The dietary isothiocyanate, sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor (SXR)

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Running Title: Identification of SFN as an antagonist ligand for SXR

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Abbreviations: SFN, sulforaphane; CYP, cytochrome P450; SXR, steroid and xenobiotic receptor; PXR, Pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor, RXR, retinoid X receptor, PPAR, peroxisome proliferator-activated receptor; RIF, rifampicin; RU486, mifepristone; CLOT, clotrimazole; PCN, pregnenolone 16α-carbonitrile.

Abstract

Sulforaphane (SFN) is a biologically active phytochemical found abundantly in broccoli. SFN has been promoted as a putative chemopreventive agent to reduce cancer and most studies have associated its anticancer effects with the induction of phase II xenobiotic metabolism enzymes via activation of the Keap1/Nrf2 antioxidant response pathway. Interestingly, SFN can significantly down-regulate cytochrome P450 3A4 (CYP3A4) expression in human primary hepatocytes. CYP3A4 is responsible for the hepatic and intestinal metabolism of numerous pro-toxicants, pharmaceutical compounds and endogenous sterols. Among the most important mediators of CYP3A4 expression is the nuclear hormone receptor, steroid and xenobiotic receptor (SXR; also called 'hPXR'). SXR functions as a xenobiotic sensor to coordinately regulate xenobiotic metabolism via transcriptional regulation of xenobiotic detoxifying enzymes and transporters. Here we report that SFN is a specific antagonist of human SXR and that it inhibits SXR-mediated induction of drug clearance. SFN can bind directly to SXR, inhibit SXR coactivator recruitment, and efficiently repress SXR activities. Furthermore, SFN inhibited SXR-mediated CYP3A4 expression and CYP3A4-catalyzed midazolam (MDZ) clearance in human primary hepatocytes. Thus, SFN is the first identified naturally occurring antagonist for SXR (hPXR). Because induction of CYP3A4 can result in adverse drug responses (e.g., lack of efficacy), which are a major public health problem, this discovery could lead to the development of important new therapeutic and dietary approaches to reduce the frequency of undesirable inducer-drug interactions.

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Introduction

Sulforaphane (SFN) is one of the most biologically active phytochemicals in the human diet (Fig. 1A), and is present at high concentrations in some cruciferous vegetables, especially broccoli (Kushad et al., 1999; Zhang et al., 1992). Epidemiologic and clinical studies have indicated that diets high in cruciferous vegetables protect against a number of cancers including non-Hodgkin's lymphoma, liver, prostate, cervical, ovarian, lung and gastrointestinal tract (Murillo and Mehta, 2001). Numerous studies in animal models and human cells support the putative chemopreventive effects of SFN (Chung et al., 2000; Conaway et al., 2002; Zhang et al., 1994). For example, SFN treatment reduced 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors (Zhang et al., 1994); inhibited benzo(*a*)pyrene-induced forestomach tumors in mice (Fahey et al., 2002); lowered the formation of colonic aberrant crypt foci in rats (Chung et al., 2000) and inhibited cell proliferation of an HT-29 colon cancer cell line (Frydoonfar et al., 2004). In addition, in a recent study with human hepatocytes in primary culture, we demonstrated that pretreatment of hepatocytes with 50 μM SFN produced more than a 90% decrease in DNA adduction of the potent hepatocarcinogen, aflatoxin B1 (Gross-Steinmeyer et al., 2005).

The mechanism(s) of action of the putative chemopreventive effects of SFN appear to be multifactorial. SFN can induce apoptosis and cell cycle arrest in human cancer cells (Gamet-Payrastre et al., 2000), and is an inhibitor of histone deacetylases (Myzak et al., 2006). However, most studies have associated the anticancer effects of SFN with the induction of phase II drug metabolism enzymes, especially the glutathione S-transferases (GST) (Talalay et al., 1995). SFN activates the Keap1/ Nrf2 transcriptional factor complex that can bind to the antioxidant response element (ARE) and induce a series of detoxification enzymes, such as NAD(P)H:quinone oxidoreductase-1 (NQO1), certain GSTs and UDP-glucuronosyltransferases (UGTs), and other

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genes involved in antioxidant response (Conaway et al., 2002; Fahey and Talalay, 1999; Gao and Talalay, 2004).

Interestingly, it has also been reported that SFN down-regulated CYP3A4 transcription and enzyme activity in cultured human hepatocytes, suggesting another mechanism that could also contribute to its anti-cancer effects (Maheo et al., 1997). Indeed, we subsequently confirmed that SFN consistently and dramatically reduced CYP3A4 mRNA content in human hepatocytes (Gross-Steinmeyer et al., 2005).

The nuclear hormone receptor, steroid and xenobiotic receptor (SXR) (Blumberg et al., 1998)(also known as pregnane X receptor (PXR) (Kliewer et al., 1998), PAR, and NR1I2), plays a central role in the transcriptional regulation of CYP3A4 (reviewed in (Dussault and Forman, 2002; Kliewer et al., 2002)). Here we use PXR to refer to the rodent form, and SXR to refer to the human form, hPXR). SXR is activated by a diverse array of pharmaceutical agents including taxol, rifampicin (RIF), SR12813, clotrimazole, phenobarbital, the herbal antidepressant St John's wort, and peptide mimetic HIV protease inhibitors such as ritonavir (Dussault and Forman, 2002; Kliewer et al., 2002). These studies indicate that SXR functions as a xenobiotic sensor (Blumberg et al., 1998) to coordinately regulate drug clearance in the liver and intestine via transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as CYP3A4 and MDR1 (Dussault and Forman, 2002; Kliewer et al., 2002). Because SFN significantly inhibited CYP3A4 expression, we tested whether down-regulation of CYP3A4 by SFN is mediated by SXR.

CYP3A4 is among the most important enzymes of the CYP family since it contributes to the metabolism of more than 50% of clinically used drugs and a corresponding number of xenobiotic chemicals (Guengerich, 1999). Surprisingly, it exhibits marked inter-individual variability in terms of its specific content and activity in the liver and small intestine, the primary sites of drug

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metabolism. Moreover, induction or inhibition of CYP3A4 is a common cause of adverse drug-drug interactions, which are a major public health problem in the US. Adverse drug reactions account for 10-17% of the medical indications for acute hospital admission of elderly patients (Beard, 1992), and may contribute to more than 100,000 deaths in the US each year. By some estimates, it represents the 4th to 6th leading cause of death in the US (Wrighton and Thummel, 2000). Thus, modification of CYP3A4 expression and activity by consumption of SFN could have important implications for drug safety.

Here we report that SFN is a specific antagonist of SXR and inhibits SXR-mediated induction of drug clearance. SFN was able to efficiently inhibit SXR-mediated transcription of the CYP3A4 gene in a concentration dependent manner. SFN bound directly to SXR and inhibited SXR-coactivator interactions. Furthermore, SFN inhibited SXR-mediated CYP3A4 expression and CYP3A4-mediated midazolam (MDZ) clearance in human primary hepatocytes. Thus, SFN is the first identified naturally occurring antagonist for SXR. This discovery could contribute to a better understanding of the mechanism of inter-individual variability in intestinal and hepatic CYP3A4-dependent drug metabolism. These findings could also lead to potentially important new therapeutic and dietary approaches to reduce the frequency of adverse drug reactions that are secondary to SXR-mediated induction of drug clearance via CYP3A4, MDR1 and other genes regulated in part by SXR. These findings also point to a novel and complementary mechanism by which SFN exerts its putative chemoprotective effects through a reduction in CYP3A4-dependent reactive metabolite formation.

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Materials and Methods

Reagents and plasmids

SFN, Rifampicin (RIF), mifepristone (RU486), and clotrimazole (CLOT) were purchased from Sigma-Aldrich; Pregnenolone 16α-carbonitrile (PCN), CITCO, WY-14643, Troglitazone, and 9-cis-retinoic acid were purchased from Bio Mol; and $1,25(OH)_2D_3$ were purchased from Calbiochem. Iberin, Cheirolin, Erucin, and PEITC were purchased from LKT, Inc. SXR, GAL4-SXR LBD, VP16-SXR, CMX-β-gal expression vectors; SXR-dependent CYP3A4 promoter reporter (CYP3A4XREM-Luciferase) and GAL4 reporter (MH100-Luciferase) have been previously described (Blumberg et al., 1998; Synold et al., 2001; Zhou et al., 2006b; Zhou et al., 2004).

Cell culture

The human intestinal epithelial cell line, LS180 were obtained from American Type Culture Collection and cultured in DMEM containing 10% FBS at 37°C in 5% CO_2. The cells were seeded into 6-well plates and grown in DMEM-10% FBS until 70-80% confluence. Twenty-four hours before treatment, the medium was replaced with DMEM containing 10% resin-charcoal stripped FBS. Immediately before treatment, the medium was removed; the cells were washed once with PBS and then treated with compounds or DMSO vehicle for appropriate times. Human primary hepatocytes were obtained from LTPADS (Liver Tissue Procurement and Distribution System, Pittsburg, PA) as attached cells in 6-well plates. The hepatocytes were maintained in hepatocyte medium (Sigma-Aldrich) for at least 24 h before treatment.

Transient Transfection and Luciferase Assay

Transfection assays and Luc and β-galactosidase were performed as described (Zhou et al., 2004). To test the ability of SFN to inhibit SXR or other nuclear receptors, HepG2 cells were seeded into 12-well plates overnight and transiently transfected with the control or SXR expression plasmid,

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together with the CYP3A4XREM-Luciferase reporter and CMX-β-galactosidase transfection control plasmids using FuGene 6 (Roche) in serum-free DMEM. Twenty-four hours post-transfection, the cells were treated with DMSO as a negative control, the known SXR ligands RIF, RU486 and clotrimazole, in the absence or presence of SFN. The cells were lysed 24 h after treatment and β-galactosidase and luciferase assays were performed as described (Grun et al., 2002). Reporter gene activity was normalized to the β-galactosidase transfection controls and the results expressed as normalized RLU per OD β-galactosidase per minute to facilitate comparisons between plates. Fold induction was calculated relative to solvent controls. Each data point represents the average of triplicate experiments +/- SEM and was replicated in 3-4 independent experiments. For mammalian two-hybrid assays, HepG2 cells were transfected with GAL4 reporter, VP16-SXR, and GAL-SRC1, GAL-PBP, GAL-ACTR, or GAL-TIF2 (kindly provided by Dr. B.M. Forman, City of Hope National Medical Institute) (Synold et al., 2001). The cells were then treated with 10 μM RIF or RU486 in the presence or absence of SFN at the indicated concentration. IC50s were calculated by curve fitting of data, assuming a competitive antagonism model, using GraphPad Prism software.

Ligand binding assays

N-terminal His_6-tagged human SXR ligand binding domain was expressed in *Escherichia coli* together with the SRC-1 receptor interaction domain and scintillation proximity assays were performed essentially as described (Zhou et al., 2004). Briefly, active protein was refolded from inclusion bodies solubilized in denaturation buffer (6 M guanidium-HCL, 50 mM HEPES pH7.4, 0.2 M NaCl, 25 mM DTT, 1% w/v Triton-X100) by rapid 10-fold dilution into binding buffer (50 mM HEPES pH 7.4, 1 M sucrose, 0.2 M NaCl, 0.1 mM DTT, 0.1% w/v CHAPS) followed by dialysis overnight at 4 ºC against binding buffer. Binding assays were performed by coating 96-well nickel chelate FlashPlates (Perkin Elmer Life Sciences) with a 10-fold molar excess of protein for one hour

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at 22 ºC in binding buffer (50 mM HEPES, pH 7.4, 200 mM NaCl, 1 M sucrose, 0.1% CHAPS). Unbound protein was removed from the wells by washing four times with binding buffer. ^{3}H-SR12813 (Amersham-Pharmacia BioSciences) was added to a final concentration of 50 nM in each well, either alone or together with competitor ligands in binding buffer as indicated. Incubation was continued for 3 hours at room temperature. Total counts were measured using a Topcount scintillation counter (Packard, Meriden, CT). Counts remaining after the addition of 10 μM clotrimazole were taken as non-specific background and subtracted from all wells. All assays were performed in triplicate and reproduced in independent experiments.

Competition binding curves were determined at constant 3H-specific ligand concentrations (50 nM SR12813, Kd = 41 nM; Zhou et al., 2004) with increasing cold competitor ligands over the range indicated in the figure. Data were analyzed in GraphPad Prism by nonlinear regression of a competitive one-site binding equation (Chang-Prusoff method) to determine Ki values ± 95% confidence intervals (n = 3).

RNA Isolation and Quantitative Real-time PCR (QRT-PCR) Analysis

Total RNA was isolated from primary hepatocytes and LS180 cells using TRIzol reagent (InVitrogen Life Technology) according to the manufacturer-supplied protocol. Quantitative real time PCR was performed using gene specific primers and the SYBR green PCR kit (Applied Biosystems) in an ABI 7900 system (Applied Biosystems). All samples were quantified using the comparative Ct method for relative quantification of gene expression, normalized to GAPDH (Livak and Schmittgen, 2001). The following primer sets were used in this study: CYP3A4 (5'-GGCTTCATCCAATGGACTGCATAAAT-3' and 5' -TCCCAAGTATAACACTCTACACAGACAA-3') Primer sites were in exon 13 of CYP3A4 which represent a unique region that distinguishes CYP3A4 from CYP3A5 and 3A7; MDR1 (5'-

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CCCATCATTGCAATAGCAGG-3' and 5'-GAGCATACATATGTTCAAACTTC-3');
UGT1A1(5'-TGCTCATTGCCTTTTCACAG-3' and 5'-GGGCCTAGGGTAATCCTTCA-3');
NQO1(5'-GGCAGAAGAGCACTGATCGTA-3' and 5'-TGATGGGATTGAAGTTCATGGC-3');
GAPDH (5'-GGCCTCCAAGGAGTAAGACC-3' and 5'-AGGGGAGATTCAGTGTGGTG-3').

MDZ clearance analysis

An internal standard mixture containing $^{15}N_3$-labeled MDZ metabolite, 1'-OH MDZ, was prepared by incubating 6 nmol of cytochrome P450 (using HL-122 microsomes) with 100 µg of $^{15}N_3$-MDZ and 12 mg of NADPH (final concentration, ~1.5 mM) in potassium phosphate buffer (0.1 M, pH 7.4, in a final volume of 8 ml) at 37°C. After 10 min, the reaction was stopped by the addition of 8 ml of Na_2CO_3 (0.1 M, pH 12). The compounds were extracted twice with 20 ml of ethyl acetate, and the solvent was evaporated to dryness under a stream of nitrogen. The remaining solid was then reconstituted in 20 ml of methanol, split into two 10-ml aliquots and stored at −80°C. To determine CYP3A4 activity, human primary hepatocytes were pre-incubated with 10 or 25 µM SFN for 24 hrs before addition of 10 µM RIF. Twenty-four hrs later, cells were rinsed with media 3 times and then incubated with new media containing 8 µM MDZ for 6 hrs. The supernatant media were collected for 1'-OH MDZ formation analysis as described before (Paine et al., 1997). Briefly, Samples were spiked with 100 µl of a 1:5 dilution of the internal standard mixture, which represented ~50 ng of $^{15}N_3$-labeled 1'-OH MDZ. The metabolites were extracted with 5 ml of ethyl acetate, the solvent was removed under nitrogen and the concentrated extracts were dissolved in 100 µl of derivatizing reagent (10% N-methyl-N-(*t*-butyl-dimethylsilyl)trifluoroacetamide in acetonitrile). The samples were then transferred to autoinjector vials and were analyzed for 1'-OH MDZ by selective ion gas chromatography-negative chemical ionization mass spectrometry (GC/NCI-MS) as previously described (Paine et al., 1997). The 1'-OH MDZ was quantified by

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comparing peak area ratios with standard curves prepared by the addition of known amounts of 1'-OH MDZ (0-160 pmol) and 100 µl of internal standard to phosphate buffer.

Statistical analysis

We used a two-sample, two-tailed Student's t test to evaluate the effect of SFN treatment on the control, RIF or RU486 mediated inductive response in the different cultured cell systems. A *P* value less than 0.05 was considered to be significant. One way ANOVA was utilized when multiple comparisons were made, followed by Dunnett's t-test for multiple comparisons to a control.

Results

SFN efficiently inhibits SXR activity

We previously observed that SFN consistently and dramatically reduced CYP3A4 mRNA content in human hepatocytes. SXR contributes importantly to both constitutive and inducible expression of CYP3A4 (Dussault and Forman, 2002; Kliewer et al., 2002) and several other genes involved in xenobiotic disposition (e.g., MDR1). SXR is activated by a diverse array of pharmaceutical agents including taxol, rifampicin (RIF), RU486, SR12813, clotrimazole, phenobarbital, and hyperforin, and enhances the transcription of its target genes (Blumberg et al., 1998; Kliewer et al., 2002; Kliewer et al., 1998). Thus, we tested the ability of SFN to inhibit ligand-mediated activation of SXR by use of transfection assays. Two different SXR ligands, RIF and RU486, were able to strongly induce SXR reporter activities in SXR-transfected cells (Fig. 1B). SFN significantly inhibited both RIF and RU486 induced reporter activities. This effect was SFN dose-dependent. Inhibition of SXR reporter activity was detected at a concentration as low as 1 µM and, at 25 µM, SFN completely blocked RU486 induced SXR reporter activity. Moreover, RIF induction was completely blocked by 50 µM SFN (Fig. 1B, C). Dose-response analysis revealed that the IC_{50}

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for SFN inhibition of 10 μM RIF-induced CYP3A4 promoter activity was about 12 μM (Fig. 1C). To further confirm that SFN inhibited SXR function, we also transfected HepG2 cells with a GAL4 reporter along with a vector expressing the SXR ligand-binding domain linked to the DNA binding domain of GAL4 (GAL4-SXR). Consistent with the results obtained using the full-length SXR, SFN elicited a similar potency of inhibition of GAL4-SXR activity (Fig. 1D), with an IC_{50} of 14 μM.

To determine whether SFN acts specifically on SXR, we evaluated the ability of SFN to inhibit ligand activation of a number of other nuclear hormone receptors, including mouse PXR (mPXR), rat PXR (rPXR), and human CAR, VDR, PPARα, and PPARγ. SFN (tested at 10 μM concentration) had little, if any, inhibitory effect on ligand activation of any of these other nuclear hormone receptors, nor did it serve as an activating ligand (Fig. 1E). Surprisingly, although 10 μM SFN can efficiently inhibit SXR activity, it had comparatively little inhibitory effect on rodent PXR (mPXR or rPXR) function. This observation is consistent with an in vivo study that found that SFN did not inhibit rat CYP3A gene expression (Hu et al., 2004). These data suggest that SFN is a species-selective antagonist of human SXR function, perhaps analogous to the known species selectivity of RIF as an effective human, but not rodent, SXR/PXR ligand (Blumberg et al., 1998).

SFN can specifically bind to SXR

Because SFN effectively inhibited human SXR activities in transient transfection assays (Fig. 1), we hypothesized that SFN works as an antagonist of SXR. Most natural and synthetic nuclear receptor agonists or antagonists act as ligands by directly binding to the nuclear receptor ligand binding domain (LBD). Thus, we next tested whether SFN can bind directly to purified SXR protein in vitro using a sensitive scintillation proximity ligand-binding assay (Zhou et al., 2004). This assay employed the high-affinity SXR ligand ^{3}H-SR12813 and recombinant histidine-6-tagged-SXR co-

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expressed with the SRC-1 receptor interacting domain. SFN as well as clotrimazole (positive control) displaced ^{3}H-SR12813 from the SXR LBD in a dose dependent manner (Fig. 2). The K_i for SFN binding to SXR was 16 μM, a value in the range of other known SXR ligands (Jones et al., 2000). In addition, the affinity was similar to the value we obtained for inhibition of SXR function in transfection experiments (Fig. 1C and D). We infer from these results that SFN binds specifically to the ligand binding domain of SXR.

SFN inhibits SXR co-activator interactions

In the absence of ligand, many nuclear receptors form a complex with corepressors that inhibit transcriptional activity of the complex through the recruitment of histone deacetylase. When a ligand binds to its nuclear receptor, a conformational change occurs, resulting in dissociation of corepressor and recruitment of coactivator proteins (Glass and Rosenfeld, 2000). Coactivator recruitment is therefore a critical part of nuclear receptor signaling pathways. Several coactivators have been shown to be important for nuclear receptor activation including the steroid receptor coactivator-1 (SRC-1), transcriptional intermediary factor (TIF2), activator of thyroid and retinoic acid receptor (ACTR), and peroxisome proliferator-activated receptor-binding protein (PBP) (Synold et al., 2001). As our data demonstrate that SFN blocks ligand binding to SXR, it was of interest to determine whether SFN also inhibits ligand-induced recruitment of co-activators to SXR. We used the mammalian two-hybrid assay to evaluate whether SFN affects the SXR and co-coactivator interaction. HepG2 cells were transfected with a GAL4 reporter, a vector expressing VP16-SXR, and an expression vector for the GAL4 DNA-binding domain or the GAL4 DNA-binding domain linked to the receptor interaction domains of the indicated coactivators. Consistent with previous reports (Synold et al., 2001), RIF strongly promoted the specific interaction of SRC-1 and PBP (Fig. 3) but,

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as expected, had no significant interaction with ACTR and TIF2 (data not shown). SFN inhibited RIF induced SRC-1 and PBP recruitment to SXR in a dose-dependent manner (Fig. 3) and again there was no interaction with ACTR and TIF2 (data not shown). Thus, although structurally distinct from previously described natural or synthetic ligands, SFN appears to antagonize SXR function via direct binding to SXR, inhibition of ligand binding, and subsequent inhibition of SXR coactivator recruitment, thereby potentially preventing ligand mediated SXR transcriptional activation of SXR-regulated genes in a concentration dependent manner.

SFN inhibits SXR-mediated CYP3A4 expression in LS180 cells and human primary hepatocytes

Given the fact that SXR is a major regulator of CYP3A4 and our observation that SFN is an effective antagonist of human SXR, we evaluated whether SFN modulates SXR-mediated CYP3A4 gene expression in human cells that express CYP3A4. Human intestinal LS180 cells and primary hepatocytes were used for CYP3A4 gene expression analysis. LS180 cells are derived from a human colonic epithelial tumor, and represent one of very few human-derived cell lines that have been demonstrated to have functional SXR and inducible CYP3A4 (Synold et al., 2001; Zhou et al., 2004). LS180 cells and human primary hepatocytes from two different donors were pre-treated with various concentrations of SFN for 24 hrs before addition of 10 μM RIF or RU486. Pretreatment of cells was included in the experimental design to be consistent with previous studies that had demonstrated that SFN pretreatment lowered CYP3A4 mRNA and aflatoxin-DNA adduct formation (Gross-Steinmeyer et al., 2005). Total RNA was isolated 24 hrs later and quantitative RT-PCR (QRT-PCR) was performed to measure CYP3A4 gene expression. As expected, both RIF and RU486 were able to induce CYP3A4 gene expression. RIF was a more potent inducer of CYP3A4 than was RU486,

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consistent with previous reports (Zhou et al., 2006a; Zhou et al., 2004). SFN caused a dose-related reduction in RIF and RU486 mediated induction of CYP3A4 in both primary hepatocytes (Fig. 4A and B) and LS180 cells (Fig.4C). Consistent with the results obtained from transfection experiments (Fig. 1), SFN was able to significantly inhibit both RIF and RU486 induced CYP3A4 expression at a 10 μM concentration, and almost completely blocked CYP3A4 induction at 25 μM. Additional experiments with LS180 cells were conducted in which the 24 hr pretreatment was eliminated, such that cells were only co-treated for 24 hr with SFN and RIF. The results were not significantly different from experiments in which pre-treatment with SFN was utilized (data not shown). Interestingly, SFN also significantly reduced the basal level of CYP3A4 expression in primary hepatocytes, as we observed previously (Gross-Steinmeyer et al., 2005). Furthermore, in LS180 cells, SFN inhibited RIF or RU486 induced expression of MDR1 (ABCB1), a gene that is also regulated by SXR (Fig. 4C). As expected, SFN induced UGT1A1 gene expression, presumably through a SXR-independent and Nrf2-dependent pathway (Basten et al., 2002). SFN significantly induced another Nrf2 target gene, NQO1, in both LS180 cells and human primary hepatocytes (Fig. 4D). The net inductive effect of SFN on UGT1A1 gene expression is intriguing, as the gene is also regulated in part by SXR. Indeed, both RIF and RU486 slightly induced UGT1A1 expression, consistent with previous studies (Xie et al., 2003; Zhou et al., 2004). Thus, it is clear that two distinct pathways are involved in SFN mediated gene regulation: SFN activates Nrf2 signaling pathway and induces ARE target genes such as NQO1 and UGT1A1, but it also acts as an antagonist of SXR, thereby inhibiting SXR-mediated CYP3A4 and MDR1 gene expression. SFN has also been shown to inhibit histone deacetylase(s), which could also contribute to changes in gene expression (Myzak et al., 2006).

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SFN suppresses constitutive and inducible CYP3A4-mediated midazolam (MDZ) clearance in human primary hepatocytes

Midazolam is a commonly used short-acting benzodiazepine that is metabolized mainly to 1'-hydoxymidazolam (1'OH-MDZ) almost exclusively by CYP3A4 (Kronbach et al., 1989). MDZ has been used successfully as an in vivo and in vitro CYP3A4 probe to measure CYP3A4 metabolic activity (Paine et al., 1997). We tested whether SFN suppresses MDZ clearance in one preparation of human primary hepatocytes. Human primary hepatocytes were pre-treated with 10 or 25 μM SFN for 24 hrs before addition of 10 μM RIF. After an additional 24 hrs of incubation with both RIF and SFN, cells were rinsed with PBS and then incubated with 8 μM MDZ for 6 hrs. Supernatant was collected and 1'OH-MDZ concentration was measured by LC-MS. Consistent with CYP3A4 gene expression analysis, RIF effectively induced MDZ clearance about 3-fold, and 25 μM SFN blocked both basal and RIF induced MDZ clearance (Fig. 5). Interestingly, SFN almost completely suppressed RIF induced MDZ clearance at a 10 μM concentration, whereas it decreased RIF induced CYP3A4 gene expression (mRNA) by only 50% at the same concentration.

Previous studies from our laboratory demonstrated that pre-treatment with SFN is required to block CYP-mediated activation of aflatoxin B1 in human hepatocytes (Gross-Steinmeyer et al., 2005), and that SFN is not a direct inhibitor of CYP3A4 catalytic activity, even at concentrations as high as 50 μM (Gross-Steinmeyer, K and Eaton, DL. unpublished observations). Therefore, the inhibition of RIF-induced MDZ clearance by SFN most likely reflects the inhibition of CYP3A4 gene expression rather than CYP3A4 enzyme activity, and quantitative differences in SFN effects most likely reflect a partial discordance in the time-course of change in mRNA and protein synthesis. Although we did not determine the effects of co-treatment only (no pretreatment) of SFN on MDZ activity in human hepatocytes, we would expect qualitatively similar results, and that the additional

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24 hr pre-treatment with SFN prior to RIF administration is not needed to block the effects of RIF on CYP3A4 catalytic activity. However, it is worth noting that 24 hr pre-treatment with 10 μM SFN, followed by 24 hr co-treatment of 10 μM SFN with RIF, abolished the RIF-mediated increase in MDZ activity, but that 48 hrs of 10 μM SFN had a much more limited repressive effect on constitutively expressed CYP3A4 activity. In contrast, SFN at the 25 μM dose level blocked RIF-mediated induction and decreased constitutive activity substantially. Although the reason for this dose-dependent SFN effect is not known with certainty, it suggests the possibility of a more complex mechanism of SXR antagonism that simple competitive binding or a mechanism of constitutive CYP3A4 expression that involves more than SXR activation.

Structural determinants of SXR antagonism by SFN

There are numerous other naturally occurring isothiocyanates present in a variety of cruciferous vegetables. Since SFN is structurally distinct from previously described natural or synthetic ligands of SXR, we tested naturally-derived phytochemicals that represent structural analogs of SFN to elucidate which part of SFN contributes to its antagonistic effect (Fig. 6). The results suggested that the isothiocyanate moiety is critical for antagonism of SXR since the nitrile breakdown product of SFN (SFN-nitrile) had no inhibitory activity at any concentration. Moreover, the methylsulfoxide part of the molecule also plays a role in SXR antagonism. Replacing the methysulfoxide moiety with a phenyl (PEITC) group resulted in a substantial loss of SFN's inhibitory effect; only the highest dose of 25 μM had a statistically significant inhibitory effect. Interestingly, the oxidation state of the methylsulfide moiety seems to be important as well. A fully reduced sulfur (Erucin) had much less inhibitory activity toward SXR function (inhibition only at the highest dose), and the fully oxidized sulfur, Cheirolin, had similar inhibitory effects with SFN at

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high concentrations while it was less potent at low concentration. Similar to Cheirolin, shortening the carbon chain from n-butyl to n-propyl (Iberin) had little effect, although linker length and rotational flexibility may be important for optimal interaction with SXR. Since all of these compounds are natural products found in varying concentrations in cruciferous vegetables, the results may have practical value in addition to helping to elucidate the structure-function relationship for SFN as an SXR antagonist.

Discussion

We have demonstrated that SFN, a naturally occurring dietary isothiocyanate, is an antagonist of SXR and inhibits SXR-mediated CYP3A4 gene expression and, subsequently, catalytic activity. To our knowledge, SFN is the first effective and relatively non-toxic SXR antagonist ever reported. Previous investigators (Synold et al., 2001) identified the natural product ecteinasidin-743 (trabecetedin; ET-743; Yondelis; a marine-derived compound from the ascidian *Ecetinascidia turbinanta*) as an effective antagonist to human SXR. However, this compound is highly cytotoxic and has additional biological effects, including binding to the minor groove of DNA, disorganization of microtubular networks, perturbation of cell cycle, and interference with DNA repair pathways (Kesteren et al., 2003). Because of its cytotoxic properties, ET-743 is being developed as a cancer chemotherapeutic agent, with some success (Jimeno et al., 2004; Zelek et al., 2006). In contrast to ET-743, SFN has been promoted as a non-toxic, putative chemopreventive agent to reduce cancer risk from pro-oxidant carcinogens (Talalay et al., 1995; Lee and Surh, 2005). We have demonstrated previously that SFN provides substantial protection against aflatoxin B1 induced genotoxicity in human primary hepatocytes (Gross-Steinmeyer et al., 2004). It is well established that SFN and other isothiocyanates are effective inducers of phase II detoxification pathways in

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animal models, and that this is thought to be the primary chemopreventive mechanism (Conaway et al., 2002; Fahey and Talalay, 1999; Gao and Talalay, 2004). Consistent with animal studies, gene expression profiling of human primary hepatocytes treated with SFN showed increased mRNA levels of several detoxification enzymes including NQO1 and Glutamate Cysteine Ligase (both GCLM and GCLC) (Fig, 4C and unpublished data). Most chemical carcinogens require CYP enzyme-mediated metabolic activation before exerting their effects (Conaway et al., 2002). Here we show that SFN inhibits SXR transactivation by directly binding to SXR and inhibiting coactivator recruitment. Thus a reduction in SXR-mediated CYP3A4 expression may also contribute to its cancer chemopreventive effects. However, in circumstances where CYP3A4 and/or MDR1, or other SXR-regulated genes, play a significant role in detoxification, SFN-mediated inhibition of basal expression could potentially enhance toxicity. It is interesting to note that the lack of effect of SFN on rodent PXR suggests that this mechanism would not contribute to chemoprevention in rodents.

SXR is expressed at high levels in the liver and intestine where it acts as a xenobiotic sensor that regulates the expression of cytochrome P450 enzymes such as CYP3A4 and CYP2C8; conjugation enzymes such as UGT1A1; and ABC family transporters such as MDR1 and MRP2 (Synold et al., 2001). SXR is, thus, a master regulator of xenobiotic clearance, coordinately controlling steroid and xenobiotic metabolism and transport. Our study showed that SFN inhibits SXR function and the expression of its CYP3A4 target gene at low micromolar concentration. SFN is very abundant in broccoli and especially broccoli sprouts, with a reported concentration of about 10 μmol/gm (Shapiro et al., 2001). In vitro data suggest that SFN is rapidly absorbed by cells, conjugated efficiently with glutathione (GSH) and excreted mainly as the GSH conjugate (Zhang and Callaway, 2002). However, the peak plasma concentration of unconjugated SFN in human subjects who ingest SFN in a “broccoli soup” can reach 4-5 μM (Gasper et al., 2005). Therefore, the

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concentration of SFN we used in our in vitro studies is potentially nutritionally relevant, and certainly achievable in vivo via pharmacological treatments.

Although SFN is structurally unlike any previously identified class of SXR ligands, it can directly bind to SXR and strongly inhibit SXR coactivator recruitment (Fig. 2 and 3). Interestingly, compared with human SXR, SFN has little or no inhibitory effect on mouse or rat PXR at the same concentration (Fig. 1E). It is known that the induction of hepatic P450 enzymes, especially CYP3A, differs across vertebrate species, and interspecies difference in the pharmacology of SXR/PXR has been identified as the basis for much of this difference (Blumberg et al., 1998; LeCluyse, 2001). There are significant differences in the xenobiotic response between humans and rodents and these are completely explained by the structure and pharmacology of SXR and PXR. For example, the antibiotic rifampicin, the anti-diabetic drug troglitazone and the cholesterol-reducing drug SR12813 were found to be effective activators of both human SXR and rabbit PXR, but had little activity on mouse or rat PXR (Jones et al., 2000). In contrast, pregnenolone 16α-carbonitrile (PCN) is a more potent activator of rat and mouse PXR than of human SXR or rabbit PXR (Jones et al., 2000), but SFN has little effect on PCN-mediated activation of rat and/or mouse PXR (Fig (1E). In addition, some highly chlorinated, non-planar polychlorinated biphenyls (PCBs) have been identified as human SXR antagonists, but act as rodent PXR agonists (Tabb et al., 2004). The crystal structure of the SXR LBD suggested which amino acid differences between SXR and PXR contribute to species differences in ligand activation of human SXR and mouse PXR and induction of CYP3A (Watkins et al., 2001). Further characterization of how SFN differentially interacts with human or rodent SXR/PXR ligand binding domains may explain the species-specific effects of SFN. From our experiments with SXR, it is clear that both ends of the molecule are critical for optimal SXR

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antagonism. This suggests bipolar anchoring of the relatively small molecule through hydrogen and possibly disulfide bonding with amino acid residues in the ligand binding domain.

Human CYP3A4 is expressed at high, but variable, levels in liver and small intestine. Large inter-individual differences in hepatic and intestinal CYP3A4 activity contribute to difficulties in safe and effective dosing of narrow therapeutic index CYP3A4 substrates. Genetic differences in CYP3A4 or its regulatory genes have not explained much of this variability. Thus, inter-individual differences in exposure to dietary or endogenous agents that modulate CYP3A4 transcription may contribute to functional CYP3A4 variability (Blumberg et al., 1998). Here we show that SFN decreased constitutive CYP3A4 mRNA levels, in addition to attenuating RIF and RU486 mediated CYP3A4 induction, in human intestinal cells and primary hepatocytes. These results suggest that dietary exposure to SFN and other dietary isothiocyanates from ingestion of cruciferous vegetables (especially broccoli or broccoli sprouts) could potentially contribute to the large inter-individual variability in basal CYP3A4 expression. It is interesting to speculate on the mechanism by which SFN reduces constitutive expression of CYP3A4. One likely possibility is that SFN effectively competes with endogenous ligands of SXR that contribute to constitutive expression. However, it is not yet clear what endogenous ligands contribute to SXR-mediated regulation of constitutive expression of CYP3A4 in human liver or intestine. In addition, in the absence of an SXR agonist, SFN may enhance the interaction between SXR and co-repressors. Further studies are necessary to determine whether inhibition of the effects of endogenous SXR ligand(s) via dietary isothiocyanates might contribute to variation in constitutive human CYP3A4 activity and / or other SXR regulated genes.

Induction of CYP3A4 is a common cause of adverse drug-drug interactions. For example, it has been well documented that administration of RIF significantly induces CYP3A4 expression

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thereby contributing to adverse drug interactions frequently associated with RIF treatment for tuberculosis. In a study of human volunteers, RIF caused a 95% decrease in the AUC (area under the curve) of the plasma concentration-time curve of orally administered MDZ (Niemi et al., 2003). Oral midazolam, triazolam, simvastatin, verapamil and most dihydropyridine calcium channel antagonists are ineffective during RIF treatment. The plasma concentrations of the antimycotics itraconazole and ketoconazole and the HIV protease inhibitors indinavir, nelfinavir and saquinavir, are also greatly reduced by rifampicin, potentially resulting in reduced drug efficacy (Niemi et al., 2003). Indeed, the use of RIF with these HIV protease inhibitors is contraindicated to avoid treatment failures. Rifampicin can also cause acute transplant rejection in patients treated with immunosuppressive drugs, such as cyclosporin (Niemi et al., 2003). Although research on the causes of drug interactions has focused primarily on pharmaceutical agents, numerous examples exist where components of the diet modify CYP activity, particularly CYP3A4. For example, St. John's wort, a widely used herbal antidepressant, is able to interact with a variety of drugs. Hyperforin, the active constituent of St. John's wort, can induce drug metabolism through activation of SXR and induction of CYP3A4 expression (Moore et al., 2000). Our results indicate that SFN, a component of the human diet, is able to antagonize SXR activity and SXR-mediated CYP3A4 expression. Thus, pharmacological doses of SFN have the potential to reduce adverse drug responses that arise through the induction of CYP3A4 and other SXR target genes.

In summary, we have shown that SFN is a selective and effective antagonist of SXR function and drug-induced activation of SXR target genes, including CYP3A4. These findings suggest a complementary mechanism by which ingestion of the naturally occurring phytochemical may reduce the risk of certain cancers through a reduction in CYP3A4-mediated reactive metabolite formation. The data also suggest the potential use of SFN as an adjuvant to prevent CYP3A4 induction and

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accompanying adverse drug-drug interactions in patients receiving chronic therapy with SXR agonists.

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References

Basten GP, Bao Y and Williamson G (2002) Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. *Carcinogenesis* **23**:1399-404.

Beard K (1992) Adverse reactions as a cause of hospital admission in the aged. *Drugs Aging* **2**:356-67.

Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* **12**:3195-205.

Chung FL, Conaway CC, Rao CV and Reddy BS (2000) Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. *Carcinogenesis* **21**:2287-91.

Conaway CC, Yang YM and Chung FL (2002) Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Curr Drug Metab* **3**:233-55.

Dussault I and Forman BM (2002) The nuclear receptor PXR: a master regulator of "homeland" defense. *Crit Rev Eukaryot Gene Expr* **12**:53-64.

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P and Lozniewski A (2002) Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. *Proc Natl Acad Sci U S A* **99**:7610-5.

Fahey JW and Talalay P (1999) Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes. *Food Chem Toxicol* **37**:973-9.

Frydoonfar HR, McGrath DR and Spigelman AD (2004) Sulforaphane inhibits growth of a colon cancer cell line. *Colorectal Dis* **6**:28-31.

Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J and Terce F (2000) Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Res* **60**:1426-33.

Gao X and Talalay P (2004) Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. *Proc Natl Acad Sci U S A* **101**:10446-51.

Gasper AV, Al-Janobi A, Smith JA, Bacon JR, Fortun P, Atherton C, Taylor MA, Hawkey CJ, Barrett DA and Mithen RF (2005) Glutathione S-transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli. *Am J Clin Nutr* **82**:1283-91.

Glass CK and Rosenfeld MG (2000) The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* **14**:121-41.

Gross-Steinmeyer K, Bradley K, Stapleton P, Liu F, Tracy J, Bammler T, Beyer R, Strom S and Eaton D (2004) Effect of six dietary phytochemicals on aflatoxinB1-mediated genotoxicity and gene expression in human hepatocytes and HepG2 cells. *Toxicological Sciences* **80:S1**.

Gross-Steinmeyer K, Liu F, Stapleton P, Tracy J, Bammler T, Storm S and Eaton D (2005) Altered transcriptional regulation of genes involved in aflatoxin genotoxicity by sulforaphane (SFN) and diindolylymethane (DIM). *Toxicological Sciences* **84(S1)**:1495.

Grun F, Venkatesan RN, Tabb MM, Zhou C, Cao J, Hemmati D and Blumberg B (2002) Benzoate X receptors alpha and beta are pharmacologically distinct and do not function as xenobiotic receptors. *J Biol Chem* **277**:43691-7.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39**:1-17.

Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, Soteropoulos P, Tolias P, Hart RP and Kong AN (2004) In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. *J Pharmacol Exp Ther* **310**:263-71.

Jimeno J, Lopez-Martin JA, Ruiz-Casado A, Izquierdo MA, Scheuer PJ, Rinehart K. (2004). Progress in the clinical development of new marine-derived anticancer compounds. *Anticancer Drugs* **15**:321-9.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA and Moore JT (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14**:27-39.

Kliewer SA, Goodwin B and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* **23**:687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73-82.

Kronbach T, Mathys D, Umeno M, Gonzalez FJ and Meyer UA (1989) Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. *Mol Pharmacol* **36**:89-96.

Kushad MM, Brown AF, Kurilich AC, Juvik JA, Klein BP, Wallig MA and Jeffery EH (1999) Variation of glucosinolates in vegetable crops of Brassica oleracea. *J Agric Food Chem* **47**:1541-8.

LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact* **134**:283-9.

Lee JS, Surh YJ (2005). Nrf2 as a novel molecular target for chemoprevention. *Cancer Lett* **224:**171-84.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402-8.

Maheo K, Morel F, Langouet S, Kramer H, Le Ferrec E, Ketterer B and Guillouzo A (1997) Inhibition of cytochromes P-450 and induction of glutathione S-transferases by sulforaphane in primary human and rat hepatocytes. *Cancer Res* **57**:3649-52.

Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL and Kliewer SA (2000) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A* **97**:7500-2.

Murillo G and Mehta RG (2001) Cruciferous vegetables and cancer prevention. *Nutr Cancer* **41**:17-28.

Myzak MC, Dashwood WM, Orner GA, Ho E and Dashwood RH (2006) Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice. *Faseb J* **20**:506-8.

Niemi M, Backman JT, Fromm MF, Neuvonen PJ and Kivisto KT (2003) Pharmacokinetic interactions with rifampicin : clinical relevance. *Clin Pharmacokinet* **42**:819-50.

Paine MF, Khalighi M, Fisher JM, Shen DD, Kunze KL, Marsh CL, Perkins JD and Thummel KE (1997) Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism. *J Pharmacol Exp Ther* **283**:1552-62.

Shapiro TA, Fahey JW, Wade KL, Stephenson KK and Talalay P (2001) Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. *Cancer Epidemiol Biomarkers Prev* **10**:501-8.

Synold TW, Dussault I and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* **7**:584-90.

Tabb MM, Kholodovych V, Grun F, Zhou C, Welsh WJ and Blumberg B (2004) Highly chlorinated PCBs inhibit the human xenobiotic response mediated by the steroid and xenobiotic receptor (SXR). *Environ Health Perspect* **112**:163-9.

Talalay P, Fahey JW, Holtzclaw WD, Prestera T and Zhang Y (1995) Chemoprotection against cancer by phase 2 enzyme induction. *Toxicol Lett* **82-83**:173-9.

van Kesteren Ch, de Vooght MM, Lopez-Lazaro L, Mathot RA, Schellens JH, Jimeno JM, Beijnen JH (2003). Yondelis (trabectedin, ET-743): the development of an anticancer agent of marine origin. *Anticancer Drugs* **14**:487-502.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA and Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* **292**:2329-33.

Wrighton SA and Thummel KE (2000) CYP3A, in *Metabolic Drug Interactions* pp 115-134, Lippincott Williams & Wilkens, Philadelphia.

Xie W, Yeuh MF, Radominska-Pandya A, Saini SP, Negishi Y, Bottroff BS, Cabrera GY, Tukey RH and Evans RM (2003) Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proc Natl Acad Sci U S A* **100**:4150-5.

Zelek L, Yovine A, Brain E, Turpin F, Taamma A, Riofrio M, Spielmann M, Jimeno J, Misset JL (2006). A phase II study of Yondelis (trabectedin, ET-743) as a 24-h continuous intravenous infusion in pretreated advanced breast cancer. *Br J Cancer* **94**:1610-4.

Zhang Y and Callaway EC (2002) High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. *Biochem J* **364**:301-7.

Zhang Y, Kensler TW, Cho CG, Posner GH and Talalay P (1994) Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc Natl Acad Sci U S A* **91**:3147-50.

Zhang Y, Talalay P, Cho CG and Posner GH (1992) A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci U S A* **89**:2399-403.

Zhou C, Assem M, Tay JC, Watkins PB, Blumberg B, Schuetz EG and Thummel KE (2006a) Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia. *J Clin Invest* **116**:1703-12.

Zhou C, Tabb MM, Nelson EL, Grun F, Verma S, Sadatrafiei A, Lin M, Mallick S, Forman BM, Thummel KE and Blumberg B (2006b) Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* **116**:2280-9.

Zhou C, Tabb MM, Sadatrafiei A, Grun F and Blumberg B (2004) Tocotrienols activate the steroid and xenobiotic receptor, SXR, and selectively regulate expression of its target genes. *Drug Metab Dispos* **32**:1075-82.

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Figure legends:

Fig. 1. SFN efficiently inhibits SXR activity.

(A) Structure of SFN (4-methylsulfinylbutyl isothiocyanate). (B) HepG2 cells were transiently transfected with full-length SXR together with a CYP3A4-luc reporter and CMX-β-galactosidase transfection control plasmid. After transfection, cells were treated with control medium or medium containing 10 μM RIF or 10 μM RU486 in the absence or presence of SFN at the indicated concentrations for 24 h. Results were presented as relative luciferase units (R.L.U.) normalized to the β-galactosidase internal control. (C) HepG2 cells were transiently transfected as described above. Cells were treated with 10 μM RIF with indicated concentrations of SFN for 24 h. (D) HepG2 cells were transiently transfected with GAL4-SXR, a GAL4 reporter fused to luciferase and CMX-β-galactosidase transfection control plasmid. Cells were then treated with 10 μM RIF and SFN at the indicated concentrations for 24 h. (E) HepG2 cells were cotransfected with GAL4 reporter and a series of GAL4 constructs in which the GAL4 DNA binding domain is linked to the indicated nuclear hormone receptor ligand binding domain. Cells were treated with the appropriated ligand or ligand plus SFN (10 μM). The ligands used were mouse PXR (10 μM PCN); rat PXR (10 μM PCN); VDR (10 nM $1,25(OH)_2D_3$); CAR (1μM CITCO). PPARα (10 μM WY-14643); PPARγ (10 μM Troglitazone); and RXR (100 nM 9-cis-retinoic acid). Results in the presence of SFN are presented as percent activation relative to the normalized luciferase values in the presence of ligands (100%). Statistically significant expression compared with control group (-SFN) respectively is marked with asterisks: **P*<0.05, ***P*<0.01, and ****P*<0.001 (n=3, Student's t test).

Fig. 2. SFN specifically binds to the purified SXR ligand binding domain.

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His_6-SXR LBD was co-expressed with the SRC-1 receptor interaction domain and purified. The receptor complex was bound to nickel chelate FlashPlates and incubated with 50 nM of ^{3}H-SR12813 in the presence of indicated concentration of SFN or clotrimazole. Values represent the average of triplicates ±S.E.M.

Fig. 3. SFN inhibits SXR co-activator interactions.

HepG2 cells were transfected with a GAL4 reporter and VP16-SXR as well as expression vector for GAL4 DNA-binding domain or GAL4 DNA-binding domain linked to the receptor interaction domains of the indicated SXR co-activators (GAL-SRC1 and GAL-PBP). Cells were then treated with control medium or medium containing 10 μM RIF in the presence or absence of SFN at indicated concentrations. Statistically significant expression compared with control group (-SFN) is marked with asterisks: ****P*<0.001 (n=3, Student's t test).

Fig. 4. SFN inhibits SXR-mediated CYP3A4 expression in human primary hepatocytes and LS180 cells

Human primary hepatocytes from two different donors (A, and B) or LS180 intestinal epithelial cells (C) were pre-treated 24h with 10 or 25 μM SFN before addition of 10 μM RIF or RU486 for 24 h as indicated. (D) Human primary hepatocytes and LS180 cells were treated with 10 or 25 μM SFN for 48h. Total RNA from each sample was isolated and the expression of indicated genes (panels A-D) was determined by QRT-PCR. Statistically significant expression compared with control group (-SFN) is marked with asterisks: **P*<0.05, ***P*<0.01, and ****P*<0.001 (n=3, Student's t test).

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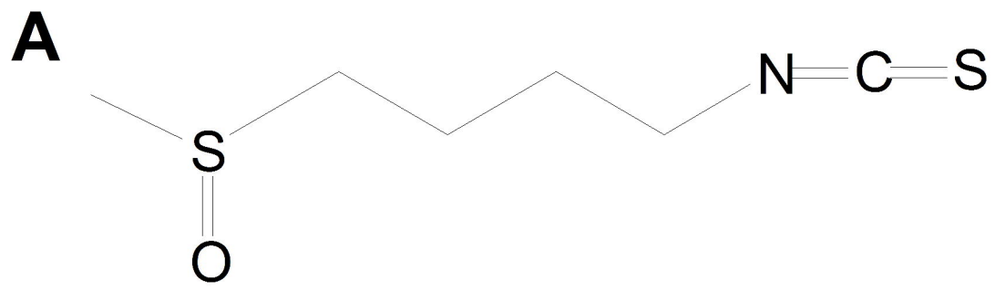
Fig. 5. SFN inhibits CYP3A4-mediated midazolam (MDZ) clearance in human primary hepatocytes.

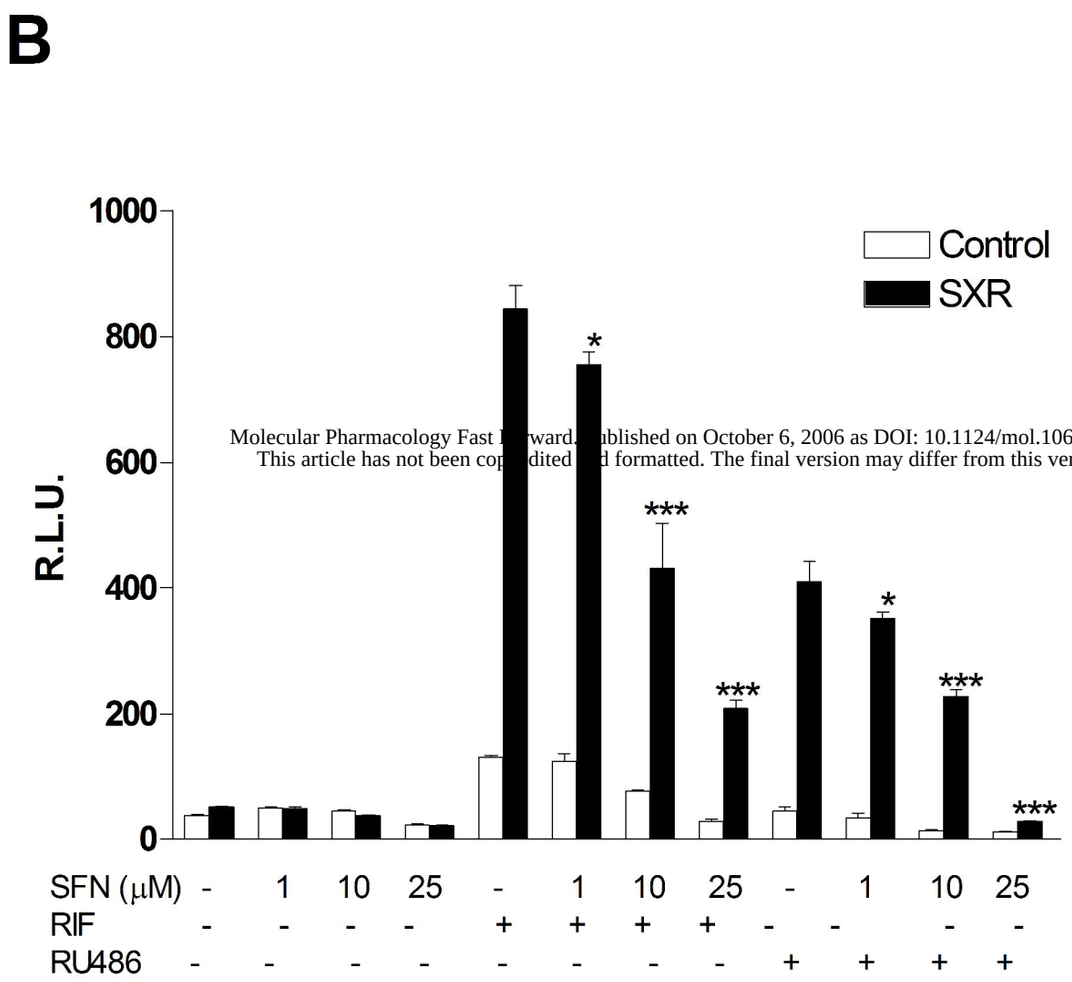
Human primary hepatocytes were pre-treated with 10 or 25 μM of SFN for 24 hrs before addition of 10 μM RIF. After 24 hrs, cells were rinsed with PBS and then incubated with 8 μM MDZ for 6 hrs. Supernatant media was collected and 1'OH-MDZ concentration was measured by LC-MS. Data represent the mean +/- S.E.M. of duplicate analyses of a single hepatocytes preparation.

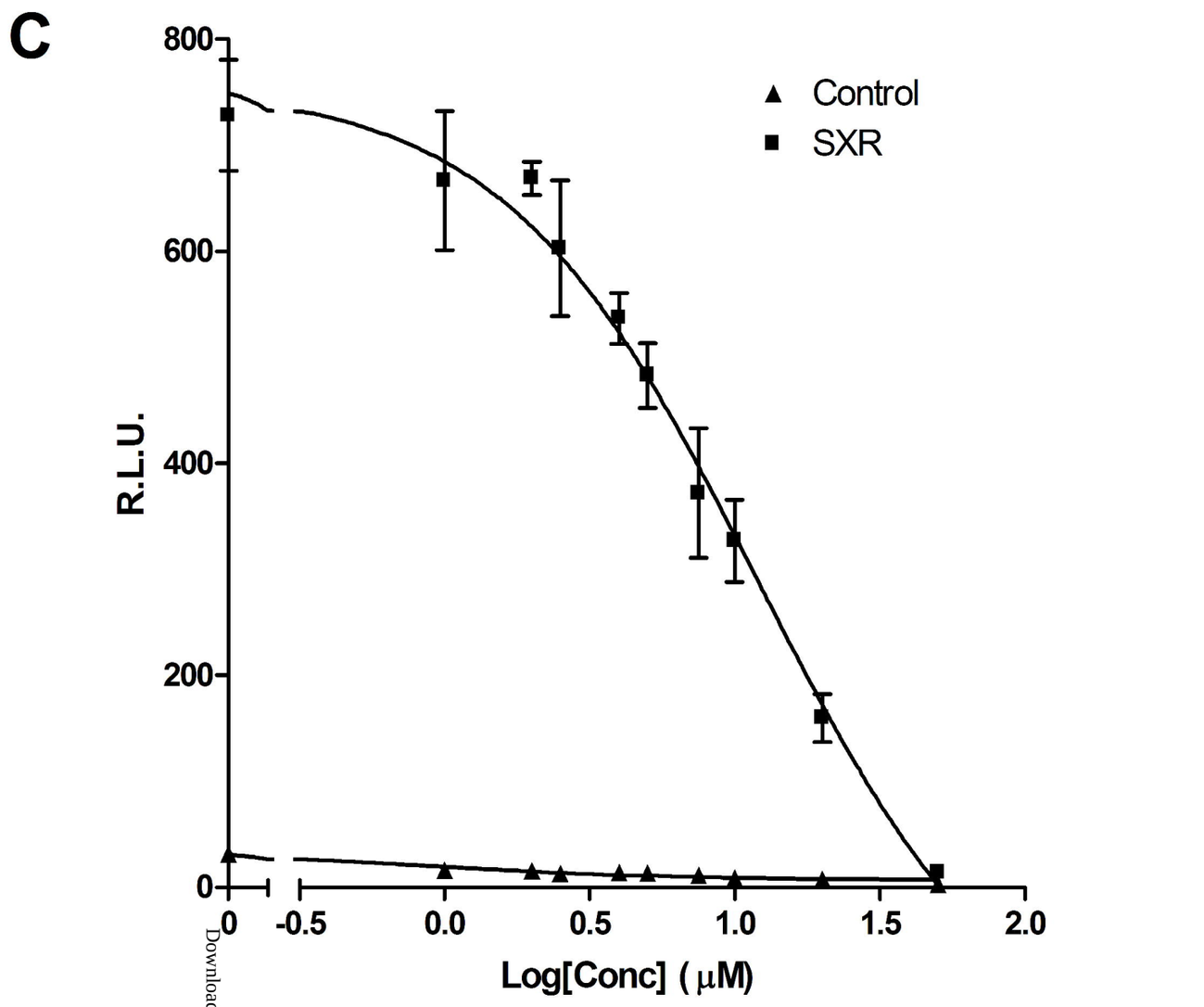
Fig.6. Structural determinants of SXR antagonism by SFN.

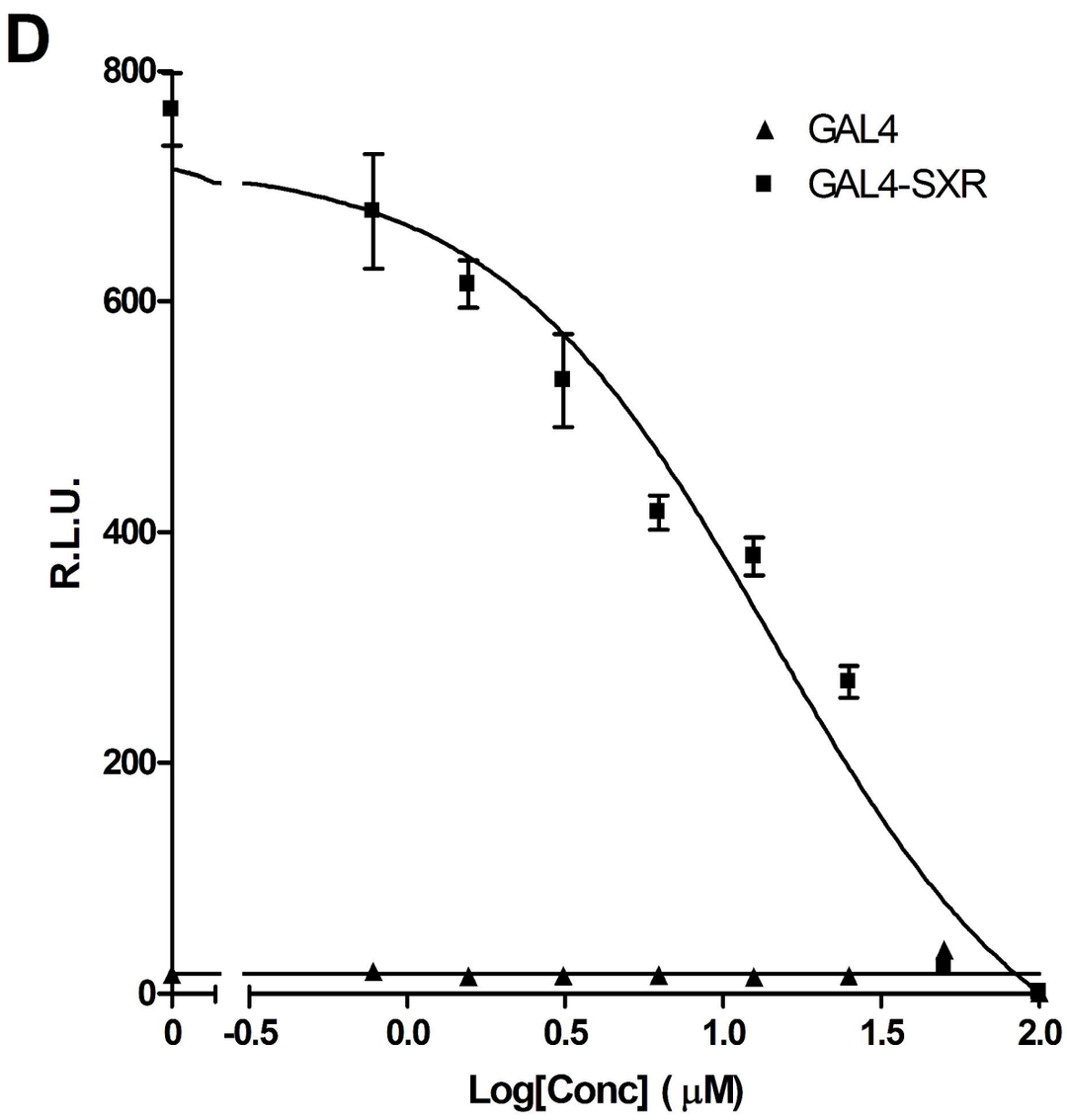
HepG2 cells were transiently transfected with full-length SXR together with a CYP3A4-luc reporter and CMX-β-galactosidase transfection control plasmid. After transfection, cells were treated with control medium or medium containing 10 μM RIF in the absence or presence of SFN analogs at the indicated concentrations for 24 h. Each bar represents the mean =/- S.E.M. of 3 experiments. A one way ANOVA was performed for each treatment group. When statistical significance was found, each SFN dose was compared to the control using Dunnett's t-test for multiple comparisons to a control. *, $p<0.05$ and **, $p< 0.01$.

Figure 1









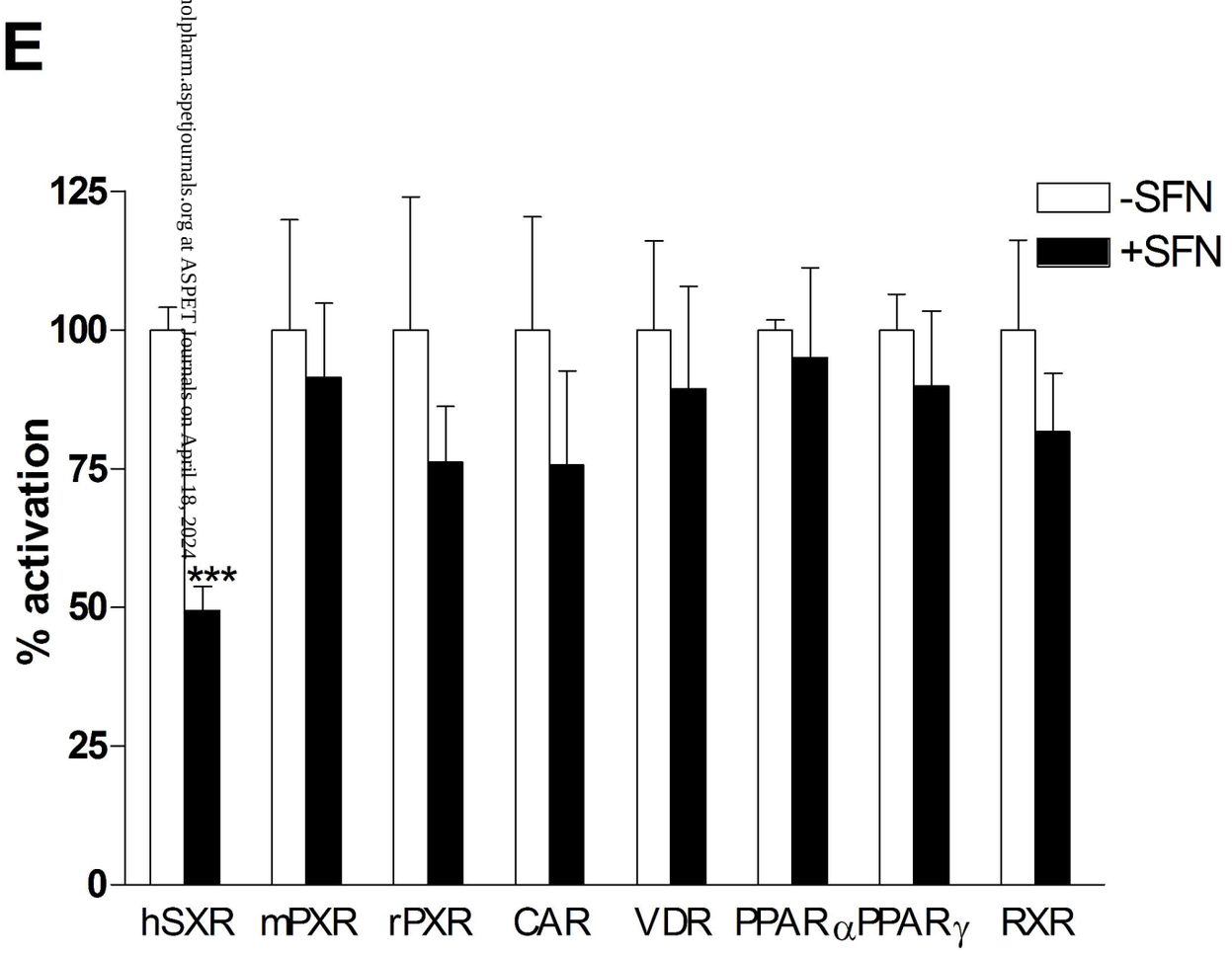


Figure 2

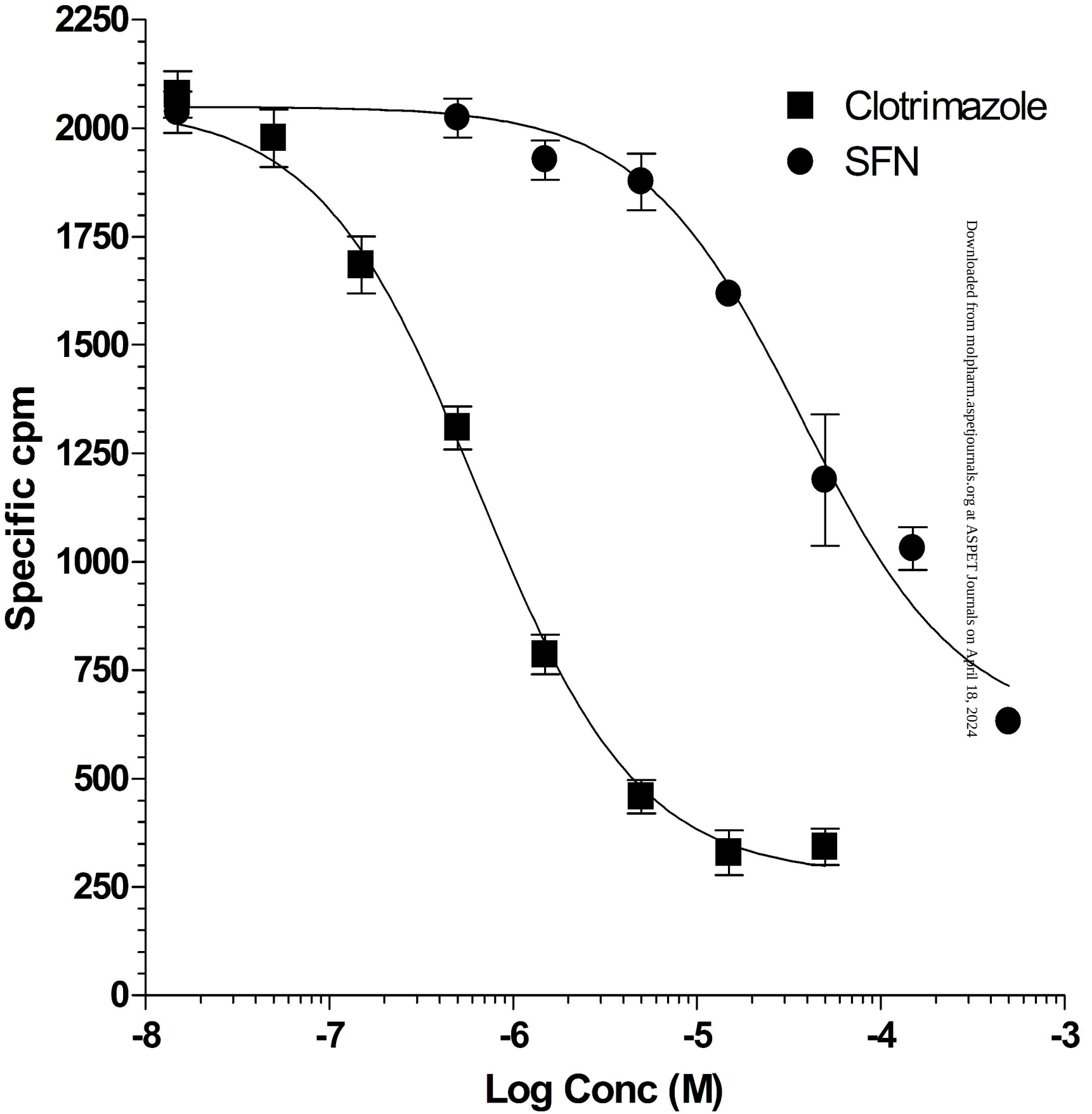


Figure 3

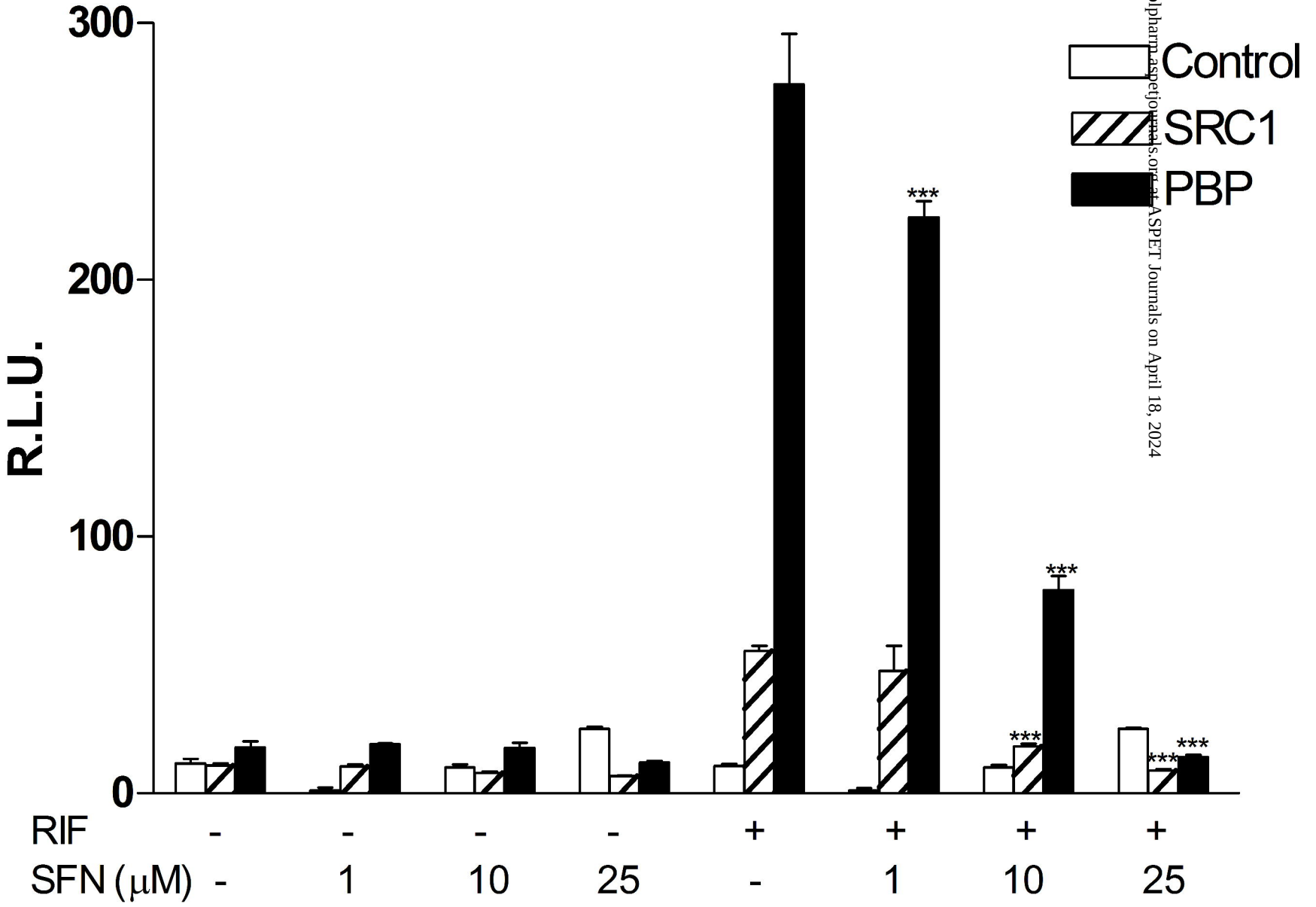
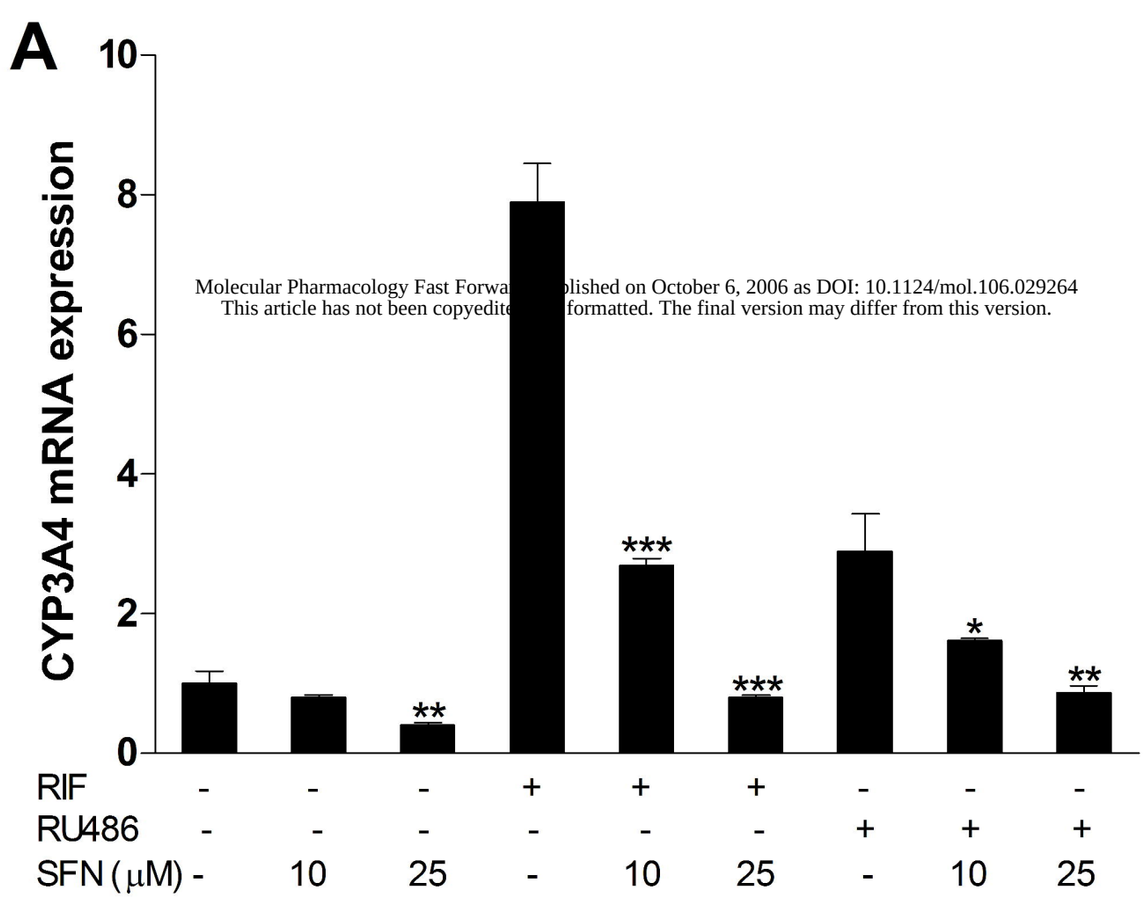
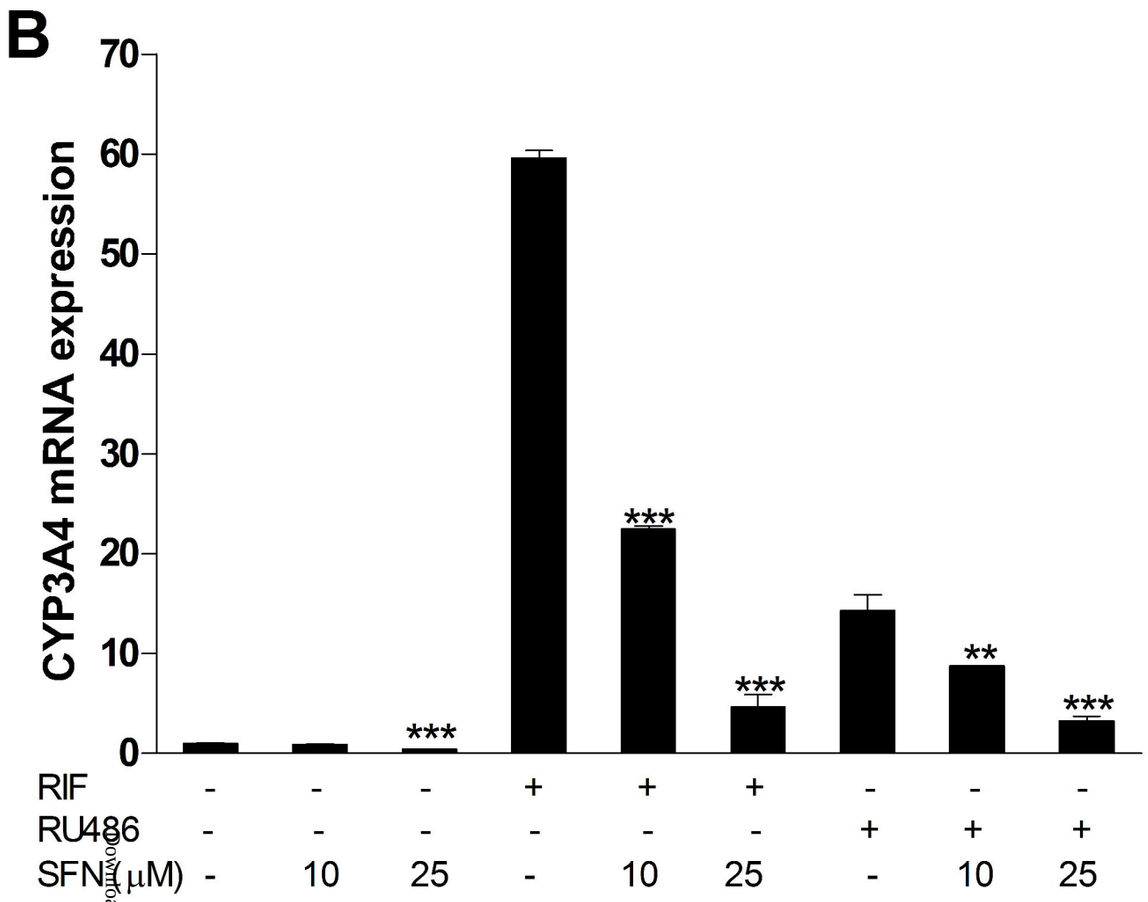
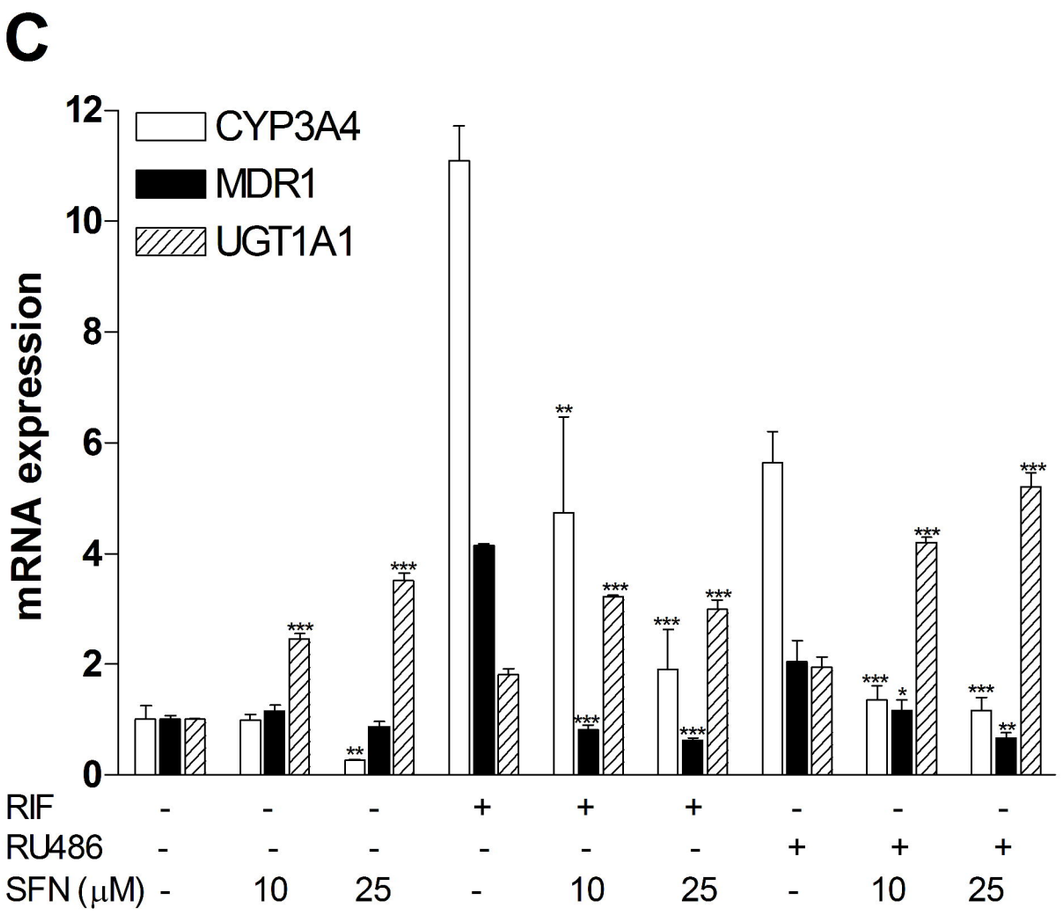


Figure 4







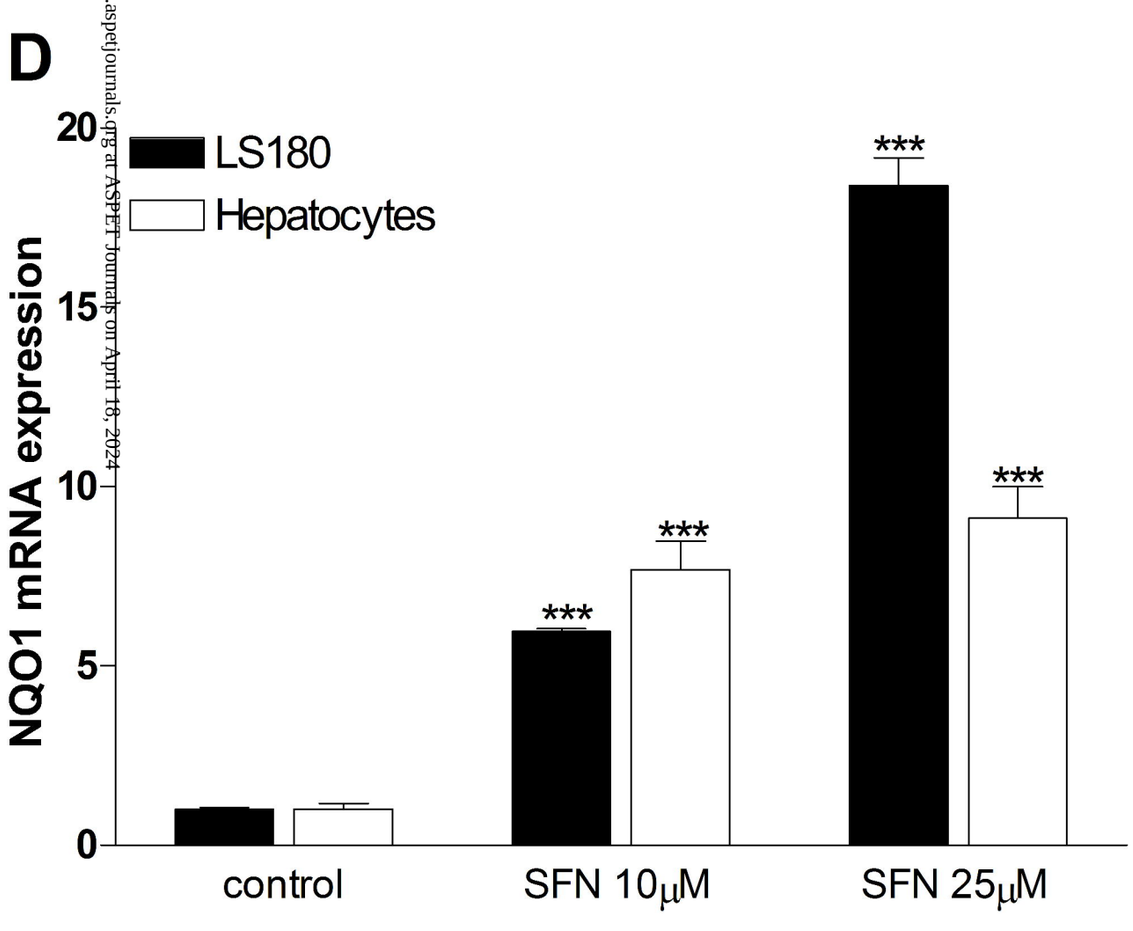
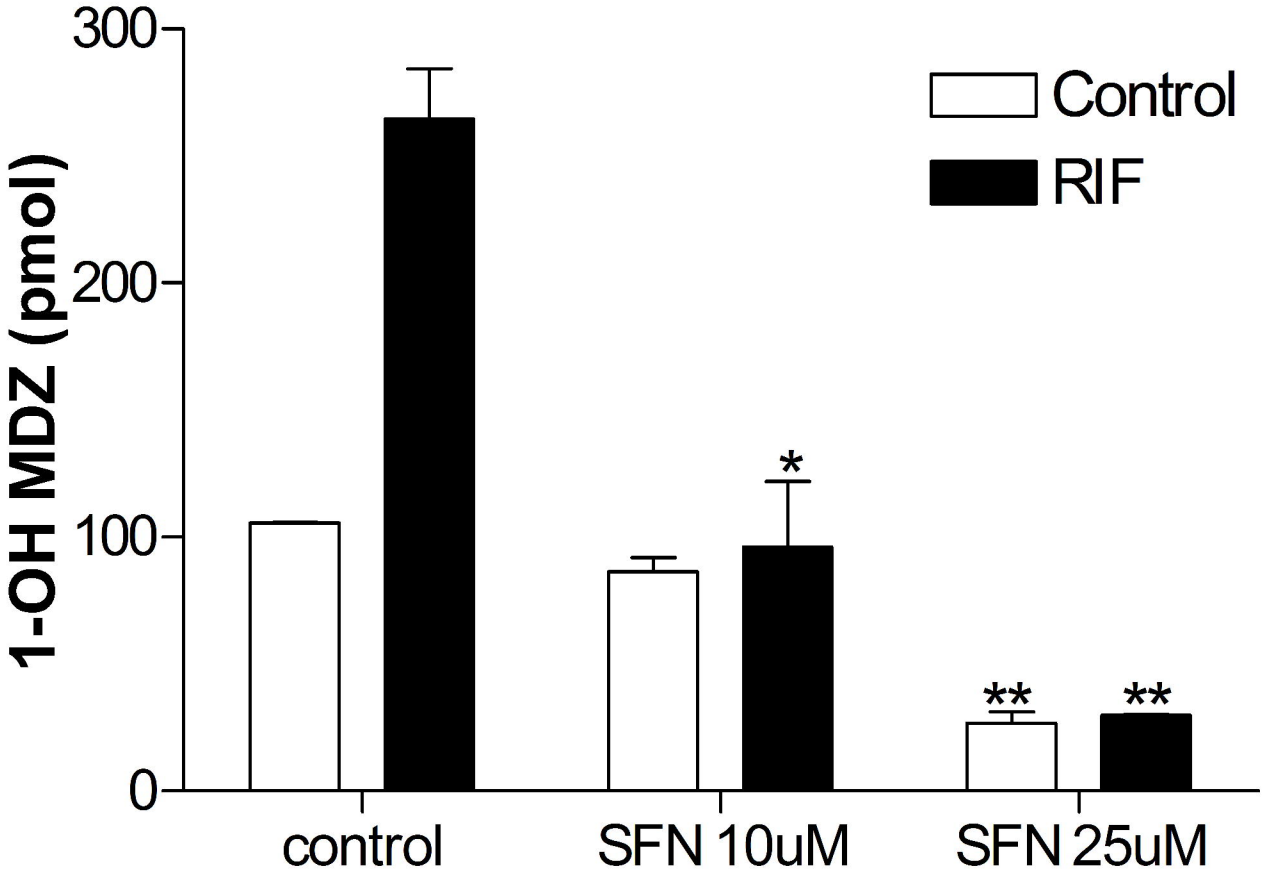


Figure 5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200
100
0
1-OH MDZ (pmol)
Control
RIF
*
**
**
control
SFN 10uM
SFN 25uM

Figure 6