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SHORT COMMUNICATION

Rosuvastatin and Atorvastatin are Ligands of the Human CAR/RXRα Complex

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Running Title Page

Running title: Statins are ligands of human CAR/RXRα complex

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Number of words in Introduction: 341

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Abbreviations: CAR - constitutive androstane receptor; hCAR – human CAR; CITCO - 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; CYP – cytochrome P450; GST - glutathione S-transferase; LBD – ligand binding domain; PBREM – phenobarbital-responsive enhancer module; PXR - pregnane X receptor; RXRα - retinoid X receptor alpha; SPR – surface plasmon resonance; SRC-1 - steroid receptor coactivator 1; XREM - xenobiotic responsive enhancer module.

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Abstract

Statins are well known lipid lowering agents that inhibit the enzyme HMG-CoA reductase. They also activate drug metabolism but their exact receptor-mediated action has not been proven so far. The aim of this study was to test if atorvastatin and rosuvastatin are direct ligands of human CAR. We measured binding activities of atorvastatin and rosuvastatin to the human CAR/RXRα-LBD heterodimer with surface plasmon resonance (SPR). Additionally, three-dimensional models of CAR/RXRα-LBD were constructed by ligand-based and structure-based *in silico* modelling. Experiments and computational modeling show that atorvastatin and rosuvastatin bind to the human CAR/RXRα-LBD heterodimer, suggesting both can modulate the activity of CAR through direct interaction with the LBD of this receptor. In conclusion, we confirm that atorvastatin and rosuvastatin are direct ligands of CAR. The clinical consequences of CAR activation by statins are in their potential drug-drug interactions, and changes in glucose and energy metabolism.

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Introduction

Statins have been recognized as drugs of choice for use in patients with high risk for developing cardiovascular disease, but also may have some side-effects, including increasing risk for diabetes (Sattar *et al.*, 2014). Transcriptome analyses have revealed that rosuvastatin and atorvastatin affect expression of many metabolic and signaling pathways in the liver, including drug metabolism (Hafner *et al.*, 2011). Several statins have also been identified as inducers of drug metabolism and potential agonists for the constitutive androstane receptor (CAR, NR1I3) (Kobayashi *et al.*, 2005; Kocarek *et al.*, 2002; Monostory *et al.*, 2009). However, discrepancies among studies exist. For example, a study using cell-based reporter assay and CAR inverse agonist identified fluvastatin, atorvastatin, simvastatin and cerivastatin as hCAR activators (Kobayashi *et al.*, 2005; Monostory *et al.*, 2009). However, in a study using a ligand-binding assay none of the tested statins (fluvastatin, atorvastatin, simvastatin) were confirmed to be activators of hCAR (Howe *et al.*, 2011). In addition, atorvastatin did not activate hCAR using two-hybrid assembly (Hoffart *et al.*, 2012). While studies suggest that statins might be ligands of this nuclear receptor, experimental evidence measuring direct binding of statins to CAR has been lacking so far.

CAR is a member of the nuclear receptor superfamily and was initially discovered as a xenosensor regulating drug metabolism (Honkakoski *et al.*, 1998; Sueyoshi *et al.*, 1999). However, CAR was implicated also in regulation of lipid, glucose and energy metabolism, proliferation, and many other pathways (Ueda *et al.*, 2002; Režen *et al.*, 2009). Therefore, the clinical relevance of statin-CAR interactions is not only in potential drug-drug interactions, but also in changes in glucose and energy metabolism induced by statins.

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In this study we have combined computational and experimental approaches to determine whether rosuvastatin and atorvastatin are ligands of the CAR/RXRα heterodimer. Using surface plasmon resonance (SPR) we measured binding of atorvastatin, rosuvastatin and CITCO to the CAR/RXRα-LBD (ligand binding domain) heterodimer. We also used ligand and structure based modeling of different statins to CAR and the pregnane X receptor (PXR, NR1I2), another drug metabolism inducing nuclear receptor.

Materials and Methods

Materials and methods are described in more detail in Supplemental Information. The GST-hCAR (gift from Jean Marc Pascussi, INSERM, France) and GST-hRXRa-LBD (Addgene, Cambridge, MA, USA) plasmids were transformed into the BL21 (DE3) *E. coli* strain. Briefly, the overnight culture was grown in Lysogeny broth (LB) media with ampicillin then protein expression was induced with the addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Cell supernatant was loaded on a GST affinity column (Clontech, Palo Alto, CA, USA) and concentrated using Microcon YM-30 centrifugal filter (Millipore, Bedford, MA, USA). Purity of isolated GST-hCAR and GST-hRXRα-LBD were checked by SDS-PAGE and western blot analysis. The interaction of rosuvastatin, atorvastatin and CITCO was measured using a Biacore X analytical system and CM5 sensor chip (Biacore, GE Healthcare, Uppsala, Sweden).10 mM stock solution of each analyte was prepared in DMSO and diluted into a series of concentrations with the running buffer to detect direct binding with immobilized GST-hCAR/GST-hRXRα-LBD. K_D values were determined by the fitting of the data to the steady state affinity model using BIAevaluation software. Previously generated pharmacophores for PXR or CAR agonists

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or Bayesian models (Ekins *et al.*, 2009) generated with steroids or diverse compounds were employed to generate predictions for statins tested in this study. The crystal structures of PXR and CAR were obtained from the Protein DataBank with codes 1M13 and 1XVP respectively and used for docking.

Results and Discussion

Since it was still not known if atorvastatin and rosuvastatin are binding to human CAR, we measured the interaction of rosuvastatin, atorvastatin and the positive control CITCO with hCAR using the surface plasmon resonance technique (SPR). The structures of the molecules are shown in Supplemental Figure 1. Recombinant proteins GST-hCAR and GST-hRXRα-LBD were isolated and their purity assessed by SDS-PAGE and western blot analysis (Supplemental Figure 2). SDS-PAGE analyses of recombinant proteins revealed high purity and western blot confirmed presence of GST proteins. The GST-hCAR/GST-hRXRα-LBD heterodimer was covalently attached to the surface of the SPR sensor chip and assayed with at least 4 different concentrations of compounds. Each concentration was injected three times to test the reproducibility. All binding responses and K_Ds were reproducible which indicates that immobilized complex did not lose activity over time. As expected, CITCO, which is known to be a direct ligand for hCAR, showed specific binding to the complex. We also observed specific concentration-dependent binding of both statins of this study (Figure 1). The measured K_D indicates that rosuvastatin binds to the CAR/RXR heterodimer with approximately two-fold higher affinity than atorvastatin, but approximately three-fold lower affinity than CITCO (Table 1). A comparison of measured maximal response with the theoretical maximal response

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indicated that several molecules of atorvastatin and CITCO bind to the hCAR/hRXRα-LBD complex (Table 1). By applying SPR we show for the first time that atorvastatin and rosuvastatin directly bind to the hCAR/RXR-LBD heterodimer. Binding of statins to hCAR is consistent with our previous transcriptome study, in which we showed that many genes were differentially expressed in human primary hepatocytes treated with atorvastatin and rosuvastatin and are under direct regulation of CAR (Hafner *et al.*, 2011). Another study used surface plasmon resonance to test binding of atorvastatin and hCAR with co-activator (Hoffart *et al.*, 2012). They pre-incubated atorvastatin or CITCO with CAR-LBD protein and injected it onto immobilized SRC-1-RID protein. Under these conditions atorvastatin did not induce binding of hCAR-LBD with co-activator SRC-1. Further, they did not observe assembly of human CAR-LBD with co-activators in COS1 cells. The difference in results obtained by SPR between our study and this was probably due to the different CAR dimerization partner and steps used. We used RXR as a dimerization partner and studies show that RXRα may also have a role in stabilizing CAR-LBD in its active conformation via direct interactions across the heterodimer interface (Xu *et al.*, 2004). RXR is also important for recruitment of co-activators, like SRC-1 (steroid receptor coactivator 1) (Dussault *et al.*, 2002; Pavlin *et al.*, 2014). Also, we tested binding of atorvastatin to the CAR/RXR dimer, while Hoffart *et al*., tested binding of the atorvastatin-CAR pair to SRC-1 co-activator. It is important to note that none of the above experiments published on statins used physiological concentrations of the drugs. All used μM concentrations, while in humans in serum only nM concentrations were measured, but certainly concentrations in the liver have not been determined so it is unclear how the *in vitro* observations relate to the *in vivo* situation.

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Our computational predictions using structure based or ligand based approach indicated that statins are good potential ligands for CAR and PXR with little difference in potential between them. Furthermore, statins docked in CAR produced scores that correlate with the K_D values determined by SPR. Our data suggested variability in binding to these different models (Table 1, Supplemental Table 1, Supplemental Figure 3). Based on the docking results the statins appeared to be good ligands for both CAR and PXR. Atorvastatin had the highest score against CAR (73.18) while rosuvastatin was lower (67.09) (Table 1). Similarly, the pharmacophores and Bayesian models suggest that all other statins will likely bind to CAR and PXR (Supplemental Table 1). These results broadly agree with published data, confirming that the majority of statins are CAR/PXR activators, however; the manner of this activation awaits experimental confirmation in the future. When comparing these scores with other studies using the same *in silico* approaches we observed that calculated scores for statins are within the same range as for known hCAR activators (Kortagere *et al.*, 2009; Lynch *et al.*, 2013). Interestingly, we and others have predicted that pravastatin is also a good potential ligand for CAR and PXR, while this statin has never been shown to have any ability to induce these two nuclear receptors or drug metabolism (Howe *et al.*, 2011). In conclusion, these findings are important in understanding the full biological consequences of statin exposure due to molecular interaction of statins with nuclear receptors. We showed *in silico* that statins are potentially ligands of CAR and PXR and confirmed *in vitro* that atorvastatin and rosuvastatin directly bind to the CAR/RXRα dimer. As CAR and PXR regulate not only drug metabolism but also glucose and energy metabolism, this leads us to the assumption that they are potentially involved in the statins' unwanted side effects such as the increase

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in developing type 2 diabetes in humans. Additionally, we conclude that computational approaches may be useful for assessing statin binding to CAR and PXR while SPR can provide valuable insights on binding to validate these predictions.

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Authorship Contributions

Participated in research design: Režen, T. and Rozman, D.

Conducted experiments: Hafner, M. performed SPR experiments together with Hodnik, V.

Contributed new reagents or analytic tools: Ekins, S. and Kortagere, S.

Performed data analysis: Režen, T., Hafner, M., Hodnik, V., Rozman, D., Ekins, S. and Kortagere, S

Wrote or contributed to the writing of the manuscript: Režen, T., Hafner, M., Kortagere, S., Ekins, S., Hodnik, V. and Rozman, D.

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REFERENCES

Dussault I, Lin M, Hollister K, Fan M, Termini J, Sherman MA, and Forman BM (2002) A structural model of the constitutive androstane receptor defines novel interactions that mediate ligand-independent activity. *Mol Cell Biol* **22**:5270–80.

Ekins S, Kortagere S, Iyer M, Reschly EJ, Lill MA, Redinbo MR, and Krasowski MD (2009) Challenges predicting ligand-receptor interactions of promiscuous proteins: the nuclear receptor PXR. *PLoS Comput Biol* **5**:e1000594.

Hafner M, Juvan P, Rezen T, Monostory K, Pascussi J-M, and Rozman D (2011) The human primary hepatocyte transcriptome reveals novel insights into atorvastatin and rosuvastatin action. *Pharmacogenet Genomics* **21**:741–750.

Hoffart E, Ghebreghiorghis L, Nussler AK, Thasler WE, Weiss TS, Schwab M, and Burk O (2012) Effects of atorvastatin metabolites on induction of drug-metabolizing enzymes and membrane transporters through human pregnane X receptor. *Br J Pharmacol* **165**:1595–1608.

Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18**:5652–5658.

Howe K, Sanat F, Thumser AE, Coleman T, and Plant N (2011) 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. *Xenobiotica* **41**:519–29.

Kobayashi K, Yamanaka Y, and Iwazaki N (2005) Identification of HMG-CoA reductase inhibitors as activators for human, mouse and rat constitutive androstane receptor.

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DMD Fast Forward. Published on May 23, 2017 as DOI: 10.1124/dmd.117.075523
This article has not been copyedited and formatted. The final version may differ from this version.

Drug Metab Dispos **33**:924–929.

Kocarek TA, Dahn MS, Cai H, Strom SC, and Mercer-Haines NA (2002) Regulation of CYP2B6 and CYP3A expression by hydroxymethylglutaryl coenzyme a inhibitors in primary cultured human hepatocytes. *Drug Metab Dispos* **30**:1400–1405.

Kortagere S, Chekmarev D, Welsh WJ, and Ekins S (2009) Hybrid scoring and classification approaches to predict human pregnane X receptor activators. *Pharm Res* **26**:1001–11.

Lynch C, Pan Y, Li L, Ferguson SS, Xia M, Swaan PW, and Wang H (2013) Identification of Novel Activators of Constitutive Androstane Receptor from FDA-approved Drugs by Integrated Computational and Biological Approaches. *Phar* **30**:489–501.

Monostory K, Pascussi J, Temesva M, Acimovic J, Kocjan D, Kuzman D, Wilzewski B, and Bernhardt R (2009) Drug Interaction Potential of 2- ((3 , 4- Dichlorophenethyl)(propyl) amino) -1- (pyridin-3-yl) ethanol (LK-935), the Novel Nonstatin-Type Cholesterol-Lowering Agent. *Drug Metab Dispos* **37**:375–385.

Pavlin MR, Brunzelle JS, and Fernandez EJ (2014) Agonist ligands mediate the transcriptional response of nuclear receptor heterodimers through distinct stoichiometric assemblies with coactivators. *J Biol Chem* **289**:24771–24778.

Režen T, Tamasi V, Lövgren-Sandblom A, Björkhem I, Meyer UA, and Rozman D (2009) Effect of CAR activation on selected metabolic pathways in normal and hyperlipidemic mouse livers. *BMC Genomics* **10**:384.

Sattar NA, Ginsberg H, Ray K, Chapman MJ, Arca M, Averna M, Betteridge DJ, Bhatnagar D, Bilianou E, Carmena R, Češka R, Corsini A, Erbel R, Flynn PD,

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Garcia-Moll X, Gumprecht J, Ishibashi S, Jambart S, Kastelein JJP, Maher V, Marques da Silva P, Masana L, Odawara M, Pedersen TR, Rotella CM, Salti I, Teramoto T, Tokgozoglu L, Toth PP, Valensi P, and Vergès B (2014) The use of statins in people at risk of developing diabetes mellitus: Evidence and guidance for clinical practice. *Atheroscler Suppl* **15**:1–15.

Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, and Negishi M (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem* **274**:6043–6046.

Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari C a, Lehmann JM, and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. *Mol Pharmacol* **61**:1–6.

Xu RX, Lambert MH, Wisely BB, Warren EN, Weinert EE, Waitt GM, Williams JD, Collins JL, Moore LB, Willson TM, and Moore JT (2004) A structural basis for constitutive activity in the human CAR/RXRalpha heterodimer. *Mol Cell* **16**:919–28.

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FOOTNOTES

a. This work was supported by the Slovenian Research Agency [Grant P1-0104, P1-0390].

b. This work was a part of Hafner's doctoral thesis.

c. Damjana Rozman, Centre for Functional Genomics and Bio-Chips, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, SI-1000 Ljubljana, Slovenia, Email: damjana.rozman@mf.uni-lj.si

d. TR and MH contributed equally to this work.

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Figure Legends

Figure 1. SPR analysis of atorvastatin, rosuvastatin and CITCO binding to hCAR/hRXRα-LBD heterodimer. Different concentrations (1.56, 3.125, 12.5, 25 and 50 μM for CITCO and 6, 12.5, 25, 50, 75 100 and 150 μM for atorvastatin and rosuvastatin) of analytes were tested for the binding (left panels). The binding curves (right panels) were generated by fitting steady state response levels at the end of association phase, versus concentration of the injected analyte. The K_Ds were obtained from fitting the data to the steady-state affinity model. For each analyte 6 or 7 independent titration experiments were performed.

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Table 1. K_D values, theoretical and measured maximal response of atorvastatin, rosuvastatin and CITCO binding to hCAR/hRXRα-LBD heterodimer as measured by surface plasmon resonance, and scores of ligand-based (CAR pharmacophore) and structure-based (CAR-score-docking) *in silico* calculations.

	K_D (μM)	**CAR-score-docking**	**CAR pharmacophore**	**Theoretical maximal response**	**Measured maximal response**
Atorvastatin	0.225±0.06	73.18	2.07	84	251
Rosuvastatin	0.144±0.03	67.09	-	70	86
CITCO	0.045±0.04	63.02	-	30	74

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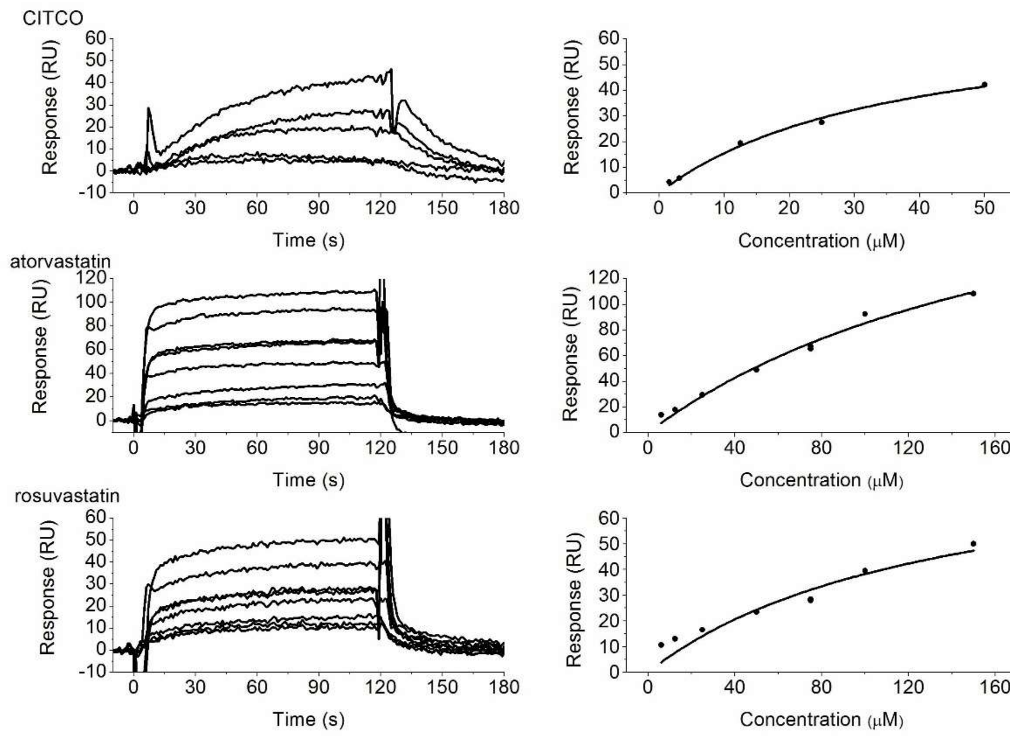

Figure 1.

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