The statin class of HMG CoA reductase inhibitors demonstrate differential activation of the nuclear receptors PXR, CAR and FXR, as well as their downstream target genes.

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Abbreviations: CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; FXR, farnesoid X-receptor; LDL, low-density lipoprotein; PXR, Pregnane X-receptor.

Abstract

1. The therapeutic class of HMG-CoA reductase inhibitors, the statins, are central agents in the treatment of hypercholesterolemia, and the associated conditions of cardiovascular disease, obesity and metabolic syndrome. Whereas statin-therapy is generally considered safe a number of known adverse effects do occur, most commonly treatment associated-muscular pain.
2. In vitro evidence also supports the potential for drug-drug interactions involving this class of agents, and to examine this a ligand-binding assay was used to determine the ability of six clinically used statins for their ability to directly activate the nuclear receptors PXR, FXR and CAR, demonstrating a relative activation of PXR>FXR>CAR.
3. Using reporter gene constructs, we demonstrate this order of activation is mirrored at the transcriptional activation level, with PXR-mediated gene activation being pre-eminent. Finally, we describe a novel regulatory loop, whereby activation of FXR by statins increases PXR reporter gene expression, potentially enhancing PXR-mediated responses.
4. Delineating the molecular interactions of statins with nuclear receptors is an important step in understanding the full biological consequences of statin exposure. This demonstration of their ability to directly activate nuclear receptors, leading to nuclear receptor cross-talk, has important potential implications for their use within a polypharmacy paradigm.

Introduction

The HMG CoA reductase inhibitors, or statins, are front-line drugs for the treatment of hypercholestraemia, reducing mortality and morbidity associated with cardiovascular disease. They achieve their therapeutic action through the inhibition of HMG-CoA reductase, the rate limiting enzyme in the cholesterol biosynthesis pathway (Dietschy and Wilson 1970). This reduction in cholesterol synthesis is accompanied by an up-regulation of LDL receptors on hepatocytes, resulting in increased LDL uptake, and thus cholesterol, from the systemic circulation. The combined effect of reducing de novo synthesis and the up-regulation of LDL receptors leads to a typical reduction in LDL cholesterol of 20-35% (Linsel-Nitschke and Tall 2005), although rosuvastatin, the most recently marketed compound in the statin class, has been reported to cause up to a 63% decrease (Olsson et al. 2001). Lipid lowering impacts positively on the stability of atherosclerotic plaques in blood vessels, probably by reducing plaque size/lipid composition, and as plaque rupture is a major cause of cardiovascular disease, this stabilisation reduces cardiovascular disease by around 30-35% (Linsel-Nitschke and Tall 2005).

Simvastatin, lovastatin and pravastatin, which are all fungal-derived compounds, share very similar structures, although additional hydroxyl groups make pravastatin more hydrophilic than either simvastatin or lovastatin. By comparison, fluvastatin, pitavastatin, cerivastatin, atorvastatin and rosuvastatin are synthetically derived compounds, again sharing structural similarities, such as a common fluoride side group (Figure 1). In general, statins are administered in their active form, and are readily absorbed by passive diffusion. However, both simvastatin and lovastatin are administered as inactive lactone prodrugs, which are lipophilic, and hydrolysed to the

active β -hydroxyacid by intracellular esterases and paraoxonases following absorption (Billeclke et al. 2000).

An important functional difference within the statin family is their mode of metabolism: The majority of statins undergo cytochrome P450-mediated metabolism, predominantly through CYP3A4, while the increased hydrophilicity of pravastatin and rosuvastatin reduces the requirement for this, with the parent compound being efficiently excreted. The net result of this is altered pharmacokinetics for pravastatin and rosuvastatin compared to the remaining chemicals in this class (Everett et al. 1991): For example, upwards of 54 % of pravastatin is eliminated from the body as the parent compound, considerably higher than 20 % typically observed with other, more lipophilic, statins (Everett et al. 1991; Lennernas and Fager 1997).

In general, statins are well tolerated, although treatment-dependent muscular pain is a relatively common side effect. In general this side effect can be mitigated through dose reduction (Sirvent et al. 2008), although in some cases it can progress to severe muscular toxicity (rhabdomyolysis), which requires cessation of treatment (Vaklavas et al. 2009). The probability of such adverse effects may be increased through alterations in statin pharmacokinetics caused via drug-drug interactions, with CYP3A4-metabolised statins being particularly susceptible victim drugs (Neuvonen et al. 2006). In addition, it has been reported that statins have the potential to induce CYP3A4 (El-Sankary et al. 2001) and ABCB1 (Yamasaki et al. 2009) expression levels in vitro, and that this effect may extend to in vivo (Watanabe et al. 2004), suggesting that statins may act as perpetrators for drug-drug interactions as well as victims. Such interactions are presumed to occur through the activation of members of the nuclear receptor superfamily, which act as ligand-activated transcription factors regulating many aspects

of cellular metabolism (Plant and Aouabdi 2009). Indeed, several statins have been identified as potential agonists for the pregnane X-receptor (PXR; NR1I2) (Raucy et al. 2002; Wagner et al. 2005), the nuclear receptor that regulates CYP3A4 and ABCB1 gene expression (Plant 2007), as well as the constitutive androstane receptor (CAR; NR1I3 (Kobayashi et al. 2005)). In addition, equivocal evidence exists to suggest that statins may activate other nuclear receptors, such as the farnesoid X-receptor (FXR; NR1H4): This interaction may occur either through direct agonism of FXR or through the pharmacological action of statins in altering bile acid levels, which act as ligands for FXR (Habeos et al. 2005; Rizzo et al. 2005).

Herein, we use a ligand-binding assay to undertake the first comprehensive analysis of the potential for statins to activate the nuclear receptors PXR, its closely related paralogue the constitutive androstane receptor (CAR; NR1I3) and FXR. In addition, we link the activation of these nuclear receptors with activation of classical target genes for PXR, CAR and FXR, demonstrating that activation is both nuclear receptor- and statin-specific. Finally, we present the novel finding that activation of a PXR reporter gene is regulated by FXR, demonstrating a feed forward pathway that is important in priming PXR-mediated responses to statins.

Materials and Methods

Reagents: Fugene-6 transfection reagent was purchased from Roche Diagnostics, Lewes, UK. Unless otherwise stated all other chemicals were of molecular biology grade and obtained from Sigma Chemical Co. (Poole, UK). The Huh7 human hepatocellular carcinoma cell line (Nakabayashi et al. 1982) was a kind gift from Dr Steve Hood (GSK, Ware, UK). All cells were routinely cultured in 75 cm² vented tissue culture flasks (Nunc,

UK) using minimal essential medium with Earle's salts supplemented with 1 % non-essential amino acids, 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin and 10 % foetal bovine serum. All cell culture medium and supplements were purchased from Invitrogen (Paisley, UK). In order to maintain phenotypic consistency, Huh7 cells were only used for three weeks (approximately 6 passages) following recovery from liquid nitrogen. Expression plasmids for nuclear receptors were kindly provided as follows: PXR, Dr Kliewer (University of Texas, Dallas, USA); CAR, Prof M. Negishi (NIEHS, USA); FXR, Dr Swales (University of Surrey, UK). The IR6 reporter gene plasmid was also a kind gift of Dr Swales. The SLC01B1 expression plasmid was constructed as follows: The coding sequence for SLC01B1 (positions 120-2246 relative to the transcription start site) was amplified from Huh7-derived cDNA using a high fidelity polymerase (PrimeSTAR, Cambrex, UK). This product was cloned into the pTriEx1.1 expression plasmid (Clontech, UK) and sequenced to ensure 100% identity to the published sequence,

In silico analysis: Ligand docking was undertaken using Autodock 4 suite and Autodock Tools for Windows (Morris et al. 2009). Molecular coordinates for the crystal structure of PXR with rifampicin bound (Chrencik et al. 2005) were obtained from the RCSB protein databank (Accession: 1SKX; <http://www.rcsb.org/pdb/home/home.do>), whereas three dimensional structures of statins were designed using Corina (<http://www.molecular-networks.com/products/corina>). Docking was undertaken using a 0.375Å grid centred upon the ligand-binding pocket of PXR, using a genetic algorithm with the following parameters: 150 iterations; 100 population size; 1E+6 energy evaluations; 1E+5 maximum generations. Local search parameters were for 500

runs, with a maximum 3000 iterations. The output from each docking procedure was analysed using the Autodock Tools software.

Reporter gene assay: Reporter gene assays were undertaken as described in Aouabdi et al (Aouabdi et al. 2006). Briefly, Huh7 cells were seeded into 96-well plates (Nunc International, Leicestershire, UK) at a concentration of 10,000 cells/well and incubated at 37 °C for 24 hrs in a humidified container for attachment. FuGENE 6-mediated DNA co-transfections, using 75ng/well reporter gene constructs and 25ng/well over-expression plasmid as appropriate, were performed as described previously (Goodwin et al. 1999), using serum-free medium for the six-hour transfection period; this was then replaced with fresh, complete medium, containing charcoal-stripped serum, for the remaining culture period.

Transfections were allowed to proceed for 48 hours, in the presence or absence of xenobiotic as indicated, and then secretory alkaline phosphatase (SEAP) activity determined. Aliquots of cell culture medium (25 µl/well) were transferred into 96-well optiplates (Canberra Packard, UK). Endogenous alkaline phosphatase activity was deactivated by heat-treatment of the medium at 65 °C for 30 minutes. SEAP activity was then assayed using the AURORA system (ICN, Thame, UK), according to the manufacturer's protocol. Chemiluminescent output was measured using a LumiCount automated plate reader (Canberra Packard, UK). SEAP activity following 48 hours culture was calculated for both reporter constructs and blank, control, plasmid, and a fold induction relative to vehicle control calculated.

Nuclear Receptor Activation Assay. Nuclear reporter activation assays were undertaken using a modification of the Checkmate mammalian two-hybrid system

(Promega, UK). Initially, an extended ligand binding region for human PXR, CAR and FXR, representing amino acids 141-434, 162-348 and 250-469 respectively, was sub-cloned into the pBIND expression plasmid, thus encoding a fusion construct with the GAL4-DBD. Next, Huh7 cells were seeded into 96-well plates (Nunc International, Leicestershire, UK) at a concentration of 20,000 cells/well and incubated at 37 °C for 24 hrs. FuGENE 6-mediated DNA co-transfections, using 25ng/well luciferase reporter plasmid (pG5luc) and 12.5ng/well NR-Gal4 fusion plasmid (pBIND-NR), using serum-free medium for the six-hour transfection period; this was then replaced with fresh, complete medium, containing charcoal-stripped serum, for the remaining culture period.

Transfections were allowed to proceed for 24 hours, in the presence or absence of xenobiotic as indicated, and then luciferase activity determined using the Dual-Luciferase reporter system (Promega, UK) according to the manufacturer's protocol. Luminescent output was measured using a LumiCount automated plate reader (Canberra Packard, UK).

Transcript level measurement: Primers and TAMRA/FAM dual labelled probe specific for SLC01B1 and 18S rRNA were designed using the Primer Express software (Applied Biosystems, Warrington, UK) and purchased from Eurofins (Wolverhampton, UK). Following knockdown and over-expression experiments, total RNA was isolated from quadruplicate samples using the RNeasy Mini kit (Qiagen) and quantified using a Nanodrop Agilent 2100 Bioanalyser.

Total RNA was treated with RNase-free DNase (Promega, Southampton, UK) to remove genomic contamination. Reverse transcription was primed with random hexamers and

carried out by Superscript II (Invitrogen) as per the manufacturer's instructions. To ensure that DNase treated samples were free from genomic contamination an RT-control (lacking enzyme) was carried out for every RNA sample. cDNA generated from 50ng (SLC01B1) or 50 pg (18S rRNA) of total RNA was amplified using TaqMan Universal PCR Master Mix with 400 nM primers and 200 nM fluorogenic probe in a total reaction volume of 25 µl. Q-PCR reactions were run on the ABI7000 SDS instrument and quantitation was carried out using the ABI proprietary software against a standard curve generated from human genomic DNA (Promega).

Protein Level Measurement

Plasma membrane protein extracts (10 µg per lane) were resolved on 12 % SDS-polyacrylamide gels and then transferred electrophoretically to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, Bucks, UK). Membranes were blocked (1 hour) in 5 % fat free dried milk and then probed with primary antibodies against SLC01B1 (sc-18433; 1:200; Autogen Bioclear, Calne, Wilts, UK) or β -actin (sc1616, 1:500; Autogen Bioclear) for one hour, followed by anti-goat (sc-2022; 1:20000) IgG for one hour (Autogen Bioclear). Bound antibodies were visualised using enhanced chemiluminescence reagents according to the manufactures instructions (Amersham Biosciences), and opposed to Fuji Super RX film (Fisher Scientific).

Results and Discussion

Differential activation of the nuclear receptors PXR, CAR and FXR by statins.

Activation of CYP3A expression levels has been previously demonstrated for the statin class of chemicals, with this being enhanced by over-expression of the nuclear receptor

PXR (El-Sankary et al. 2001). However, both pravastatin and rosuvastatin are reported to be unable to induce CYP3A4 reporter gene activity, consistent with their lack of requirement for CYP3A-mediated metabolism prior to elimination. The reported lack of activation of CYP3A by rosuvastatin and pravastatin is consistent with these statins being poor agonists for PXR, despite their close structural similarity to other statins, although this has not been clearly demonstrated. To examine this, an *in silico* docking approach was undertaken, using Autodock to determine Gibbs energy for the most likely conformations of pravastatin, lovastatin and simvastatin docking into the PXR ligand binding domain. As expected, both lovastatin and simvastatin were able to dock efficiently into the ligand binding domain of PXR, with mean Gibbs energy values of -11.5 kJ and -9.5 kJ for their most common conformations. Unexpectedly, pravastatin was also able to efficiently dock into the ligand binding domain of PXR, with a mean Gibbs energy for the most common conformation of -11 kJ, which is highly similar to that seen for both lovastatin and simvastatin. In addition, Figure 2A clearly demonstrates that the orientation of pravastatin allows acceptor/donor atoms to localise within 2 Å of amino acids within the ligand binding pocket of PXR known to be central to ligand docking: Suitable within of Ser247 (1.85 Å), Gln285 (1.75 Å) and His407 (2.12 Å) (Ekins and Erickson 2002).

As *in silico* analysis was not inconsistent with pravastatin being a ligand for PXR, despite its apparent lack of efficacy as a CYP3A inducer, we next examined this interaction *in vitro*. A fusion construct of the PXR LBD, plus activation domain, and the GAL4 DBD was generated and used to determine the ability of multiple statins to act as agonists against PXR. In addition, we examined for interaction of statins with CAR, a paralogue of PXR in higher mammals (Maglich et al. 2003) and FXR, another nuclear

receptor that has been implicated in statin-mediated responses (Habeos et al. 2005; Handschin and Meyer 2005; Rizzo et al. 2005). As can be seen from figure 2b, atorvastatin, fluvastatin, simvastatin (acid only) and lovastatin (both lactone and acid forms) are all able to elicit a statistically significant activation of PXR in a dose-dependent manner. Activity of the lactone pro-drug may represent either real activity of this chemical form, or conversion to the pharmacologically active β -hydroxysteroid within the assay system. For the hydrophilic statins pravastatin and rosuvastatin, activation of PXR was less marked, although a significant, dose dependent, activation of PXR was observed with pravastatin (Figure 2a), consistent with the *in silico* docking.

In general, statins were poor agonists of CAR, with significant activation of the ligand-binding construct only occurring at the highest dose studied, if at all (Figure 2b): Only lovastatin (β -hydroxyacid) and rosuvastatin produced statistically significant, activations of the CAR ligand-binding assay, and then only at the highest dose tested (10 μ M), suggesting that this class of compounds are, generally, poor activators of CAR.

Statin-mediated activation of the FXR fusion construct was observed with several of the tested statins, namely fluvastatin, lovastatin (acid), pravastatin and rosuvastatin; however, this activation was generally of lower potency than observed against PXR (Figure 2b). It is of note that both rosuvastatin and pravastatin were able to elicit significant activation of FXR within this system, again suggesting that these more hydrophilic statins may be nuclear receptor ligands.

Statins demonstrate differential PXR-mediated target gene activation.

CYP3A4 is a major drug metabolising enzyme in human liver, and is predominantly under the control of PXR, although other nuclear receptors may also influence the

transcription rate. It would hence be logical that those statins that are PXR agonists would also be activators of CYP3A4 transcription. To examine this, a reporter gene construct of the CYP3A4 regulatory regions (both proximal promoter and XREM) was transfected into the human hepatoma cell line Huh7, along with an expression plasmid for PXR, and challenged with a variety of statins, as well as the classical PXR and CAR agonists rifampicin and CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) respectively. As can be seen from Figure 3, dose-dependent activation of the CYP3A4 reporter gene expression following exposure of Huh7 cells to atorvastatin, fluvastatin, lovastatin and simvastatin was demonstrated. In the case of lovastatin and simvastatin this activation was greater for the lactone pro-drug compared to the active β -hydroxyacid, presumably reflecting increased cellular access of the lactone form, with subsequent activation representing either direct activity of this lactone, or the β -hydroxyacid formed following absorption of the lactone prodrug. No activation of the CYP3A4 reporter gene was evident in cells exposed to pravastatin or rosuvastatin. This difference between the ligand-activation and reporter gene assay results could be as a result of differential sensitivity between the two assays, or reflect that the agonism of PXR by pravastatin observed within the ligand binding assay was insufficient to elicit a transcriptional response of PXR-target genes. This transcriptional response was shown to be PXR-dependent, as lack of co-transfection with the PXR expression plasmid led to an attenuated response (data not shown and (El-Sankary et al. 2001)). The derived EC₅₀ and I_{max} values for each of the statins against CYP3A4 reporter gene activation are presented in table 1. These data compare well to those previously reported, with statins showing I_{max} values in excess of the classical CYP3A4 inducing agent rifampicin (El-Sankary et al. 2001), suggesting that they are

potent activators of CYP3A4 expression in vitro. All dose response curves were fitted with a variable slope model, and the associated Hill slopes are given in table 1: These values are generally greater than one, suggesting co-operativity in the activation of the CYP3A4 reporter gene, which is perhaps not surprising given the multi-step nature of the induction pathway being measured herein, and could reflect nuclear receptor interactions.

Whereas PXR is the main nuclear receptor responsible for transcriptional regulation of CYP3A4, other nuclear receptors are able to influence this control, either through binding to unique response elements (e.g. HNF4), or through response element sharing (e.g. CAR, VDR). The potent human-specific CAR agonist CITCO was able to activate CYP3A4 reporter gene expression, demonstrating that CAR-mediated regulation of CYP3A4 gene expression can occur in Huh7 cells in vitro (Figure 3). In comparison to the observed PXR-mediated effects on CYP3A4 transcription, only minimal CAR-mediated activation of the CYP3A4 reporter gene by statins was observed. A two-fold activation by atorvastatin, simvastatin (lactone) and lovastatin (lactone) was observed at the top dose examined (50 μ M), although these did not reach significance, whilst no increase in reporter gene expression was observed following exposure to simvastatin (acid), lovastatin (acid), pravastatin and rosuvastatin. Such data is consistent the findings of Kobayashi et al., who used a PBREM-based reporter construct, and observed a maximal two fold increase in reporter gene assay following exposure of HepG2 cells to 30 μ M simvastatin, atorvastatin and fluvastatin, but no increase elicited by pravastatin (Kobayashi et al. 2005). Interestingly, in the same paper, PXR-mediated activations of a XREM reporter gene construct were, on average, an order of magnitude higher than

those seen with the CAR reporter construct, which is again consistent with the findings observed herein.

Whereas the majority of statins possess a high degree of lipophilicity ($\log D_{7.0} > 1.5$), both pravastatin ($\log D_{7.0} = 0.5$) and rosuvastatin ($\log D_{7.5} = 0.5$) are relatively hydrophilic, resulting in the requirement for SLCO-mediated uptake into hepatocytes (Hirano et al. 2005; Holdgate et al. 2003; Ishigami et al. 2001): It is hence possible that the lack of activation of the CYP3A4 reporter gene by rosuvastatin and pravastatin may be due to lack of compound access. To examine this possibility, we modulated expression of SLCO1B1 (Figure 4), the main uptake transporter for pravastatin and rosuvastatin in hepatocytes (Ho et al. 2006; Nakai et al. 2001), and re-examined the impact of these statins on CYP3A4 reporter gene expression. Transfection of Huh7 cells with an expression plasmid encoding SLCO1B1 resulted in a statistically significant increase in SLCO1B1 at both the transcript and plasma membrane protein levels (figure 4a), consistent with the production of increased SLCO1B1 in the membrane, although this was not demonstrated to be functional. Increased levels of membrane-localised SLCO1B1 had no impact on CYP3A4 induction by either rifampicin or simvastatin, which is expected as neither of these compounds are substrates for SLCO1B1. In addition, no increased expression of the CYP3A4 reporter gene was observed with either pravastatin or rosuvastatin despite them being SLCO1B1 substrates (Figure 4b), suggesting that compound access was not the reason for the observed lack of response. However, further experimentation would be required to conclusively demonstrate that increased levels of membrane-localised SLCO1B1 do indeed increase pravastatin and rosuvastatin access into Huh7 cells.

Statins demonstrate differential FXR-mediated target gene activation.

As demonstrated in Figure 2, statins are able to act as FXR agonists, albeit with generally lower potencies than against PXR. We, thus, examined the potential for activation of FXR-regulated target genes by statins through the use of an IR6 reporter gene construct in cells over-expressing FXR. As can be seen from figure 5a, fluvastatin, simvastatin and lovastatin were able to activate expression of the IR6 reporter gene plasmid, although with a lower potency than the classical FXR agonist chenodeoxycholic acid (CDCA); in comparison, rosuvastatin appeared to inhibit expression of the reporter gene construct at higher doses, an effect that was not due to toxicity (data not shown). For simvastatin and lovastatin, the lactone forms were more potent activators of the IR6 reporter gene construct, consistent with the increased cellular access of this form compared to the β -hydroxyacid. This activation of the IR6-reporter gene is consistent with the agonist action of these statins against FXR (Figure 2), although no increase in FXR-mediated gene expression is seen for either rosuvastatin or pravastatin, despite their ability to agonise FXR in a ligand binding assay. This transcriptional response was shown to be FXR-dependent, as lack of co-transfection with an FXR expression plasmid led to an attenuated response (data not shown). Again, the use of an over-expression plasmid for SLC01B1, the uptake transporter for rosuvastatin and pravastatin, was used to examine if lack of uptake was a limiting factor in reporter gene response. As can be seen from figure 5b, over-expression of SLC01B1 did not increase allow IR6-reporter gene activation by rosuvastatin and pravastatin, suggesting that cellular uptake is not the limiting factor.

It is becoming increasingly clear that nuclear receptors interact as part of a regulatory signal network, and it is through these interactions that the refinement of cellular response to chemical challenge may occur (Plant and Aouabdi 2009). Given that statins

are able to act as agonists of both PXR and FXR, albeit with differing potencies, it is of interest to see if these two nuclear receptors interact at the transcriptional level. To examine this, we used a reporter gene construct containing the 2.2kb proximal PXR promoter, as described previously in Aouabdi et al (Aouabdi et al. 2006). As can be seen from figure 6, activation of FXR by the classical agonist CDCA was able to increase expression of the PXR reporter gene assay, demonstrating that FXR may regulate transcriptional activation of this PXR reporter gene. This effect was also seen with fluvastatin, simvastatin (lactone) and lovastatin (lactone), which were also able to activate the IR6-reporter gene, albeit at a relatively low level. Interestingly, *in silico* analysis of this proximal promoter region did not indicate the presence of an IR6 element, to which FXR could bind and mediate its action; however, given that nuclear receptors are known to cross-talk at the level of response element selection, it is possible that FXR may be interacting through another site, with further experimentation being required to confirm this. Neither rosuvastatin nor pravastatin were able to significantly increase PXR reporter gene activity (Figure 5c).

Conclusion

The co-ordination of biological responses to xenobiotic insult represents a highly developed network designed to bring about the most efficient response to any given stimulus (Plant 2004). The central mediators of this response are, generally, members of the nuclear receptor superfamily, which number 48 in humans and regulate and whose gene products regulate a large number of anabolic and catabolic processes within the body (Bookout et al. 2006). It has been long established that cross-talk exists between nuclear receptors, both in terms of their ligand promiscuity (Ding and

Staudinger 2005; El-Sankary et al. 2001), activation of target genes (Maglich et al. 2002), as well as cross-regulation of their own transcriptional regulation (Aouabdi et al. 2006; Pascussi et al. 2003). As the networks of response to xenobiotic exposure become better delineated it can be seen that the relative levels of different nuclear receptors impacts upon the biological response elicited to any given stimuli (Bonofiglio et al. 2005; Ding and Staudinger 2005). It is thus important to consider not only the presence of a putative target nuclear receptor within a tissue, but also the potential presence of other factors that may compete for the stimulating signal. We present herein an examination of the activation of PXR, CAR and FXR by the statin-group of nuclear receptor agonists, demonstrating that they capable of activating all three nuclear receptors, albeit to differing degrees. Such information is important in determining the molecular actions following statin-exposure, and hence predicting the biological response observed.

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References

- Aouabdi S, Gibson GG, Plant N. (2006) Transcriptional Regulation of Pxr: Identification of a Ppre within the Proximal Promoter Responsible for Fibrate-Mediated Transcriptional Activation of Pxr. *Drug Met. Disp.* 34(1):138-44.
- Billeclke S, Draganov D, Counsell R, Stetson P, Watson C, La Du BN. (2000) Human Serum Paraoxonase (Pon1) Isozymes Q and R Hydrolyze Lactones and Cyclic Carbonate Esters. *Drug Met. Disp.* 28(11):1335-42.
- Bonofiglio D, Gabriele S, Aquila S, Catalano S, Gentile M, Middea E, Giordano F, Ando S. (2005) Estrogen Receptor Alpha Binds to Peroxisome Proliferator-Activated Receptor Response Element and Negatively Interferes with Peroxisome Proliferator-Activated Receptor Gamma Signaling in Breast Cancer Cells. *Clin. Cancer Res.* 11(17):6139-47.
- Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. (2006) Anatomical Profiling of Nuclear Receptor Expression Reveals a Hierarchical Transcriptional Network. *Cell* 126(4):789-99.
- Chrencik JE, Orans J, Moore LB, Xue Y, Peng L, L. CJ, Wisely GB, Lambert MH, Kliewer SA, Redinbo MR. (2005) Structural Disorder in the Complex of Human Pregnane X Receptor and the Macrolide Antibiotic Rifampicin. *Mol. Endocrinol.* 19(5):1125-34.
- Dietschy JM, Wilson JD. (1970) Regulation of Cholesterol Metabolism. *N. Engl. J. Med.* 282(20):1128-&.
- Ding X, Staudinger JL. (2005) The Ratio of Constitutive Androstane Receptor to Pregnane X Receptor Determines the Activity of Guggulsterone against the Cyp2b10 Promoter. *J. Pharmacol. Exp. Ther.* 314(1):120-7. .
- Ekins S, Erickson JA. (2002) A Pharmacophore for Human Pregnane X Receptor Ligands. *Drug Met. Disp.* 30(1):96-9.
- El-Sankary W, Gibson GG, Ayrton A, Plant N. (2001) Use of a Reporter Gene Assay to Predict and Rank the Potency and Efficacy of Cyp3a4 Inducers. *Drug Met. Disp.* 29(11):1499-504.

- Everett DW, Chando TJ, Didonato GC, Singhvi SM, Pan HY, Weinstein SH. (1991) Biotransformation of Pravastatin Sodium in Humans. *Drug Met. Disp.* 19(4):740-8.
- Goodwin B, Hodgson E, Liddle C. (1999) The Orphan Human Pregnane X Receptor Mediates the Transcriptional Activation of Cyp3a4 by Rifampicin through a Distal Enhancer Module. *Mol. Pharm.* 56(6):1329-39.
- Habeos I, Ziros PG, Psyrogiannis A, Vagenakis AG, Papavassiliou AG. (2005) Statins and Transcriptional Regulation: The Fxr Connection. *Biochem. Biophys. Res. Commun.* 334(2):601-5.
- Handschin C, Meyer UA. (2005) Regulatory Network of Lipid-Sensing Nuclear Receptors: Roles for Car, Pxr, Lxr, and Fxr. *Arch. Biochem. Biophys.* 433(2):387-96.
- Hirano M, Maeda K, Matsushima S, Nozaki Y, Kusuhashi H, Sugiyama Y. (2005) Involvement of Bcrp (Abcg2) in the Biliary Excretion of Pitavastatin. *Mol. Pharm.* 68(3):800-7.
- Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, Wang Y, Kim RB. (2006) Drug and Bile Acid Transporters in Rosuvastatin Hepatic Uptake: Function, Expression, and Pharmacogenetics. *Gasteroenterol.* 130(6):1793-806.
- Holdgate GA, Ward WHJ, McTaggart F. (2003) Molecular Mechanism for Inhibition of 3-Hydroxy-3-Methylglutaryl CoA (Hmg-CoA) Reductase by Rosuvastatin. *Biochem. Soc. Trans.* 31:528-31.
- Ishigami M, Honda T, Takasaki W, Ikeda T, Komai T, Ito K, Sugiyama Y. (2001) A Comparison of the Effects of 3-Hydroxy-3-Methylglutaryl-Coenzyme a (Hmg-CoA) Reductase Inhibitors on the Cyp3a4-Dependent Oxidation of Mexazolam in Vitro. *Drug Met. Disp.* 29(3):282-8.
- Kobayashi K, Yamanaka Y, Iwazaki N, Nakajo I, Hosokawa M, Negishi M, Chiba K. (2005) Identification of Hmg-CoA Reductase Inhibitors as Activators for Human, Mouse and Rat Constitutive Androstane Receptor. *Drug Met. Disp.* 33(7):924-9.
- Lennernas H, Fager G. (1997) Pharmacodynamics and Pharmacokinetics of the Hmg-CoA Reductase Inhibitors - Similarities and Differences. *Clin. Pharmacokinet.* 32(5):403-25.

- Linsel-Nitschke P, Tall AR. (2005) Hdl as a Target in the Treatment of Atherosclerotic Cardiovascular Disease. *Nat. Rev. Drug Discov.* 4(3):193-205.
- Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM, Moore JT. (2003) Identification of a Novel Human Constitutive Androstane Receptor (Car) Agonist and Its Use in the Identification of Car Target Genes. *J. Biol. Chem.* 278(19):17277-83.
- Maglich JM, Stoltz CM, Goodwin B, Hawkins-Brown D, Moore JT, Kliewer SA. (2002) Nuclear Pregnane X Receptor and Constitutive Androstane Receptor Regulate Overlapping but Distinct Sets of Genes Involved in Xenobiotic Detoxification. *Mol. Pharm.* 62(3):638-46.
- Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. (2009) Autodock4 and Autodocktools4: Automated Docking with Selective Receptor Flexibility. *J. Comput. Chem* 30(16):2785-91.
- Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. (1982) Growth of Human Hepatoma Cell Lines with Differentiated Functions in Chemically Defined Medium. *Cancer Res.* 42:3858-63.
- Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T, Nishimura K. (2001) Human Liver-Specific Organic Anion Transporter, Lst-1, Mediates Uptake of Pravastatin by Human Hepatocytes. *J. Pharmacol. Exp. Ther.* 297(3):861-7.
- Neuvonen PJ, Niemi M, Backman JT. (2006) Drug Interactions with Lipid-Lowering Drugs: Mechanisms and Clinical Relevance. *Clinical Pharmacology & Therapeutics* 80(6):565-81.
- Olsson AG, Pears J, McKellar J, Mizan J, Raza A. (2001) Effect of Rosuvastatin on Low-Density Lipoprotein Cholesterol in Patients with Hypercholesterolemia. *Am. J. Cardiol.* 88(5):504-8.
- Pascussi JM, Busson-Le Coniat M, Maurel P, Vilarem MJ. (2003) Transcriptional Analysis of the Orphan Nuclear Receptor Constitutive Androstane Receptor (Nr1i3) Gene Promoter: Identification of a Distal Glucocorticoid Response Element. *Mol. Endocrinol.* 17(1):42-55.

- Plant N. (2004) Interaction Networks: Coordinating Responses to Xenobiotic Exposure. *Toxicol.* 202:21-32.
- Plant N. (2007) The Human Cytochrome P450 3a Sub-Family: Transcriptional Regulation, Inter-Individual Variation and Interaction Networks. *Biochim. Biophys. Acta* 1770(3):478-88. .
- Plant N, Aouabdi S. (2009) Nuclear Receptors: The Controlling Force in Drug Metabolism of the Liver? *Xenobiotica* 39(8):597-605.
- Raucy J, Warfe L, Yueh MF, Allen SW. (2002) A Cell-Based Reporter Gene Assay for Determining Induction of Cyp3a4 in a High-Volume System. *J Pharmacol Exp Ther* 303(1):412-23.
- Rizzo G, Renga B, Mencarelli A, Pellicciari R, Fiorucci S. (2005) Role of Fxr in Regulating Bile Acid Homeostasis and Relevance for Human Diseases. *Curr. Drug Targets Immune Endocrin. Metabol, Disorders* 5:289-303.
- Sirvent P, Mercier J, Lacampagne A. (2008) New Insights into Mechanisms of Statin-Associated Myotoxicity. *Curr. Opin. Pharmacol.* 8(3):333-8.
- Vaklavas C, Chatzizisis YS, Ziakas A, Zamboulis C, Giannoglouc GD. (2009) Molecular Basis of Statin-Associated Myopathy. *Atherosclerosis* 202(1):18-28.
- Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, Denk H, Trauner M. (2005) Car and Pxr Agonists Stimulate Hepatic Bile Acid and Bilirubin Detoxification and Elimination Pathways in Mice. *Hepatology* 42(2):420-30.
- Watanabe H, Kosuge K, Nishio S, Yamada H, Uchida S, Satoh H, Hayashi H, Ishizaki T, Ohashi K. (2004) Pharmacokinetic and Pharmacodynamic Interactions between Simvastatin and Diltiazem in Patients with Hypercholesterolemia and Hypertension. *Life Sci*, 76(3):281-92.
- Yamasaki D, Nakamura T, Okamura N, Kokudai M, Inui N, Takeuchi K, Watanabe H, Hirai M, Okumura K, Sakaeda T. (2009) Effects of Acid and Lactone Forms of 3-Hydroxy-3-Methylglutaryl Coenzyme a Reductase Inhibitors on the Induction of Mdr1 Expression and Function in Ls180 Cells. *Eur. J. Pharm. Sci.* 37(2):126-32.

Statin-tested	EC50 (μM)	I _{max}	Hill Slope
Rifampicin	0.46±1.12	4.13±0.92	1.76
Atorvastatin	1.95±2.17	15.44±1.17	3.75
Fluvastatin	9.92±1.37	5.78±0.74	4.24
Lovastatin – acid	26.85±1.42	3.04±0.72	6.59
Lovastatin – lactone	1.86±2.06	6.48±0.76	2.04
Simvastatin – acid	6.53±1.18	2.27±0.32	3.86
Simvastatin - lactone	10.32±1.24	15.91±2.22	2.12
Pravastatin		Did Not Fit	
Rosuvastatin		Did Not Fit	

Table 1: PXR-mediated activation of CYP3A4 reporter gene by statins

CYP3A4 reporter gene expression was measured following 48 hours exposure to varying doses of indicated chemical (0-50μM) in the presence of a co-transfected PXR expression plasmid. A non-linear fit for log (agonist) vs response, variable slope, was undertaken using GraphPad Prism v5, generating EC50, maximal induction (I_{max}) and hill coefficient values

Figure 1: Structures of statins used in the current study

Figure 2: Dose- and chemical-specific agonism of the nuclear receptors PXR, FXR and CAR by statins. (A) Autodock 4/ADT for Windows was used to dock pravastatin into the ligand binding domain of PXR (crystal structure of PXR with rifampicin bound; 1SKX) using a genetic algorithm approach (150 GA runs). Pravastatin (white) docking

overlapped rifampicin (dark grey) conformation, and produced donor/acceptor pairs with Ser247, Gln284 and His 407. (B) Nuclear receptor LBDs fused to the Gal4 DBD were used to examine statin-mediated activation of PXR, CAR and FXR. Each data point represents n=6, and statistical analysis was via one-way ANOVA; *=p<0.01, **=p<0.05 and ***=p<0.001 relative to vehicle control. Graph is representative of triplicate repeat experiments.

Figure 3: Dose- and chemical-specific activation of CYP3A4 reporter gene

expression by statins is dependent on PXR but not CAR. Huh7 cells were transiently

transfected with a SEAP reporter gene under the control of the CYP3A4 regulatory regions, plus either an expression plasmid for PXR or CAR, and then incubated with the indicated concentration of statin for 48 hours. SEAP activity was measured, corrected for transfection efficiency, and presented as fold induction relative to vehicle control. Each data point represents n=6, and statistical analysis was via one-way ANOVA; *=p<0.01, **=p<0.05 and ***=p<0.001 relative to vehicle control. Graph is representative of at least triplicate repeat experiments.

Figure 4: Over-expression of SLC01B1 does not permit pravastatin- or rosuvastatin-mediated activation of CYP3A4 reporter gene expression. (A) Huh7 cells were transiently transfected with an expression plasmid for SLC01B1 and incubated for 24 hours, whereupon RNA and protein levels of SLC01B1 were measured. Specificity of SLC01B1 antibody was demonstrated by comparison to TNT-produced SLC01B1. (B) Huh7 cells were transiently transfected with a SEAP reporter gene under the control of the CYP3A4 regulatory regions, plus expression plasmids for FXR and SLC01B1. Cells were then incubated with statin for 48 hours, SEAP activity measured, corrected for transfection efficiency, and presented as fold induction relative to vehicle control. Each data point represents n=6, and statistical analysis was via one-way

ANOVA; ***= $p < 0.001$ relative to vehicle control. Graph is representative of at least duplicate repeat experiments.

A **B**

Figure 5: Dose- and chemical-specific activation of an IR6-reporter gene by statins is dependent on FXR. Six wells of Huh7 cells were transiently transfected with a SEAP reporter gene under the control of the [IR6]₃ concatamer, plus either (A) an expression plasmid for FXR alone, or (B) expression plasmids for FXR and SLC01B1. Cells were then incubated with the indicated concentration of statin for 48 hours. SEAP activity was measured, corrected for transfection efficiency, and presented as fold induction relative to vehicle control. Each data point represents n=6, and statistical

analysis was via one-way ANOVA; $*=p<0.01$, $**=p<0.05$ and $***=p<0.001$ relative to vehicle control. Graph is representative of at least triplicate repeat experiments.

Figure 6: Dose- and chemical-specific activation of PXR reporter gene expression

by statins is dependent on FXR. Six wells of Huh7 cells were transiently transfected

with a SEAP reporter gene under the control of the PXR regulatory regions, plus an

expression plasmid for FXR and then incubated with the indicated concentration of

statin for 48 hours. SEAP activity was measured, corrected for transfection efficiency,

and presented as fold induction relative to vehicle control. Each data point represents

n=6, and statistical analysis was via one-way ANOVA; *=p<0.01 and ***=p<0.001

relative to vehicle control. Graph is representative of at least triplicate repeat

experiments.