin (RIF; ≥97%), SR12813 (SR; ≥98%), and ketoconazole (KET; ≥98%) were purchased from Sigma-Aldrich. Gefitinib (GEF); (≥99%) was purchased from Selleck Chemicals.

Cell Culture. HepG2 human hepatocellular carcinoma cells were purchased from the American Type Culture Collection (ATCC) and grown in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS) and the other additives (HyClone), as described previously.[39−41] The assay media for HepG2 experiments were comprised of phenol red-free DMEM supplemented with 5% charcoal/dextran-treated FBS (HyClone). Cryopreserved human primary hepatocytes were purchased from Corning, Triangle Research Labs, or Invitrogen. The hepatocyte culture media were procured from Invitrogen, and the hepatocytes were cultured using the manufacturer's specifications altered slightly with our published protocol.[13,39] The hepatocytes' donor information is given in Table 1. Cryopreserved hepatocells and hepatocells' media were purchased from Corning. The hepatocells were

Table 1. Identification Number, Gender, Race, and Age of the Hepatocyte Donors

identification number	gender	race	age (years)
thermo fisher scientific			
Hu8210	male	caucasian	51
Hu8164	male	caucasian	23
triangle research labs			
HUM4275B	male	caucasian	29
HUM4122B	male	asian	35
corning			
cat. no. 454,551 (lot # 385)	male	caucasian	39

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cultured using the manufacturer specifications altered slightly with our published protocol.[13] While the human primary hepatocytes were treated with the vehicle or drugs for 24 h, the hepatocells were treated with the vehicle or drugs for 12 h before harvesting the cells for gene expression studies.

Luciferase Reporter Gene Assays. Luciferase reporter gene assays were performed in HepG2 cells as previously described.[13,39−41] HepG2 cells were transiently transfected with pcDNA3-hPXR and pGL3-CYP3A4-luc plasmids using jetPRIME (Polyplus Transfection). After 24 h transfection, the cells were plated in 96-well assay plates (PerkinElmer) and treated with DMSO or the compounds for 24 h. A Neolite Reporter Gene Assay System (PerkinElmer) was used to determine the luciferase activity using a FLUOstar Optima microplate reader (BMG Labtech).

Cell Viability Assays. Cell viability assays were performed in primary hepatocytes and HepG2 cells. The cells were treated with DMSO or compounds for 24 h before measuring cell viability using CellTiter-Glo Luminescent Cell Viability Assays (Promega).[13,39−42]

RNA Isolation and Quantitative RT-PCR Analysis. RNA isolation, reverse transcription, and the quantitative polymerase chain reaction were performed as previously described.[13] Quality control of RNA was assessed using a NanoVuePlus Spectrophotometer (GE Healthcare). Transcripts of the housekeeping genes GAPDH and CYP3A4 were amplified using the gene-specific primers (Table 2). The comparative $\Delta\Delta C_t$ method was used to analyze the relative gene expression.

Table 2. Forward (F) and Reverse (R) Primers Used for RT-qPCR of GAPDH and CYP3A4

gene/primer sequence	amplified segment (bp)	gene bank accession no.
GAPDH	265	NM_002046
F: 5′-ACCACAGTCCATGCCATCAC-3′		
R: 5′-GCTTCACCACCTTCTTGATG-3′		
CYP3A4	265	NM_017460
F: 5′-TTGGAAGTGGACCCAGAAAC-3′		
R: 5′-CTGGTGTTCTCAGGCACAGA-3′		

Competitive Ligand-Binding Assays. LanthaScreen TR-FRET hPXR competitive binding assays were performed to examine the binding affinity of GEF to the hPXR ligand-binding domain (Thermo Fisher Scientific).[13] Briefly, the assays were performed in 384-well solid black plates with a 10 nM GST−hPXR ligand-binding domain, 40 nM fluorescent-labeled hPXR agonist (Fluomore hPXR Green), 10 nM terbium-labeled anti-GST antibody, and GEF or SR. The reaction mixtures were incubated at room temperature in the dark for 1 h, and fluorescent emissions of each well were measured at 490 and 520 nm using a SpectraMax iD5 microplate reader (Molecular Devices), and the 520/490TR-FRET ratio was calculated.

Human hPXR Molecular Docking Studies. Ensemble-based molecular docking studies were conducted using GOLD suite version 5.5.0 (CCDC, Cambridge, U.K.), as described previously.[13,43,44] GOLD uses a genetic algorithm (GA) to explore the conformational flexibility of the ligand and receptor side chains in the binding pocket. Overall, 30 centroid conformations of *apo* hPXR generated using an RMSD-based clustering algorithm, obtained from previous work, were used for the docking.[44] In all of the protein conformations, water and ions were removed prior to docking. For the docking purpose, a binding site was defined by considering all atoms within 12 Å from the geometrical center of the docking site. For each of the 30 independent GA runs, a maximum number of 200 GA operations were performed. The docked complexes were ranked with the *goldscore* and then rescored using a *chemscore* fitness function.[43] GEF, KET, and belinostat[13] were docked at multiple sites, whereas SR12813 and RIF were docked at the ligand-binding pocket (LBP) as controls.[13] The scoring functions account for the hydrogen bonding, van der Waals (vdW) interactions, and steric complementarity between the ligand and the receptor. For each ligand, the best-ranked docked pose with the corresponding *chemscore* is considered for further analysis.

Data and Statistical Analysis. Data are shown as mean values ± standard deviation (SD). Analyses were performed using GraphPad Prism 9.0 (La Jolla, CA). Significance ($P < 0.05$, 0.01, 0.001, or 0.0001) was evaluated by ANOVA, followed by Tukey's multiple comparison test.

RESULTS

Gefitinib (GEF), at Its Therapeutically Relevant Concentrations, Inhibits Rifampicin (RIF)-Induced CYP3A4 Gene Expression. In a small-scale preliminary screening approach using hepatocells,[45] we sought to identify clinical drugs with the capability to modulate hPXR-mediated target gene expression. In total, 29 compounds were selected to form a biased library of commercially available FDA-approved drugs made up of diverse chemical structures and used to treat a variety of diseases. Preliminary screening was performed to determine drug candidates with the potential to inhibit RIF-induced CYP3A4 gene expression. We identified GEF as a drug candidate capable of antagonizing the hPXR agonist RIF-induced CYP3A4 gene expression (Figure 2A). We, therefore, hypothesized that GEF, at its therapeutic concentrations, could antagonize hPXR agonist-induced upregulation CYP3A4 expression in human hepatocytes.

To determine the experimental concentrations of GEF that are therapeutically relevant, we have collected the pertinent plasma pharmacokinetic parameters from previous studies in humans (Table 3). In humans, after a single oral dose of GEF at the recommended dosage of 250 mg,[46] the mean peak plasma concentration (C_{max}) of GEF could reach 0.84 (0.37−1.75) μM.[47−50] The FDA treatment regimen for relevant NSCLC is a daily oral dosage of GEF 250 mg once daily until disease progression or unacceptable toxicity. However, in combination with certain medications such as carboplatin and paclitaxel, 500 mg daily can be given.[46,51] Plasma concentrations of GEF will increase to ∼2 μM when using dosages in the 500 mg range.[48] However, dosages of up to 1500−3500 mg can be given without causing significant adverse events,[46,52] so the usage of higher concentrations is also feasible. GEF plasma concentrations have been measured with dosages of 700 mg/mL daily at 2.6 μM.[46] For our study, GEF concentrations (1, 3, 10 μM) were chosen based on previous PK ranges, as well as the commonly used experimental concentrations. We, there-

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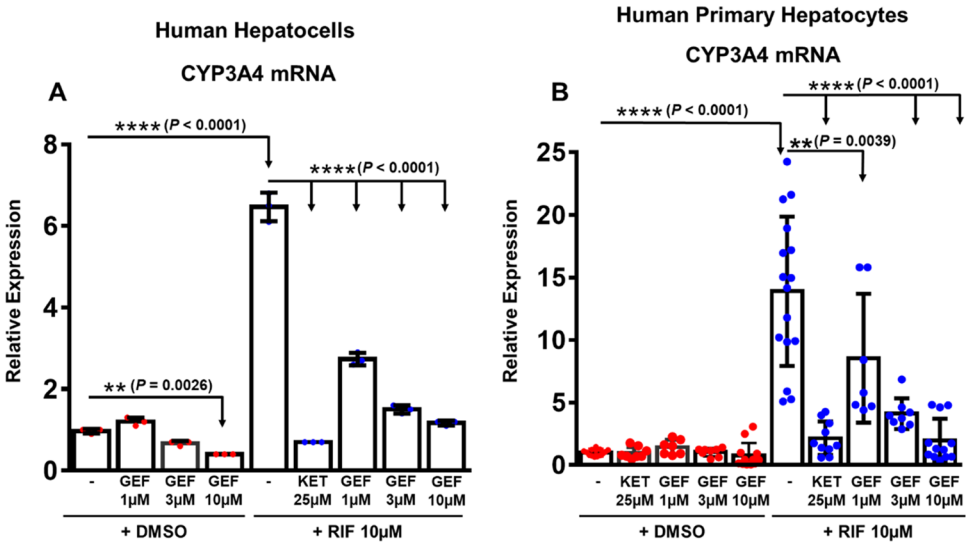

Figure 2. Effect of GEF on hPXR agonist-induced CYP3A4 gene expression. CYP3A4 mRNA expression in human hepatocells (A) and human primary hepatocytes (B) after treatment with vehicle DMSO, RIF, GEF ± RIF, or KET ± RIF for 12 h in the hepatocells and 24 h in the hepatocytes. Results are shown as the fold change over DMSO treatment. Data are expressed as the mean ± SD values from three to five donor hepatocytes and three experimental repeats performed on single-lot hepatocells. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P <0.0001 by ANOVA with Tukey's multiple comparison test.

fore, examined the effect of GEF, at its C_{max} achievable under therapeutic dosages, on agonist-induced endogenous hPXR target gene expression in human hepatocytes.

Ketoconazole (KET), a known antagonist of hPXR,[53] inhibited the hPXR agonist RIF-induced CYP3A4 mRNA expression in human primary hepatocytes (Figure 2B). Similarly, therapeutically relevant concentrations of GEF repressed RIF-induced CYP3A4 mRNA levels in human primary hepatocytes (Figure 2B). These results suggest that GEF can antagonize hPXR agonist-induced CYP3A4 gene expression at its C_{max} achievable under therapeutic dosages in human primary hepatocytes (Figure 2A,B).

GEF Antagonizes RIF Induction of hPXR-Mediated CYP3A4 Promoter Activity. To determine whether GEF could effectively inhibit the agonist-activated hPXR transcriptional activity, we examined the effect of GEF on hPXR-regulated CYP3A4 promoter activity in human HepG2 cells (Figure 3). The cells were transiently transfected with CYP3A4-luc and hPXR and treated with DMSO, RIF, GEF ± RIF, or KET ± RIF. KET decreased RIF-induced hPXR-mediated transactivation of CYP3A4 promoter activity (Figure 3). Although not as strongly as the known hPXR antagonist KET, GEF, at its therapeutically relevant concentrations, was able to significantly inhibit RIF-induced hPXR-mediated transactivation of CYP3A4 promoter activity (Figure 3). These results suggest that GEF can antagonize the agonist-activated hPXR.

GEF is Moderately Cytotoxic at Concentrations Effective for Inhibiting RIF Induction. In CellTiter-Glo Luminescent cell viability assays, while 1 and 3 μM alone did not exert noticeable cytotoxicity in the human primary hepatocytes (Figure 4A) or HepG2 cells (Figure 4B), 10 μM GEF was significantly cytotoxic (Figure 4A,B). In combination with RIF, 10 μM GEF was modestly cytotoxic to the human primary hepatocytes (Figure 4A). RIF by itself was cytotoxic to HepG2 cells (Figure 4B). Similarly, RIF in combination with GEF (1, 3, or 10 μM) was also modestly cytotoxic to HepG2 cells (Figure 4B). Collectively, these data suggest that GEF can antagonize RIF-induced hPXR target gene expression at its C_{max} achievable under therapeutic dosages, with modest cytotoxicity.

Molecular Docking Studies Predict that GEF Could Bind to Multiple Sites at hPXR. Several mechanisms could contribute to the GEF inhibition of RIF-induced CYP3A4 gene expression, including GEF binding to hPXR at multiple functionally important sites. The docking score from ensemble-based docking studies predicted that GEF could bind to the LBP, AF2 region, and α8 pocket of hPXR (Figure 5A). At the LBP, GEF possesses different binding predicted modes (Figure 5B), with the docking score comparable to that of known hPXR LBP-binding compounds, RIF, SR12812, and belinostat,[13] suggesting that GEF could act as an agonist/antagonist by direct interaction with the hPXR LBP. Similar to KET and belinostat,[13] known hPXR antagonists, GEF was predicted to bind to the AF2 region, which was reflected in their docking score (Figure 5A). GEF was predicted to interact with residues at the AF2 region that are essential for SRC-1 interaction,[54] potentially hindering the binding of the coactivator SRC-1 peptide (Figure 5C,D). It is interesting to compare GEF binding to hPXR with that of our previously published antagonist, belinostat.[13] Primarily, GEF with a rigid bicyclic ring is constrained in its conformational space (Figure 1). On the other hand, belinostat with monocyclic rings is more flexible and has the capacity to form strong hydrogen bonds with the terminal N═O group and with the SO_2 group on the other end of the molecule, which is easily accessible to the polar/charged groups of PXR groups (Figure 5 in 13). Thus, belinostat interaction at the AF2 site is more dominated by hydrogen bonds and that of GEF is dominated by hydrophobic interactions, resulting in a slightly higher docking score of belinostat. This feature is also evident in interactions at the α8 site (Figure 5C). It is interesting to note that the predicted binding of GEF at the AF2 site is remarkably similar to that of BEL, in spite of the differences in their chemical features. As in the case of BEL, K259 of α3 hydrogen bonds with the oxygen atom attached to a bicyclic ring of GEF

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Table 3. Published Plasma Pharmacokinetic Parameters of GEF in Human Beings

human subjects' disease state and experimental design	dosage of GEF	C_{max} μM	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{(0-t)}$ (μg·h/mL)	reference
patients with non-small-cell lung, adenocarcinoma, squamous, poorly differentiated adenocarcinoma, colorectal, head and neck, and breast cancer were given a single oral dose of 225 mg for 1 day (1)[a] or a single oral dose of 225, 400, 525, or 700 mg daily for 14 days (14)[a]	(1)[a] 225 mg (oral)	0.34	4 (3−12)	30	4.4	48
	(14)[a] 225 mg (oral)	0.76	5 (3−7)	40	11.6	
	(14)[a] 400 mg (oral)	1.75	3 (3−7)	45	25.5	
	(14)[a] 525 mg (oral)	1.90	5 (3−7)	59	36.5	
	(14)[a] 700 mg (oral)	2.59	5 (3−7)	55	48.2 (0−24 h)	
Healthy humans were given a single oral dose of 250 or 500 mg for 1 day (1)	(1) 250 mg (oral)	0.18 (0.10−0.42)	5 (3−8)	31.5 (9.6−62.1)	2.4 (0.89−6.55)	49
	(1) 500 mg (oral)	0.45 (0.20−1.19)	3 (3−7)	31.3 (9.8−74.5)	5.9 (2.11−13.09) (0−24 h)	
Patients with advanced solid tumors of the colon/rectum, lung, stomach, ovary, liver, pancreas, and kidney were given a single oral dose of 250 mg for 1 day (1)[a]	(1)[a] 250 mg (oral)	0.35 (0.10−0.72)	3 (1−8)	50.5 (15.6−111)	4.87 (1.86−10.0) (0−24 h)	49
Healthy human subjects were given a single oral dose of 250 mg for 1 day (1)[a]	(1)[a] 250 mg (oral)	0.29 (0.11−0.55)	5 (3−24)	26.9 (20.0−45.6)	8.61 (3.87−20.41) (0−24 h)	47
Patients with advanced non-small-cell lung cancer were given a single oral dose of 250 mg for 1 day (1)[a]	(1)[a] 250 mg (oral)	0.84 (0.37−1.75)	na	na	10.95 (1.56−31.31)	50

[a]Number in the parenthesis represents the number of days the human subjects were given a single dose of GEF.

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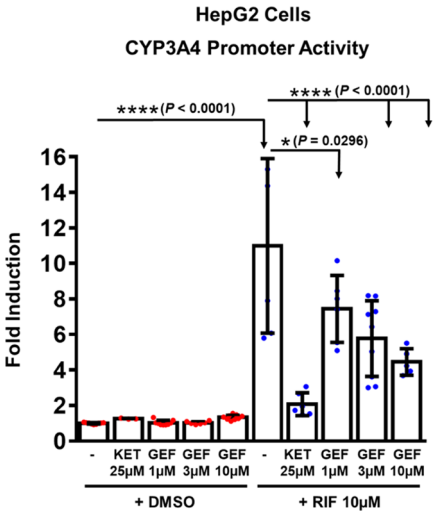

Figure 3. Effect of GEF on hPXR-mediated CYP3A4 promoter activity. HepG2 cells were transiently cotransfected with pcDNA3-hPXR and pGL3-CYP3A4-luc plasmids and treated with DMSO, GEF, RIF, or GEF ± RIF. CYP3A4 promoter activity was measured 24 h after the treatments. The luciferase activity was normalized to the number of live cells, and the data of three experimental repeats are expressed as a fold induction ± SD over DMSO treatment. Differences were tested using one-way ANOVA with Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

(Figure 5C,D), anchoring one side of SRC-1 binding site (Figure 5D). Collectively, the docking study results suggest that GEF could exert its antagonistic activity either by directly binding to the LBP and/or by allosterically interacting with the AF2 and/or α8 pocket.

GEF Binds to the Ligand-Binding Domain (LBD) of hPXR. Computational molecular modeling docking studies predicted that GEF could bind to the ligand-binding domain of hPXR (Figure 5). Using a cell-free hPXR ligand-binding assay, we tested whether GEF could bind to the ligand-binding domain of hPXR at its C_{max} achievable under therapeutic dosages. The hPXR agonist SR12813 exhibited successful binding to the LBD of hPXR (Figure 6). Similarly, GEF was capable of binding to the LBD of hPXR (Figure 6). The ability of GEF to bind the LBD of hPXR suggests the potential of GEF to act as a direct antagonist.

DISCUSSION

Our results show for the first time that gefitinib (GEF) antagonizes the hPXR agonist-induced CYP3A4 gene expression in human hepatocytes at its clinically relevant therapeutic concentrations. Many of the known hPXR antagonists are inadequate for clinical utility as they are unable to achieve the concentrations required to modulate hPXR *in vivo* without causing unacceptable toxicity.[11,55] For example, the required concentration of KET needed to inhibit hPXR is 10−25 μM, which is unlikely to be achieved safely. Similarly, sulforaphane effectively antagonized hPXR in *in vitro* studies, but concentrations needed for sulforaphane to antagonize hPXR could not be achieved *in vivo*.[56] Recently, a novel hPXR antagonist has been identified. However, the pharmacokinetics and safety profile of this compound in humans are unknown.[15] GEF is a clinically used anticancer drug with validated pharmacokinetics and safety profile. While adverse events resulting from the therapeutic use of GEF have been described, these were mild to moderate (grade 1/2) skin rash, diarrhea, and nausea. These were further determined to be manageable and noncumulative.[57] Therefore, GEF may have the potential to be used to antagonize hPXR as an adjuvant therapy with manageable adverse events at its relevant therapeutic concentrations.

While GEF inhibits EGFR with an inhibitory concentration (IC_{50}) of 2.12 ± 0.25 nM in cell-free assays,[58] it is expected to inhibit EGFR at its *in vivo* therapeutic concentration (0.3 to 3 μM).[47−50] It is important to note that although the effective *in vivo* therapeutic concentration may go up to 3 μM, the maximum C_{max} reported at more typical dosing ranges of 250−500 mg is 2 μM. However, increasing the dosage to 700 mg in patients has increased the C_{max} to 2.7 μM. It may be possible to increase the dosage to increase C_{max} to 3 μM, as additional patient studies have shown that dosages up to 3500 mg are possible without significant toxicity.[46,52] Further studies are needed to determine the feasibility of using higher dosages of GEF. Our studies show that GEF can also antagonize hPXR at its therapeutically relevant concentration of 1 μM, with increasing effectiveness at potentially achievable concentrations

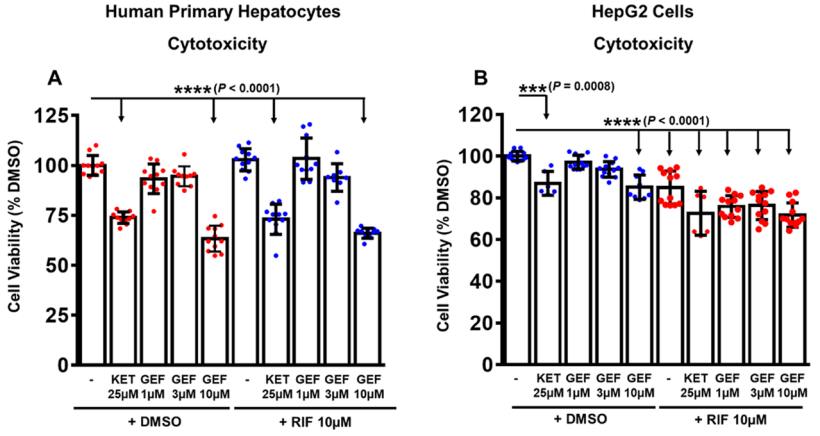

Figure 4. Effect of GEF on the viability of the human primary hepatocytes and HepG2 cells. The viability of the human primary hepatocytes (A) and HepG2 cells (B) was determined under the same experimental conditions indicated in gene expression studies. The viability of DMSO-treated cells was expressed as 100%. Results are presented as the mean ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by ANOVA and Tukey's multiple comparison test.

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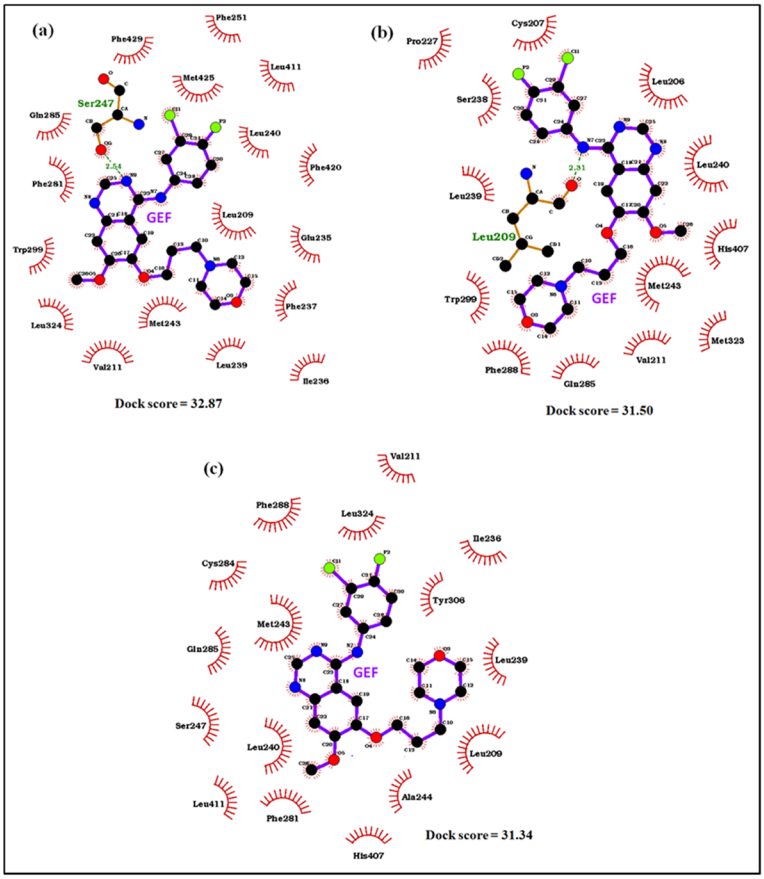
A

Compound	LBP	AF2	α8
Gefitinib	32.87	23.36	22.87
Belinostat	32.43	30.24	28.43
Ketoconazole	32.01	14.96	16.73
SR12813	35.01		
Rifampicin	31.49		

B



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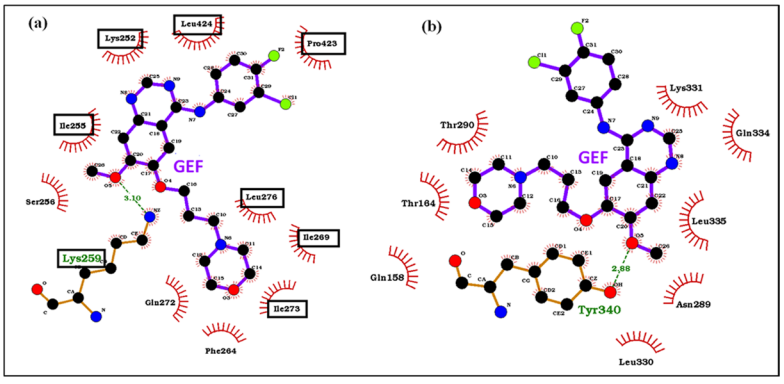

Figure 5. continued

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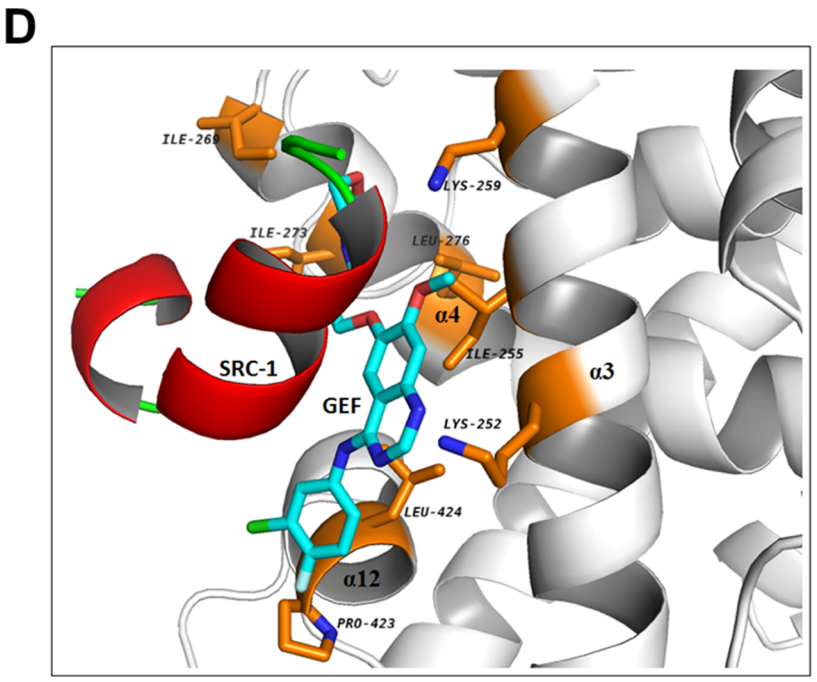

Figure 5. hPXR molecular docking studies. (A) Computational modeling with ensemble-based molecular docking studies predicts that GEF could bind to the multiple distinct sites of hPXR. The score of the top-ranked docked pose of ligands at different sites in hPXR was obtained from docking of ligands against an ensemble of hPXR conformations. (B) (a–c) Top three ranked docked poses of GEF at LBP of hPXR, with the corresponding docking score. Dotted lines denote the H-bonding interaction, and the protein residues involved in hydrophobic interactions are shown by red spikes. The H-bond distance is also shown alongside. (C) Mode of the interaction of GEF at (a) AF2 region as well as (b) α8 pocket. Dotted lines indicate the H-bonding interaction, and red spikes indicate the protein residues involved in hydrophobic interactions. The amino acids that are crucial for the interaction of SRC-1 at the AF2 region are displayed in a rectangle. (D) Superposition of SRC-1 and GEF interaction at the hPXR AF2 region. The interacting amino acids common to both GEF and SRC-1 are highlighted.

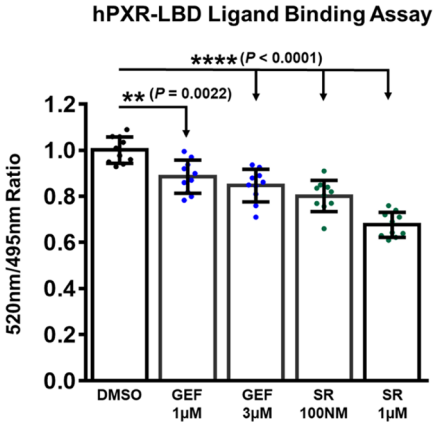

Figure 6. GEF binds to the hPXR-LBD in the competitive ligand-binding assay. hPXR-LBD, a fluorescein-labeled hPXR ligand tracer, and Tb-anti-GST antibody were incubated in the presence of the vehicle control (DMSO), a test compound (GEF), or a known hPXR agonist SR12813 (SR). The TR-FRET ratio denotes the binding of the fluorescein-labeled hPXR ligand tracer to the hPXR-LBD, and a reduction of the TR-FRET ratio denotes the binding of antagonists or agonists to the hPXR-LBD by outcompeting the binding of the fluorescein-labeled hPXR ligand tracer. Data are presented as mean ± SD values from three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by ANOVA with Tukey's multiple comparison test.

of 3 μM. Although higher therapeutic concentrations of GEF may be possible, the feasibility and safety of using higher dosages of GEF to achieve a sustainably higher therapeutic concentration require future studies. Computational molecular docking predictions reveal that GEF likely interacts with hPXR at multiple sites, including the ligand-binding pocket, α8 pocket, and AF2 regions of hPXR. Cell-free LBD binding assays confirmed that GEF acts as an antagonist by binding to the LBD of hPXR (Figure 6). GEF may also inhibit the agonist-activated hPXR *via* allosteric interaction with the binding to the AF2 region as well as the α8 pocket of hPXR. However, the potential allosteric binding of GEF to the AF2 region and the α8 pocket of hPXR has not been investigated.

The net effect of GEF may vary by tissue and cancer type. hPXR activity has previously been shown to be regulated in a tissue-specific and context-dependent manner.[27,59] Indeed, in LS180 human colon cancer cells, GEF appears to activate hPXR and induce hPXR-mediated MDR1 gene expression.[60] However, CYP3A4 expression was not evaluated in this study. Furthermore, as hPXR and its coregulators are also expressed differentially in a tissue-specific manner,[59] a compound such as GEF, capable of inhibiting the interaction between hPXR and its coactivators, may only be effective in some cancer types and moreover so in a context-dependent manner. hPXR is known to be regulated in a promoter and ligand-dependent manner, thereby different ligands can influence hPXR to bind to different response elements and influence different genes.[61] Indeed, differing ligands were shown to result in PXR binding to different promoters, resulting in ligand-dependent promoter activity of CYP3A4 and MDR1 (P-gp). This is, in part, due to different ligand structures having unique hPXR ligand-binding conformations, resulting in differential interaction with hPXR cofactors,[61] which may explain any differential effects of GEF compared to other TKIs. Furthermore, hPXR modulation by the same drug can have differential effects in the same tissue

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type depending on the disease state.[27] In liver hepatocellular carcinoma HepG2 cells, a known hPXR agonist buprenorphine, increased hPXR-mediated gene expression. However, the same study revealed that exposure of noncancerous human primary hepatocytes to buprenorphine resulted in no induction of hPXR-mediated gene expression.[62] It is therefore possible that GEF may modulate hPXR differentially depending on tissue or scenario in a context-dependent manner.

Our previous work has demonstrated that it is possible to overcome hPXR agonist-mediated chemoresistance by repurposing a clinical anticancer drug to antagonize the agonist-activated hPXR at therapeutic concentrations during combination chemotherapy.[13] Based on the ability of GEF to antagonize the RIF-activated hPXR-mediated upregulation of CYP3A4, it is feasible to use GEF to overcome chemoresistance in some cases; however, this remains to be studied. Our hypothesis would be relevant only to cancers in which chemoresistance occurs because of drug activation of hPXR-mediated overexpression of CYP3A4.[27,63,64]

In conclusion, our results show that GEF, at its C_{max} achievable under therapeutic dosages, can antagonize the RIF-activated hPXR target CYP3A4 gene expression in human hepatocytes. Thus, GEF could serve as a novel candidate for use in precision combinational chemotherapies to combat hPXR-mediated chemoresistance in relevant cancers where the hPXR agonist-induced upregulation of CYP3A4 contributes to chemotherapy resistance.

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Notes

The authors declare no competing financial interest.

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