Peptide Mimetic HIV Protease Inhibitors Are Ligands for the Orphan Receptor SXR*

Received for publication, July 3, 2001, and in revised form, July 19, 2001
Published, JBC Papers in Press, July 20, 2001, DOI 10.1074/jbc.C100075200

Isabelle Dussault‡, Min Lin‡, Kevin Hollister‡, Eric H. Wang‡, Timothy W. Synold§, and Barry Marc Forman¶

From the §Division of Molecular Medicine, The Gonda Diabetes and Genetic Research Center and the §Department of Medical Oncology and Therapeutics Research, Beckman Research Institute, City of Hope National Medical Center, Duarte, California 91010

The orphan nuclear receptor SXR coordinately regulates drug clearance in response to a wide variety of xenobiotic compounds. This signaling system protects the body from exposure to toxic compounds; however, it can also pose a severe barrier to drug therapy. We now demonstrate that the human immunodeficiency virus (HIV) protease inhibitor ritonavir binds SXR and activates its target genes. This represents an example of a commonly used therapeutic agent that effectively activates SXR. We also show that other protease inhibitors are weaker (saquinavir) or unable to activate SXR (nelfinavir, indinavir) thus defining analogs that fail to induce SXR-regulated clearance pathways. Interestingly, HIV protease inhibitors are distinct from previously known SXR ligands in that they are peptide mimetic compounds. This expands the ligand specificity of SXR to include this unique chemical class whose pharmacological significance is expanding. Finally, we show that SXR ligands activate expression of multiple resistance protein 2, a critical regulator of bile flow and biliary drug excretion. These findings have important implications for the role of SXR in regulating drug clearance and hepatic disorders associated with impaired bile flow.

HIV protease inhibitors have been a powerful tool in extending the lives of people infected with HIV. Although these drugs directly target the HIV protease, their in vivo efficacy can be limited by efficient drug clearance pathways (1). HIV protease inhibitors are administered orally, and their intestinal uptake is reduced by P-glycoprotein (ABCB1), a broad-specificity efflux pump that is the product of the MDR1 gene (2). Any protease inhibitor that escapes P-glycoprotein-mediated efflux enters the portal blood where it can ultimately be degraded by the hepatic cytochrome P450 CYP3A4. HIV protease inhibitors may also serve as substrates for MRP2 (ABCC2) (3, 4), an export pump expressed on the bile canalicular membrane (5). It is thus possible that protease inhibitors may also be cleared by MRP2-dependent biliary secretion. Optimization of HIV treatment requires a thorough understanding of how protease inhibitors interact with and regulate these xenobiotic clearance pathways.

Recent studies have demonstrated that the orphan nuclear receptor, SXR (also known as PXR, PAR, PRR, NR1I2), plays a central role in regulating CYP3A4 and MDR1 transcription (6). SXR is activated by a diverse array of pharmaceutical agents including taxol, rifampicin, SR12813, clotrimazole, phenobarbital, and hyperforin (7–10). These studies indicate that SXR is a xenobiotic sensor that coordinately regulates drug clearance in the liver and intestine. Indeed, gene knockout studies have confirmed a role for SXR in regulating xenobiotic accumulation (11, 12). However, most of the drugs that are known to activate SXR are not commonly used clinically, thus it remains unclear whether SXR activation represents a widespread pharmacologic problem.

In this report we show that SXR activates expression of MRP2. This extends the repertoire of SXR target genes to include those involved in biliary drug excretion. We also demonstrate that certain HIV protease inhibitors bind to and activate SXR at pharmacologically relevant concentrations. Previously known ligands for SXR and other nuclear receptors are derived from steroids, lipids, or traditional small molecule compounds. As HIV protease inhibitors are peptide mimetic compounds, our findings widen the diversity of SXR ligands to include this unique chemical class of pharmaceutical agents. Thus, the pharmacologic implications of SXR action are much broader than originally imagined.

MATERIALS AND METHODS

Plasmids and Reagents—A cytomegalovirus expression vector was used to express the following proteins: Gal-SXR (human SXR ligand binding domain, GenBank‡ accession number AF031814); Ser443), Gal-PBP (human PBP, AF283812; Val 574-Ser649), and VP-SXR (human SXR ligand binding domain; Lys107-Ser443). Gal-PXR containing the ligand binding domain of mouse PXR (AF031814) was obtained from Bruce Blumberg. Gal4 fusions contained the indicated fragments fused to the C-terminal end of the yeast Gal4 DNA binding domain; VP16 fusions contained the 78-amino acid herpesvirus VP16 activation domain. All other constructs were as described previously (6). Ritonavir was obtained from Moravek Biochemicals (Brea, CA); saquinavir, nelfinavir, and indinavir were obtained from the NIH AIDS Research and Reference Reagent Program; rifampicin and pregnenolone-16α-carbonitrile were obtained from Sigma.

Transient Transfection Assays—CV-1 cells were grown and transfected as described (6).

Northern Blot Analysis—Primary human hepatocytes were obtained from Clonetics (Walkersville, MD) and cultured according to the vendor instructions. Cells were treated with the indicated compounds for 24 h, and total RNA was isolated using the Trizol reagent. Human LS180 cells were maintained in Eagle’s minimum Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM l-glutamine, non-essential amino acids, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. One day prior to treatment LS180 cells were switched to phenol red-free medium containing 10% resin charcoal-stripped fetal bovine serum and then treated for 24 h with the indicated compounds. Northern blots were prepared from total RNA
and analyzed with the following probes: MRP2 (GenBank™ accession number NM_000392; nt 3960–4441), MDR1 (NM_000927; nt 843–1111), CYP2C8 (NM_000770; nt 700–888), CYP3A4 (M18807; nt 1521–2058), and GAPDH (NM_002046; nt 101–331). Note that the CYP2C8 probe was specific as it did not cross-hybridize to the two most closely related members of the CYP2C family, CYP2C9 and CYP2C19 (data not shown).

**Ligand Binding Assays**—The human SXR ligand binding domain was expressed in *Escherichia coli* with an N-terminal His tag and purified. For binding assays 0.25 μg of His-tagged SXR was added per well of a 96-well nickel chelate flash plate (PerkinElmer Life Sciences) and incubated at room temperature for 30 min in binding buffer (50 mM Tris, pH 8, 50 mM KCl, 1 mM CHAPS, 0.1 mg/ml bovine serum albumin, and 0.1 mM dithiothreitol). After 30 min the well was washed three times with binding buffer, and 37.5 nM [3H]SR12813 (7) was added in 100 μl of binding buffer. Where indicated, unlabeled competitor ligands were added, and the incubation was continued for 75 min at room temperature with shaking. Readings were taken using a Topcount scintillation counter (Packard, Meriden, CT).

**RESULTS AND DISCUSSION**

To identify whether SXR regulated other genes involved in xenobiotic clearance, a number of candidate genes were analyzed for their ability to respond to SXR ligands (data not shown). As seen in Fig. 1A, several SXR ligands including hyperforin, rifampicin, and SR12813 induced expression of MRP2 in primary human hepatocytes. MRP2 (ABCC2, cMOAT), a member of the ATP binding cassette transporter family is a multispecific organic anion transporter expressed on the hepatocyte bile canalicular membrane (5). This protein is responsible for the biliary excretion of a number of drug metabolites. Interestingly, HIV protease inhibitors serve as substrates for MRP2 (3, 4), as well as for other SXR target genes including CYP3A4 and P-glycoprotein (1).

Our previous studies with taxol demonstrated that this drug is both a CYP3A4/P-glycoprotein substrate and an activator of SXR (6). This overlap in specificity among SXR and its target genes prompted us to explore the possibility that HIV protease inhibitors may also serve as SXR ligands. CV-1 cells were transfected with a Gal4 reporter along with a vector expressing the ligand binding domain of human SXR linked to the DNA binding domain of yeast Gal4 (Gal-SXR). As expected, this chimeric receptor was activated by SXR ligands such as hyperforin and rifampicin, but not by PCN, a specific agonist of PXR, the mouse ortholog of SXR (13) (Fig. 1B). Surprisingly, the HIV protease inhibitor ritonavir also activated SXR. Dose response analysis (Fig. 1C) indicated that activation by ritonavir occurred at clinically relevant concentrations (EC50 ~ 2 μM). In contrast, the same doses of ritonavir were less effective at activating PXR and failed to activate RXR (Fig. 1C), the heterodimeric partner of SXR. Activation of SXR by ritonavir was specific as this compound had no effect on other nuclear receptors including mouse PXR, estrogen receptor, TGR, vitamin D receptor, TR, retinoic acid receptor, RXR, retinoid X receptor, LXR, liver X receptor, FXR, PPARα, peroxisome proliferator-activated receptor, CAR, constitutive androstene receptor, and/or P-glycoprotein and MRP2-mediated excretion. Thus, we examined the ability of other HIV protease inhibitors to activate SXR. As indicated in Fig. 2A, ritonavir and indinavir (5 μM) both activated Gal-SXR, but other protease inhibitors including saquinavir, nelfinavir, and indinavir failed to activate at concentrations as high as 10 μM. However, at 30 μM saquinavir activated SXR 7-fold whereas nelfinavir and indinavir remained inactive (data not shown). Thus, the hierarchy of response of SXR to HIV protease inhibitors is ritonavir > saquinavir > nelfinavir and indinavir. None of the protease inhibitors were effective activators of PXR, the mouse ortholog of SXR (Fig. 2B). The inability of nelfinavir and indinavir to activate SXR (“SXR-transparent” drugs) suggests that these protease inhibitors may be less likely to promote drug-drug interactions or to autoinduce their own clearance.

Given the potential utility in generating SXR-transparent drugs, we sought to delineate the molecular mechanism underlying the SXR-transparent activity of nelfinavir and indinavir. Ligands for nuclear hormone receptors activate transcription by promoting receptor association with coactivator proteins including members of the p160 family (SRC-1, ACTR, GRIP) and PBP (DRIP205, TRAP220) (16). We utilized a mammalian two-hybrid assay to compare the effects of HIV protease inhibitors on coactivator recruitment. CV-1 cells were transiently transfected with a Gal4 reporter construct, a vector expressing a VP16-SXR ligand binding domain fusion (VP-SXR), and an expression vector for the Gal4 DNA binding domain or Gal4 linked to the coactivator interaction domain of PBP (Gal-PBP). In this system, reporter expression is activated if the herpes-
Ritonavir is the most potent HIV protease inhibitor to bind to and activate SXR. A, activation of human SXR by various HIV protease inhibitors. CV-1 cells were transiently transfected with Gal-SXR as in Fig. 1B above. After transfection, cells were treated with control medium or medium containing 10 μM rifampicin, 5 μM ritonavir, or 10 μM saquinavir, nelfinavir, or indinavir. Fold activation was determined relative to untreated cells. Note that at 30 μM saquinavir activated SXR 7-fold whereas nelfinavir and indinavir were inactive at any concentration tested (data not shown). B, HIV protease inhibitors fail to activate mouse PXR. CV-1 cells were transiently transfected with Gal-PXR as in Fig. 1B above. After transfection, cells were treated with control medium or medium containing 10 μM PCN, 5 μM ritonavir, and 10 μM saquinavir, nelfinavir, and indinavir. Note that saquinavir, nelfinavir, and indinavir remained inactive at any concentration tested up to 30 μM (data not shown). C, coactivator recruitment by various HIV protease inhibitors SXR. CV-1 cells were transiently transfected with a Gal4 reporter and an expression vector containing the VP16 transactivation domain linked to the ligand binding domain of SXR (VP-SXR). In addition, cells were also transfected with expression vectors for the Gal4 DNA binding domain linked to the receptor interaction domains human PBP. After transfection, cells were treated with control medium or medium containing 10 μM rifampicin, 5 μM ritonavir, or 10 μM saquinavir, nelfinavir, or indinavir. Similar effects were seen with other coactivators (data not shown), and no recruitment was seen with a Gal4 control. D, ritonavir binds directly to SXR. Bacterially expressed SXR was incubated with 37.5 nM [3H]SR12813 in the absence or presence of 5 μM (data not shown). Error bars indicate the range of duplicate data points. All experiments were repeated three or more times with similar results.

Fig. 3. Ritonavir stimulates SXR target gene expression. A, ritonavir activates expression of SXR target genes in hepatocytes. Primary human hepatocytes were treated with control medium (None) or medium containing 10 μM rifampicin, 2.5 μM hyperforin, or 3 μM ritonavir. After a 24-h exposure to the compounds, total RNA was prepared and analyzed by Northern analysis using probes for CYP3A4, CYP2C8, MRP2, and the GAPDH control. B, ritonavir activates expression of SXR target genes in intestinal cells. LS180 cells were treated with control medium (None) or medium containing 10 μM rifampicin, 2.5 μM hyperforin, or 3 μM ritonavir. After a 24-h exposure to the compounds, total RNA was prepared and analyzed by Northern analysis using probes for MDR1 and the GAPDH control. All experiments were repeated three or more times with similar results.

Previous studies have demonstrated that SXR is a xenobiotic sensor that activates expression of CYP3A4, CYP2C8, and MDR1 (6–12, 17). SXR is thus a master regulator of xenobiotic clearance that coordinates metabolic clearance in the liver and absorption in the intestine. Like its target genes, SXR displays a broad specificity for a variety of xenobiotic compounds including rifampicin, SR12813, clotrimazole, phenobarbital, and the progestin/glucocorticoid antagonist RU486 (7–10). This broad specificity facilitates a form of chemical immunity that allows the body to detect and then remove foreign compounds. It has been suggested that this broad specificity could limit the pharmacologic activity of potentially large numbers of therapeutic agents. However, the most effective SXR agonists described to date are either not widely used in the clinic (e.g. rifampicin, SR12813, phenobarbital) or fail to activate SXR at pharmacologically relevant concentrations (clotrimazole, RU486). We now demonstrate that a commonly used HIV protease inhibitor, ritonavir, is an agonist ligand for SXR. HIV protease inhibitors are members of a chemical class known...
as peptide mimetics. These compounds are designed to simulate the structure of active protein moieties and thus are appealing substrates for future drug discovery. Our demonstration that peptide mimetics can activate SXR has widespread implications for discovery efforts involving peptide mimetic agents.

We find that ritonavir binds and activates SXR with an EC_{50} of ~2 μM. This concentration is pharmacologically relevant as it matches both the circulating and 90% effective concentrations of this drug (18, 19). Thus, activation of SXR is an unavoidable side effect of reaching effective antiviral concentrations. This implies that SXR target genes such as CYP3A4, CYP2C8, and MDR1 will be up-regulated in patients taking this drug. Induction of MDR1 is worrisome as HIV protease inhibitors are administered orally, and their intestinal uptake is limited by P-glycoprotein (1). Similarly, protease inhibitors are metabolized by CYP3A4 (1), so induction of this enzyme could result in autoinduced metabolism in the liver. These mechanisms are likely to underlie the clinical observation that ritonavir induces its own metabolism during the first 2 weeks of therapy (19).

HIV-infected individuals are often simultaneously treated with multiple drugs to limit viral loads and/or to treat associated infections. Drugs that activate SXR can be particularly problematic in such situations. For example, protease inhibitors are often administered with azidothymidine, a reverse transcriptase inhibitor that can be metabolized by CYP2C8/9 (20). We show that ritonavir can enhance expression of CYP2C8, which in turn could metabolize and inactivate azidothymidine. Our findings may help to predict and thus limit potentially dangerous drug interactions that are associated with ritonavir.

Because HIV protease inhibitors can interact with SXR, CYP3A4, and P-glycoprotein, their overall effects on drug clearance will be determined by their relative potency to activate SXR versus their ability to competitively inhibit target genes such as CYP3A4 and P-glycoprotein. To minimize adverse drug-drug interactions, it will be necessary to identify protease inhibitors that fail to modulate the activities of both SXR and its target genes. We demonstrate that analogs such as nelfinavir and indinavir do not bind SXR. This provides further “proof-of-principle” that SXR can be used as a tool to identify drugs that fail to induce SXR-regulated pathways. As has been described with other compounds, we find that human SXR and its mouse ortholog (PXR) display distinct responses to peptide mimetic protease inhibitors. This indicates that rodent models cannot be used to accurately predict the pharmacokinetic properties of HIV protease inhibitors.

Finally, we found that MRP2 mRNA is induced within 24 h of exposure to a variety of SXR ligands. It is not known whether this represents a direct effect mediated by an SXR response element. Whatever the mechanism, it is clear that MRP2 is a downstream target of SXR activation. MRP2 is a multispecific organic anion transporter expressed on the hepatocyte bile canalicular membrane and is responsible for the biliary excretion of a number of drug metabolites. The ability of SXR to activate this gene implies that SXR can regulate biliary excretion of xenobiotic compounds and their conjugates. Thus, SXR appears to coordinately regulate multiple drug clearance pathways including intestinal uptake (P-glycoprotein), hepatic metabolism (CYP3A4 and CYP2C8), and biliary excretion. MRP2 has also been implicated in the biliary secretion of bile acid sulfates, bilirubin conjugates, and glutathione (5). Once in the bile, the tripeptide glutathione can be hydrolyzed into its component amino acids. This produces an increase in osmotic pressure, which is a driving force for enhanced bile flow. Our demonstration of a link between SXR and MRP2 provides a molecular basis to account for the 25-year-old observation that bile flow is enhanced by compounds now known to activate PXR (21, 22). Taken together, these findings raise the possibility that SXR agonists may be useful for treating cholestatic liver disease, i.e. hepatic disorders resulting from impaired bile flow.

REFERENCES
1. Piscitelli, S. C., and Gallicano, K. D. (2001) *N. Engl. J. Med.* 344, 984–996
2. Ambukar, S. V., Dey, S., Hrynevych, C. A., Ramachandra, M., Pastan, I., and Gottstein, M. M. (1999) *Annu. Rev. Pharmacol. Toxicol.* 39, 361–398
3. Miller, D. S., Nohmann, S. N., Gutmann, H., Toeroek, M., Drew, J., and Fricker, G. (2000) *Mol. Pharmacol.* 58, 1357–1367
4. Gutmann, H., Fricker, G., Drew, J., Toeroek, M., and Miller, D. S. (1999) *Mol. Pharmacol.* 56, 383–389
5. Keppeler, D., and Konig, J. (2000) *Semin. Liver Dis.* 20, 265–272
6. Synold, T. W., Dussault, I., and Forman, B. M. (2001) *Nat. Med.* 7, 584–590
7. Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeChuyse, E. L., Lambert, M. H., Willson, T. M., Kliwer, S. A., and Moore, J. T. (2000) *Mol. Endocrinol.* 14, 27–39
8. Moore, L. B., Goodwin, B., Jones, S. A., Wisely, G. B., Serabjit-Singh, C. J., Willson, T. M., Collins, J. L., and Kliwer, S. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 75.00
9. Wentworth, J. M., Agustini, M., Love, J., Schwabe, J. W., and Chatterjee, V. K. (2000) *J. Endocrinol.* 166, R1–R6
10. Blumberg, E., Sabbaah, W. Jr., Jaugilun, H., Belado, J., Jr., van Meter, C. M., Ong, E. S., and Evans, R. M. (1998) *Genes Dev.* 12, 3195–3205
11. Xie, W., Barbwick, J. L., Downes, M., Blumberg, B., Simon, C. M., Nelson, J. M., Neuschwander-Tetri, B. A., Brunet, E. M., Guzelian, P. S., and Evans, R. M. (2000) *Nature* 406, 435–439
12. Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Kliwer, B. H., and Kliwer, S. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 3369–3374
13. Kliwer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H., Perlmann, T., and Lehmann, J. M. (1998) *Cell* 92, 73–82
14. Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seel, W., Evans, R. M., and Moore, D. D. (1998) *Nature* 395, 612–615
15. Moore, L. B., Parks, D. J., Jones, S. A., Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Willson, T. M., Collins, J. L., and Kliwer, S. A. (2000) *J. Biol. Chem.* 275, 15121–15127
16. Glass, C. K., and Rosenfeld, M. G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 1357–1367
17. Geick, A., Eichelbaum, M., and Burk, O. (2001) *J. Biol. Chem.* 276, 14581–14587
18. van Heewijk, R. P., Veldkamp, A. I., Mulder, J. W., Meinhorst, P. L., Lange, J. M., Beijnen, J. H., and Hoetelmans, R. M. (2000) *AIDS* 14, 103–110
19. Hsu, A., Granneman, G. R., Witt, G., Locke, C., Denissen, J., Molla, A., Valdes, J., Smith, J., Erdman, K., Lyons, N., Niu, P., Decourt, J. P., Fourtillan, J. B., Girault, J., and Leonard, J. M. (1997) *Antimicrob. Agents Chemother.* 41, 898–905
20. Pan-Zhou, X. R., Cretton-Scott, E., Zhou, X. J., Yang, M. X., Lasker, J. M., and Sommadossi, J. P. (1998) *Biochem. Pharmacol.* 55, 757–768
21. von Bergmann, K., Schwarz, H. P., and Puamgurtner, G. (1975) *Naunyn Schmiedebers Arch. Pharmacol.* 287, 33–45
22. Zaimgrand, G., and Solymos, B. (1974) *Proc. Soc. Exp. Biol. Med.* 145, 631–635
Peptide Mimetic HIV Protease Inhibitors Are Ligands for the Orphan Receptor SXR
Isabelle Dussault, Min Lin, Kevin Hollister, Eric H. Wang, Timothy W. Synold and Barry Marc Forman

J. Biol. Chem. 2001, 276:33309-33312.
doi: 10.1074/jbc.C100375200 originally published online July 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100375200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 9 of which can be accessed free at http://www.jbc.org/content/276/36/33309.full.html#ref-list-1