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CINPA1 binds directly to constitutive androstane receptor and inhibits its activity

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Abstract

The constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are xenobiotic sensors that regulate the expression of drug-metabolizing enzymes and efflux transporters. CAR activation promotes drug elimination, thereby reducing therapeutic effectiveness, or causes adverse drug effects via toxic metabolites. CAR inhibitors could be used to attenuate these adverse drug effects. CAR and PXR share ligands and target genes, confounding the understanding of the regulation of receptor-specific activity. We previously identified a small-molecule inhibitor, CINPA1, that inhibits CAR (without activating PXR at lower concentrations) by altering CAR-coregulator interactions and reducing CAR recruitment to DNA response elements of regulated genes. However, solid evidence was not presented for the direct binding of CINPA1 to CAR. In this study, we demonstrate direct interaction of CINPA1 with the CAR ligand-binding domain (CAR-LBD) and identify key residues involved in such interactions through a combination of biophysical and computational methods. We found that CINPA1 resides in the ligand-binding pocket to stabilize the CAR-LBD in a more rigid, less fluid state. Molecular dynamics simulations, together with our previously reported docking model, enabled us to predict which CAR residues were critical for interactions with CINPA1. The importance of these residues for CINPA1 binding were then validated by directed mutations and testing the mutant CAR proteins in transcription reporter and coregulatory interaction assays. We demonstrated strong hydrogen bonding of CINPA1 with N165 and H203 and identified other residues involved in hydrophobic contacts with CINPA1. Overall, our data confirm that CINPA1 directly binds to CAR.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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Graphical Abstract

Keywords

CAR; xenobiotic receptor; transcriptional regulation; ligand-receptor interaction

1. INTRODUCTION

The constitutive androstane receptor (CAR, NR1I3 [1]) and pregnane X receptor (PXR, NR1I2 [2]) are ligand-activated nuclear receptors that are the main actors in regulating the expression of metabolic enzymes, transferases, and membrane transporters [3]. They have a profound effect on the detoxification, biotransformation, and elimination of xenobiotics, including drugs in clinical use. This can lead to critical complications involving adverse drug effects, such as hazardous drug-drug interactions and reduced efficacy [3–6]. Therefore, there has been a surge in the quest to find chemicals that can alter the activity of these xenobiotic sensors, particularly those with inhibitory properties.

CAR and PXR upregulate overlapping sets of metabolic and transporter genes [7–10], as both can bind the xenobiotic response element (XRE) of target genes. Among the products of those target genes, cytochrome P450 3A4 (CYP3A4) is recognized as the principal and most significant player in the CYP family, which has more than 50 members that are, collectively, responsible for the bulk of drug metabolism [11]. Although the objective of discovering modulators that can abrogate the function of both nuclear receptors simultaneously is attractive, CAR and PXR also each upregulate a distinct set of genes that are involved in independent physiologic pathways [12]; therefore, receptor-selective chemical probes have become invaluable tools.

The major hindrance to the development of selective inhibitors is that CAR and PXR are highly promiscuous, being activated by diverse chemicals. CAR shares some ligands with PXR, including various xenobiotics and endobiotics [13]. This commonality can be attributed to the existence of a fail-safe metabolic safety net to protect against toxic accumulation [14]. Chemicals that inhibit one receptor but activate the other could lead to the confounding of gene expression. For instance, the CAR inhibitors PK11195, clotrimazole, meclizine, and androstanol [13, 15–20] each activate PXR function to different degrees [15, 17, 21, 22].

The ligand promiscuity of CAR and PXR is attributed to the large and dynamic ligand-binding domain (LBD), which is sufficiently flexible to accommodate ligands that range widely in size and chemical structure. The LBD is connected to the N-terminal DNA binding domain (DBD) through a flexible hinge. Ligand binding induces conformational

changes that allow the receptor to differentially recruit coregulators, including co-repressors such as nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid or thyroid hormone receptors (SMRT) and co-activators such as steroid receptor co-activator 1 (SRC-1) and transcriptional mediator/intermediary factor 2 (TIF2) [23].

We recently identified SPA70 as a selective and potent inhibitor (antagonist) of PXR [24]. This finding was the result of our screening of a large chemical library followed by hit optimization and detailed mechanistic characterization. Similarly, we identified CINPA1 as a small-molecule inhibitor that selectively attenuates the activity of CAR with an IC_{50} of 70 nM and does not activate PXR within similar concentration ranges [25, 26], although it was subsequently shown to activate PXR in different cell contexts when used at higher concentrations [27]. The crystal structure of PXR in complex with an SPA70 analog (albeit an agonist) provided evidence of direct binding in the PXR LBD, along with a wealth of valuable information for antagonist optimization. The continued lack of such structural input for CINPA1 would hinder further inhibitor improvement.

In this study, we present evidence of the direct interaction of CINPA1 with the ligand-binding pocket of human CAR1 (hCAR1) by using biophysical techniques. Aided by computational and mutagenesis studies, we have identified key residues that functionally compromise the effect of CINPA1 on CAR activity when mutated. These findings should assist the development of a more potent and selective CAR inhibitor and help rationalize the structure-activity relationship (SAR).

2. MATERIALS AND METHODS

2.1. Cell lines and chemicals

Human embryonic kidney cell line HEK293 (ATCC CRL-1573) and liver hepatocellular cell line HepG2/C3A (ATCC CRL-10741) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cell lines, which have been authenticated by ATCC by using short tandem repeat DNA profiling, were expanded immediately upon receipt within three passages and frozen for future use. The cells were tested routinely for mycoplasma contamination and always tested negative. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). HepG2/C3A and HEK293 cell lines were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified incubator at 37 °C with 5% CO₂. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). CITCO was obtained from Tocris Bioscience (Bristol, UK). CINPA1 was obtained from ChemDiv (San Diego, CA) and PK11195 was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Plasmids

The CAR expression vector (FLAG-hCAR1 WT in the pcDNA3.1 vector) has been described previously [25, 28] and is referred to herein as "hCAR1-WT" or simply "WT." CYP2B6-luciferase (CYP2B6-luc) in pGL3 was generously provided by Dr. Hongbing Wang [5]. All FLAG-hCAR1 mutants were in the pcDNA3.1 vector and were prepared by Mutagenex Inc. (Suwanee, GA). The mutated sequences were confirmed by sequencing and

included in Table 1. The TK–*Renilla* luciferase plasmid was purchased from Promega (Madison, WI).

For the mammalian two-hybrid assays, CheckMate™ pG5-Luc, pBIND, and pACT vectors were purchased from Promega (Madison, WI). The pACT-hCAR1, pBIND-SRC-1, pBIND-TIF2, pBIND-NCoR, and pBIND-SMRTα plasmids have been described previously [25]. pBIND plasmids all express TK–*Renilla* luciferase. All pACT-hCAR1 mutant plasmids were prepared using polymerase chain reaction (PCR) amplification of the pACT-hCAR1 plasmid with oligonucleotide primers containing the mutation at the residue of interest. Colonies were selected after transformation, and successful insertion was confirmed by sequencing (Table 1). All transfections were performed using FuGENE 6 transfection reagent (Promega, WI) in accordance with the manufacturer's recommendations.

For the purified hCAR-LBD protein used in all biochemical assays, an *E. coli* expression plasmid was constructed. The pET-His-MBP-TEV-HIS-LIC-hCAR1-LBD expression plasmid was a gift from Dr. Elias Fernandez of the Department of Biochemistry, Cellular & Molecular Biology at the University of Tennessee, Knoxville. The expression plasmid was modified by mutagenesis with the Quick Change II Site-Directed Mutagenesis kit (New England Biolabs, cat no. 200521) to remove the intermediate His-tag and LIC cleavage sequence. For this process, the forward primer was 5' - ATGCCTGTGCAACTGAGTAAGGA and the reverse primer was 5' - GGATTGGAAGTACAGGTTTTCT. The plasmid thus derived was designated pET-His-MBP-hCAR1-LBD and was confirmed by sequencing to contain the hCAR1-LBD cDNA sequence: 5' -

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ATGCCTGTGCAACTGAGTAAGGAGCAAGAAGAGCTGATCCGGACACTCCTGGGG
GCCACACCCGCCACATGGGCACCATGTTTGAACAGTTTGTGCAGTTTAGGCCTC
CAGCTCATCTGTTTCATCCATCACCAGCCCTTGCCACCCCTGGCCCCCTGTGCTGCCT
CTGGTCACACACTTCGCAGACATCAACACTTTCATGGTACTGCAAGTCATCAAGTT
TACTAAGGACCTGCCCCGTCTTCCGTTCCTGCCCATTGAAGACCAGATCTCCCTTC
TCAAGGGAGCAGCTGTGGAAATCTGTACATCGTACTCAATACCACTTTCTGTCTC
CAAACACAAAACCTCCTCTGCGGGCCTCTTCGCTACACAATTGAAGATGGAGCCC
GTGTGGGGTTCCAGGTAGAGTTTTTGGAGTTGCTCTTTCACTTCCATGGAACACTA
CGAAAACCTGCAGCTCCAAGAGCCTGAGTATGTGCTCTTGGCTGCCATGGCCCTCT
TCTCTCCTGACCGACCTGGAGTTACCCAGAGAGATGAGATTGATCAGCTGCAAGA
GGAGATGGCACTGACTCTGCAAAGCTACATCAAGGGCCAGCAGCGAAGGCCCCG
GGATCGGTTTCTGTATGCGAAGTTGCTAGGCCTGCTGGCTGAGCTCCGGAGCATT
AATGAGGCCTACGGGTACCAAATCCAGCACATCCAGGGCCTGTCTGCCATGATGC
CGCTGCTCCAGGAGATCTGCAGC-3'.
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2.3. hCAR-LBD protein expression and purification

The pET-His-MBP-hCAR1-LBD plasmid was used to transform *E. coli* strain BL21(DE3) (Novagen™, EMD Millipore, Boston, MA), and colonies were selected on LB plates, with 30 µg/mL kanamycin being used for selection.

Transformed bacterial cells were grown in an incubator at 37 °C with shaking to a cell density of 0.7 to 0.9 at 600 nm in 6-L flasks in 2× YT medium (20 g tryptone, 10 g yeast

extract, 5 g NaCl) containing 30 µg/mL kanamycin. Next, 0.2 mM IPTG was added, and the cells were grown for a further 20 h at room temperature. Cells were harvested by centrifugation, suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM AEBSF, 1 mM TCEP), and disrupted by passage through a microfluidizer. This lysate was further centrifuged, and the supernatant was incubated with 6 mL high-density nickel-agarose beads (Gold Biotechnology Inc., St. Louis, MO) at 4 °C for 1 h with stirring. The beads were washed with 50 mM imidazole to remove loosely bound impurities. His-tagged TEV (approximately 12 mg; produced by the St. Jude Protein Production Facility) was added to the protein, and the mixture was incubated overnight at 4 °C. The digested hCAR-LBD was then eluted from the nickel beads with lysis buffer. The CAR-LBD was separated from the maltose binding protein (MBP) by applying the solution to a 5-mL MBP Trap HP column (GE Healthcare) and collecting the flow-through. An equal volume of buffer containing 10 mM Tris-Cl at pH 8.0, 100 mM NaCl, 1 mM TCEP, and 0.1 mM EDTA was added to the purified protein. The final buffer contained 30 mM Tris, pH 8.0, 200 mM NaCl, 5% glycerol, 1 mM TCEP, 0.5 mM AEBSF, and 0.05 mM EDTA. By using this method, approximately 12 mg of purified hCAR-LBD was generated for use in the various biochemical assays.

2.4. Thermal shift assay

A 10 µL solution of 10 µM hCAR-LBD in assay buffer (50 mM HEPES pH 8.0, 200 mM NaCl) was transferred to standard 384-well RT-PCR assay plates in triplicate. Compounds dissolved in DMSO were subsequently transferred with a pin-tool device, with final compound concentrations ranging from 3.3 to 210 µM and a final DMSO content of 2%. The protein-compound mixture was pre-incubated at room temperature for 30 min. Subsequently, 10 µL of a 1:1000 dilution of SYPRO Orange dye (Sigma-Aldrich, St. Louis, MO) in assay buffer was added to the mixture, resulting in a final hCAR-LBD concentration of 5 µM. The plate was covered with clear film and centrifuged prior to performing thermal shift assay using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The temperature was increased from 25 °C to 95 °C at a rate of 1 °C/min, and the fluorescence of the protein-bound SYPRO orange dye was monitored (λ_{ex} 492 nm and λ_{em} 610 nm). The raw relative fluorescence unit (RFU) data was processed and fitted with the Boltzmann Sigmoidal equation using Prism software (GraphPad, La Jolla, CA) to determine the melting temperature at the inflection point of the curves. Protein samples in 2% DMSO (without any test compound) were used as negative controls.

2.5. Surface plasmon resonance

Surface Plasmon Resonance (SPR) was used to probe direct interactions of ligands to hCAR-LBD, which was tethered using primary amines to a Ni-NTA biosensor (Pall FortéBio, Fremont, CA) with a capture couple method. SPR measurements were carried out on a SensiQ Pioneer instrument (Pall FortéBio, Fremont, CA). Prior to immobilization, 10 µM hCAR-LBD in running buffer (50 mM Hepes pH 8.0, 200 mM NaCl, 0.01% Triton X-100 and 2% DMSO) was pre-incubated with 30 µM PK11195 for 30 min at room temperature. Both the reference and ligand surfaces were conditioned using 5 min pulses of 0.5 M EDTA at a flow rate of 25 µL/min. Subsequently, a 5 min pulse of 50 µM NiCl₂ was applied to only the ligand surface. The sensor surface was activated using a 5 min pulse of

1:1 mixture of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) followed by injection of the pretreated hCAR-LBD at a flow rate of 10 μ L/min until a signal of 4000 response units (RUs) was reached. The sensor surface baselines were allowed to stabilize overnight. Freshly prepared DMSO stock solutions of CINPA1, CITCO and PK11195 were diluted in 1.02X buffer to a final compound concentration of 100 μ M and DMSO concentration of 2%. The compounds were injected in triplicate at a flow rate of 150 μ L/min to the ligand and reference surfaces of the chip using the OneStep dynamic injection method. The resulting data was double referenced and fitted with a 1:1 kinetic model using the Qdat data analysis tool.

2.6. Molecular docking (MD) simulations

All molecular dynamics simulations, visualizations, and docking were performed using Schrödinger Suites Release 2017-2. For PK11195 and ligand-free simulations, the structure of the CAR/RXR heterodimer (PDB: 1XVP) was imported and the following components were excised: RXR dimer, SRC-1 peptide, and ligand CITCO. Only RXR and SRC-1 were excised for the CITCO-bound model. The CINPA1-bound model has been published previously [26]. All structures were prepared using the Protein Preparation Wizard (Schrödinger) for energy minimization. All model systems were constructed using the System Builder panel. Molecular dynamics simulations were run for 2.0 ns at a temperature of 300 K and a pressure of 1.013 Bar, with water as the solvent.

2.7. Hydrogen/deuterium exchange mass spectrometry analysis

Hydrogen/deuterium exchange mass spectrometry (HDX-MS) experiments were conducted in a similar manner to those described previously [29, 30]. Tandem mass spectrometry (MS/MS) with an Orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Waltham, MA) was used to identify the peptides. Product ion spectra were obtained in a data-dependent mode with the five most abundant ions being selected for the product ion analysis for each scan event. Peptides were identified by submitting the MS/MS data files to Mascot (Matrix Science, Boston, MA), and the peptides included in the HDX analysis peptide set had a Mascot score greater than 20, with the MS/MS spectra being verified by manual inspection. The Mascot search was repeated against a decoy (reverse) sequence, whereby ambiguous identifications were ruled out and excluded from the HDX peptide set.

For the HDX-MS analysis, hCAR LBD (0.1 mg/mL in 20 mM Tris-HCl, pH 7.8, 250 mM NaCl, 5 mM DTT) was incubated with CINPA1 at a 1:10 molar ratio (protein:ligand) for 1 h or further incubated with NCoR1-3 (ASNLGLEDIIRKALMGsFD; synthesized by the Hartwell Center at St. Jude Children's Research Hospital, Memphis, TN) cofactor peptide at a 1:10 molar ratio (protein complex:cofactor peptide) for 1 h before the HDX reactions. A 5- μ L sample of protein or protein complex with CINPA1 or NCoR1-3 was diluted in 20 μ L D₂O ion-exchange buffer (20 mM Tris-HCl, pH 7.8, 250 mM NaCl, 5 mM DTT). The HDX reaction mixture was incubated for various times (e.g., 0, 10, 60, 300, and 900 s) at room temperature then quenched by mixing with 25 μ L of ice-cold 3 M urea, 1% trifluoroacetic acid. After quenching, the tubes containing the samples were immediately placed on dry ice until the samples were injected into the HDX platform. The protein was then passed through an immobilized pepsin column (2 mm $\times$ 2 cm) at 200 μ L/min, and the digested peptides

were captured on a 2 mm × 1 cm C8 trap column (Agilent, Santa Clara, CA) and desalted. Peptides separation was accomplished using a 2.1 mm × 5 cm C18 column (1.9 µL Hypersil Gold; Thermo Fisher Scientific, Waltham, MA) with a linear gradient of 4%–40% acetonitrile (CH₃CN), 0.3% formic acid, over 5 min. Protein digestion and peptide separation were conducted at 4 °C. Mass spectrometric data were acquired using an Orbitrap mass spectrometer (Q Exactive; Thermo Fisher Scientific, Waltham, MA) with a measured resolving power of 65,000 at m/z 400. HDX analyses were performed in triplicate with single preparations of each protein-ligand complex. The intensity-weighted mean centroid m/z value of each of the peptide envelopes was calculated and subsequently converted to the percentage of deuterium incorporation. The statistical significance of the differential HDX data was determined by an unpaired *t*-test for each time point; this is part of the protocol integrated into the HDX Workbench software [31]. The deuterium level was calculated, and corrections for back-exchange were made based on an estimated 70% deuterium recovery, accounting for the known 80% deuterium content of the deuterium exchange buffer.

2.8. Reporter gene assays

HepG2/C3A or HEK293 cells were plated in six-well plates and transfected using FuGENE 6 with either the FLAG-hCAR1 or a FLAG-hCAR mutant and CYP2B6-luc plasmids (in a 1:3 ratio), along with 0.2 µg of TK-*Renilla* plasmid. Cells were incubated for 24 h in 3 mL phenol red-free Dulbecco's modified Eagle's media (DMEM) supplemented with 10% FBS and penicillin/streptomycin (P/S). Phenol red-free DMEM was used to avoid the possible interference of phenol red on the reporter assay.

Cells were trypsinized, resuspended in DMEM + 10% FBS, plated in 96-well white wall clear-bottom plates (Corning 3903) at a density of 30,000 cells/well, and incubated for 24 h with the indicated treatments (CITCO or CINPA1). The final concentration of DMSO was maintained at 0.1% in all wells in all assays. The Dual-Glo Luciferase Assay System (Promega) was used to detect reporter-gene transcription. Plates were incubated with mild shaking for 15 to 20 min at room temperature, and luciferase activity was detected using an EnVision plate reader (PerkinElmer). The relative luciferase expression was calculated by correcting the reading for each well for the TK-*Renilla* and pcDNA3.1 (DMSO) luminescence signals and normalizing the results to the hCAR1-WT (DMSO) luminescence signal. The percentage of CAR-mediated activation was calculated and reported. The expression of FLAG-hCAR (WT or mutant) was detected using anti-FLAG M2 antibody (Sigma, St. Louis, MO) and anti-mouse IRDye secondary antibody (LI-COR Biosciences, Lincoln, NE) (*n* = 2; a representative gel is shown). The specificity of the antibody was demonstrated by using the empty vector as a negative control. Actin (as the loading control) was detected using anti-β-actin antibody (Sigma, St. Louis, MO). The intensity of each protein band was quantified and normalized to that of β-actin to generate the relative intensity, as described previously [32].

2.9. Mammalian two-hybrid assays

HEK293 cells were grown in flasks, plated in EMEM + 10% FBS + P/S in six-well plates at a density of 0.5×10^6 cells/well, and incubated for 24 h. We chose HEK293 cell because of its higher transfection efficiency. Cells were co-transfected with 1 µg of each of pACT-

hCAR1 (WT or mutant), pBIND-coregulator plasmids, and pG5-luc and further incubated for 24 h. Cells were trypsinized, treated, plated at a density of 40,000 cells/well in flat-bottom 96-well plates containing treatment reagents, and incubated for 24 h. The Dual-Glo Luciferase Assay System was used to detect the relative luciferase activity, as described above. Each experiment included plasmid expressing pBIND vector alone and/or WT hCAR1 for standardization. The relative luciferase activity was determined by normalizing the firefly luciferase activity to the *Renilla* luciferase activity. The fold-change for the interaction was calculated by normalizing the result with each pBIND-coregulator plasmid to that with the pBIND vector alone.

2.10. Statistical analysis

Data from the CAR activity and mammalian two-hybrid assays were expressed as the mean $\pm$ standard deviation of at least three independent experiments. Significance was established if the *P*-value was less than 0.05. One-way analysis of variance (ANOVA) was used to analyze data sets with multiple comparisons between treatments, and the experiment-wide significance level α was set to 0.05. Following ANOVA, post-hoc Dunnett's test was performed for multiple comparisons to identify significant differences.

3. RESULTS AND DISCUSSION

3.1. Direct interaction of CINPA1 with the hCAR-LBD

CINPA1 was initially identified as a potent inhibitor of CAR, with the results of a co-activator recruitment assay indirectly suggesting that the site of CINPA1 binding was most likely the hCAR-LBD [25]. We conducted experiments using biophysical and computational techniques to demonstrate direct binding of CINPA1 to the hCAR-LBD and examine its structural effect.

3.1.1 Thermal shift assay—This assay determines the thermal stability of a protein and the increase in protection due to its denaturation in the presence of a ligand by quantifying the difference in the melting temperatures. The SYPRO Orange dye binds to hydrophobic surfaces nonspecifically as the protein begins to denature under a thermal gradient [33], producing a sigmoidal melt profile from which the melting temperature (inflection point) is determined. An increase in melting temperature indicates increased protein stability upon binding of a compound. We explored the effect of the agonist CITCO, the inverse agonist PK11195, and CINPA1 on the thermal stability of the hCAR-LBD at various concentrations (Figure 1). Remarkably, in the absence of ligand, no apparent melting curve for the CAR-LBD could be observed, suggesting a very unstructured protein state in which SYPRO Orange is saturated in the exposed hydrophobic regions of the protein. However, in the presence of each of the three ligands tested here, the prototypical stability curve could be discerned (for samples at low compound concentrations, there can be a significant subpopulation of unstructured protein as in the DMSO-only samples that increases background signal or noise, but cannot be discerned in the normalized curves). CITCO appeared to provide little thermal protection, as the melting point increased by only 1 °C. In contrast, the melting temperature increased by approximately 6.7 °C in the presence of PK11195, and CINPA1 proved able to increase the thermal denaturation temperature by

roughly 8 °C. Even though melting temperatures do not necessarily correlate with binding affinity, the fact that the hCAR-LBD is stabilized to a greater degree by CINPA1 than by the other known ligands is indicative of its direct and robust interaction with the hCAR-LBD.

3.1.2 Surface plasmon resonance—We examined the kinetics of CINPA1 binding to the hCAR-LBD by using SPR, a label-free technique that enables real-time monitoring of binding events (Figure 2). The quality of the association phase (k_{on}) in the sensogram could be improved considerably by pre-incubating hCAR-LBD with PK11195 to enable the formation of a stable protein-ligand complex. We presume that the pre-incubation prevented the coupling of reactive amines in protein regions that play a role in proper protein folding or ligand binding. After the coupling step, the PK11195 used in the pre-treatment was washed out to obtain the apo-protein for use in kinetic studies with CINPA1, CITCO, and PK11195. The signals (expressed in response unit, RU) arising from PK11195 and CINPA1 interaction with the hCAR-LBD were clearly discernible. In contrast, the corresponding signal from CITCO was inverted, indicating higher interactions of CITCO with the reference surface compared to the ligand surface. The association phase of the sensogram could be reliably fitted for PK11195 and CINPA1 using a 1:1 simple binding model [34], with association constants (K_{aa} or k_{on}) of $7.7 \pm 0.05 \text{ e}^4$ and $4.9 \pm 0.1 \text{ e}^3 \text{ M}^{-1}\text{s}^{-1}$, respectively. However, the dissociation constants (K_d or k_{off}) for CINPA1 and PK11195 could not be reliably determined because of the rapid dissociation signal. Therefore, a dependable binding affinity constant (K_D) could not be calculated, as this constant takes into account the dissociation constant ($K_D = k_{off}/k_a$): off rates were in excess of the instrument's resolvable limit of 1 sec^{-1} . Taking into consideration that the dissociation constants cannot be computed with confidence, the K_D would be estimated to be 33 μM and 548 μM for PK11195 and CINPA1, respectively. However, further assay optimization is required to improve the confidence of the dissociation constants for accurate K_D calculations, which then can be used for comparison with potencies obtained from other assays. It is likely that the pre-incubation of hCAR-LBD with PK11195 did not protect all primary amines in or around the binding site from coupling, which resulted in heterogeneous population that affects binding kinetics. Nevertheless, the SPR data demonstrate direct binding of CINPA1 to the hCAR-LBD with reasonable association rates and show that a compact and well-structured hCAR-LBD is necessary for appropriate association as a result of pre-incubation of the protein with PK11195.

3.1.3 Molecular dynamics simulations—We conducted molecular dynamics (MD) simulations to gather additional information about the stability of the protein in the presence of CINPA1 and to examine the dynamic changes in those hCAR regions that make contact with CINPA1 (Figure 3). Figure 3A compares the root mean square fluctuation (RMSF) of the hCAR-LBD in its unliganded form to that of the hCAR-LBD in the presence of CITCO, CINPA1, or PK11195. The RMSF profile characterizes the differential local changes in the protein at each residue over a period of 2 nanoseconds (ns). A drastic conformational change is generally noted only when there is a change of more than 3 Å, which was not seen with the ligands tested in our system. Nevertheless, the most destabilized residues in all cases (G126, Q146, L151, Q300, P305, and F307) correspond to residues present in unstructured loop regions, which are highly flexible segments of the proteins. This is consistent with the

results of our secondary structure element (SSE) analysis (data not shown), which indicated the absence of significant instability in the α -helix and β -strand motifs of the hCAR-LBD.

3.1.4 HDX-MS studies of CAR in the presence of CINPA1 and an NCoR1-3 peptide

—Binding of CINPA1 to hCAR confers protein stability, as evidenced by the thermal shift assay data. To obtain deeper insights into the structural dynamic perturbations due to CINPA1 binding, we employed hydrogen-deuterium exchange (HDX) coupled with mass spectrometry (MS), which enables the analysis of protein conformation changes induced by partner ligands in solution.

Comparisons between the HDX data obtained in the presence of CINPA1 and the data obtained with the apo-form of the protein provide a fingerprint of areas of the protein that are affected by ligand binding (Figures 4A and 5). Residues in these altered regions could be involved directly in ligand interactions; however, these perturbations can also be perceived at some distance from the ligand-binding site [35]. The presence of CINPA1 was seen to provide structural compactness in sections formed by α -helices, including H2, H3, H4, H5, H9, H10, and the short Hx. However, the C-terminus H12, which is part of the activation function 2 (AF-2) domain and is important for mediating the binding of coregulatory protein partners, was not affected by CINPA1.

The HDX experiments conducted in the presence of CINPA1 with or without the subsequent addition of a co-repressor NCoR1-3 peptide (ASNGLGLEDIIRKALMGSD) revealed a very different fingerprint from that resulting from differential HDX studies of CAR in the presence or absence of CINPA1 (Figures 4B and 5). Several notable regions were destabilized by the presence of the co-repressor peptide, corresponding mainly to portions of helices H1, H2, H3, H6, H7, H8, and H9 and a β -sheet. Some of these areas of greater structural fluidity corresponded to the interface with the binding partner retinoid X receptor (RXR). However, increased rigidity was observed in H10, Hx, and H12, the last of which is believed to interact directly with the NCoR1-3 peptide. Curiously, a region without defined SSEs between H2 and H3 displayed greater stability upon NCoR1-3 binding.

The short helix Hx was reported to be important for the constitutive activity of CAR [36] and has reduced flexibility in the presence of CINPA1, which is further stabilized by NCoR1-3. The two differential HDX profiles presented here provide additional support for the direct binding of CINPA1 to the hCAR-LBD and indicate how the presence of CINPA1 affects the dynamics of the hCAR-LBD upon NCoR1-3 binding.

3.2. Mutagenesis to probe the effects of CINPA1 on hCAR activity

We have previously described our studies of docking models of the hCAR-LBD bound to CINPA1, its metabolites [26], and its analog compound 72 [37], which were conducted to explain the observed differences in inhibitory potency. In this latest study, we performed MD simulations to further explore the interactions of CINPA1 with the various residues lining the ligand-binding pocket. Unlike docking experiments, which provide a static snapshot description of a protein-ligand interaction, MD simulations take into account the dynamic nature of these interactions and the intrinsic protein flexibility over a specified time. Figure 3B shows the various residues contacting CINPA1 over 2.0 ns, with regions of darker color

indicating stronger interactions. Only residues with contact of at least 30% over the entire simulation are shown: this default cutoff value from the software package was acknowledged as a good balance between increasing the chances for true positives and reducing false positives. Based on the findings from these MD simulations and our previous docking studies, we identified residues for further functional validation by mutagenesis followed by cell-based assays, as described below.

3.2.1 Role of N165 in the effect of CINPA1 on hCAR activity—Based on the report that CINPA1 docked with the hCAR-LBD [26], we predicted potentially important hydrogen-bond interactions between the ethyl carbamate of CINPA1 and N165 or H203, along with other prominent contacts with the protein. Hydrogen-bonding interactions at N165 appear to be important for the binding of all the ligands tested, as has been observed in previous simulations and experiments by others [38, 39].

Figure 6A displays the change in transactivation by CAR when N165 is mutated to alanine (N165A), as revealed by the transcription reporter assay. N165 was detrimental to the constitutive activity of hCAR1, as the N165-to-alanine mutation (N165A) resulted in an approximately 70% loss of activity, which is consistent with the findings of previous studies [40]. Additionally, N165 is critical for CINPA1 binding, as we postulated based on our docking studies, because CINPA1 was unable to inhibit CAR with the N165A mutation. However, the binding of CITCO was not affected, as it remained an activator of the receptor. This is consistent with the reported crystal structure of the hCAR-LBD in complex with CITCO, which shows a weak electrostatic interaction between N165 and CITCO [36]. We saw a more drastic loss of CAR activity when a negative charge was introduced at that site with an N165D mutation (Figure 6A).

3.2.2 Role of H203 in the effect of CINPA1 on hCAR activity—The results of our MD simulation suggest that H203 is the only residue that comes into contact with CINPA1 via hydrogen bonding (Figure 3B), with the other predicted contacts resulting from hydrophobic interactions. In cell assays, mutating H203 to alanine or to the other aromatic-containing residue tyrosine was not sufficient to significantly reduce constitutive CAR activation, but it resulted in reduced CINPA1 inhibition by comparison with the wild-type (WT) (Figure 6A). This suggests that distant hydrogen bonding, albeit in a weaker form than that observed for N165, remains possible. H203 does not appear to be critical for CITCO-mediated activation, which is consistent with the reported weak interactions observed in the crystal structure [36].

3.2.3 Role of N165 and H203 in the effect of CINPA1 on co-activator and co-repressor recruitment—Consistent with other reports, CAR interacted with the co-activator peptides SRC-1 and TIF2 in the absence of ligand, as the activity from WT hCAR with or without CITCO treatment was the basically the same, as revealed in the mammalian two-hybrid assay (Figures 6B and 6C). When compared to DMSO, whereas CINPA1 reduced the interaction of WT hCAR with SRC-1 (Figure 6B) and TIF2 (Figure 6C), it caused no significant reduction in the interactions of the mutants (N165A, N165D, H203A, and H203Y) with co-activators, presumably because there was less binding of CINPA1 with the mutated hCAR-LBD. On the contrary, the interaction of N165A with both co-activators

increased marginally in the presence of CINPA1 compared to DMSO-only samples. However, it is noteworthy to point out that the interaction of N165A with co-activators is substantially lower than that of WT hCAR. Concomitantly, CINPA1 enhanced interactions with the co-repressors SMRT and NCoR in WT hCAR, and this increase was not observed with the mutants (Figures 6D and 6E).

The asparagine at position 165 is important for increased co-activator interactions, because it forms a hydrogen bond with Y326, which in turn stabilizes L343 in the AF-2 helix [40]. Replacement of the polar residue asparagine by the negatively-charged glutamic acid would probably allow for a hydrogen bond between the mutant at position 165 and Y326, but the presence of the charge would most likely disrupt a cluster of hydrophobic residues around N165D, leading to destabilization of the region involved for coactivator interaction as observed in the mammalian two-hybrid assays.

H203 mutants displayed much-reduced overall interaction with co-activator or co-repressor peptides. The presence of CINPA1 appeared to have no effect on the interactions of the mutants with any of the four coregulator peptides tested.

3.2.4 Role of M168, L206, and F234 in the effect of CINPA1 on hCAR activity—

From our previous docking model, we identified three hydrophobic residues within the hCAR-LBD as important interacting partners of CINPA1, namely M168, L206, and F234 (Figure 7A). Of these, MD simulations showed F234 to have the strongest effect (Figure 3B). The mutation of any of these residues to alanine resulted in a noticeable loss of CINPA1-mediated inhibition of CAR activity, without substantial disruption of the constitutional activity or CITCO-induced activation of the receptor. Because the binding site has a highly hydrophobic character, it is unlikely that a single mutation of a hydrophobic residue would abolish CINPA1 binding completely; instead, the size reduction due to alanine replacement provides CINPA1 with more space in which to operate.

The ability of CINPA1 to repress the interactions between hCAR and the co-activators SRC-1 and TIF2 was compromised by mutating M168, L206, or F234 to alanine (Figures 7B and 7C). Interestingly, F234A by itself (i.e., in DMSO only) appeared to enhance co-activator recruitment when compared to WT hCAR.

Among the three mutants, CINPA1 enhanced SMRT recruitment to F234A alone (Figure 7D); however, CINPA1 had the weakest discernible effect on the inhibition of the CAR activity of F234A (Figure 7A). None of the mutants prevented the association of hCAR with NCoR in the presence of CINPA1 (Figure 7E), which suggests that CAR has a stronger affinity for NCoR than for SMRT. These findings would appear to indicate differences in co-repressor enlistment under diverse circumstances.

3.2.5 CINPA1 does not influence the loss of CAR constitutive activity due to mutations of key residues—A distinguishing feature of CAR is its ligand-independent constitutive activity, and several structural factors have been proposed to account for this. One such element is the existence of a barrier in front of the ligand pocket that fixes the AF-2 helix and the short helix X in the active conformation [36]. By using computational

tools, Jykkärinne *et al.* identified several residues that are important for the basal activity [40]. Based on the results of those studies, we selected the residues V199, F217, Y224, Y326, I330, and L343, in addition to N165, and examined their potential functional interactions with CINPA1 by mutagenesis followed by cell-based assays. In contrast to Jykkärinne and co-workers (who used the LBD of CAR in a GAL4-driven luciferase assay), we used a full-length hCAR1 construct to regulate the expression of a luciferase reporter controlled by the CYP2B6 enhancer/promoter region. Because CYP2B6 is the natural transcriptional target of hCAR, the receptor activity (WT or mutant) is proportional to its direct binding to the enhancer/promoter, as revealed by the luciferase activity.

As indicated in Figure 8A, N165A, V199A, Y326A, I330A, and L343A have drastically decreased basal activity, but the activity could be enhanced by adding CITCO. L343 forms part of the AF-2 helix, and not surprisingly, L343A showed the most dramatic loss of constitutive activity and the weakest reactivation by CITCO. Even though I330A lost most of its basal activity, it could still be activated to a considerable extent by CITCO. Only a mutation at N165 could abolish the inhibitory effect of CINPA1.

From the ligand-binding information obtained from the MD simulations, we concluded that F217 and Y224 are the most critical residues for CINPA1 binding (Figure 3B). We observed a complete loss of constitutive activity with the F217A and Y224A mutants, and CITCO was unable to rescue CAR activity (Figure 8B). Because of the loss of constitutive or agonist-induced activity of the F217A or Y224A mutants, we were unable to investigate the functional effect of mutating F217 or Y224 on the interaction of hCAR with CINPA1, therefore no conclusion can be drawn about the possible interaction between CINPA1 and F217 or Y224. This is an example of MD simulations demonstrating the possible importance of these residues in CINPA1 binding; they would go unnoticed if there was a complete loss of CAR activity.

In the mammalian two-hybrid assays with the F217A and Y224A mutants, we observed an almost complete loss of interaction with the co-activators SRC-1 and TIF2 (Figures 8C and 8D) and 50% and 75% losses of interaction with the co-repressors SMRT and NCoR, respectively (Figures 8E and 8F). Neither CINPA1 nor CITCO had any effect on coregulator recruitment to F217A and Y224A mutants. Interestingly, even though these two mutants almost completely blocked CAR constitutive and CITCO-induced activity (Figure 8B), there was still significant recruitment of SMRT.

In summary, we have demonstrated that CINPA1 interacts directly with the hCAR-LBD and that the binding of CINPA1 to the hCAR-LBD stabilizes the protein and assists in its transition from a fluid state to a more structured and compact globular state. This can be clearly deduced from thermal shift assays, and the local regions that become more structured are revealed by HDX-MS. The contributions by the potential hydrogen bond-forming residues N165 and H203 are important, as this type of interaction can be exploited to improve potency. The results of the functional assays of the mutants provide much-needed support for structure-based studies to understand SAR and pursue analogs with enhanced properties.

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Figure 1. Thermal shift assay of the hCAR-LBD at various concentrations (3.3–210 μM) of (A) CITCO, (B) PK11195, and (C) CINPA1. The average melting point values (in °C) are tabulated along with the standard errors of the mean. RFU, relative fluorescence unit.

Figure 2.
Surface plasmon resonance sensograms of hCAR-LBD interaction with PK11195 (light blue), CINPA1 (red), and CITCO (green). The signal is reported in response unit (RU).

Figure 3.

Molecular dynamics simulation of the hCAR-LBD. (A) Root mean square fluctuation (RMSF) depicting local changes in the protein in its apo-form (green) and in the presence of CINPA1 (blue), CITCO (pink), and PK11195 (maroon). (B) Protein-ligand contact profile for the hCAR-LBD with CINPA1 over a period of 2.0 ns showing total number of contacts from all residues (blue) and by individual residues (orange).

Figure 4. Results of HDX studies indicating perturbations in the structural conformation of the hCAR-LBD due to the presence of CINPA1 and NCoR1-3. The crystal structure of the hCAR-LBD (PDB code 1XVP) is overlaid with the differential HDX data for (A) CINPA1 and (B) CINPA1 with the subsequent addition of NCoR1-3. The structures are color coded according to the color bar at the bottom of the figure, where the colors correlate with differences in percentage of deuterium incorporation (%D). Regions that were not covered are shown in white.

Figure 5.

Differential HDX data for CINPA1 and for CNPA1 with the subsequent addition of NCoR1-3. The sequence of the covered peptides is reported along with the charge state of the ion (z), the secondary structural element of which the peptide is a part, and the hCAR starting and ending residue numbers. The values for each sample indicate the averaged difference in the percentage of deuterium incorporation for the corresponding peptide in two different states across all exchange time points (i.e., 10 s, 60 s, 300 s, and 900 s), and the values in parentheses represent the standard deviation calculated from three replicates. The smooth-color gradient key at the bottom of the figure represents the related protein regions with statistically significant differential deuterium incorporations, correlated with regions that show protection against solvent exchange. The statistical summary was derived from a two-way ANOVA for each pairwise experiment ($P < 0.05$). A negative value represents decreased deuterium incorporation or stabilization in the corresponding region of the hCAR-LBD when a binding event occurs. Peptides exhibiting statistically insignificant or undetectable changes are colored gray, and white regions indicate that the peptide was not detected in that experiment.

Figure 6.

Interactions of CINPA1 with the potentially hydrogen bond-forming residues N165 and H203. (A) HepG2 cells were transfected transiently with expression plasmids for hCAR1-WT, hCAR1-mutants, or pcDNA3.1 as the vector control, along with CYP2B6-luc reporter and TK-*Renilla* plasmids. Transfected cells were subsequently treated for 24 h with 0.1% DMSO, 1 μ M CINPA1, or 0.1 μ M CITCO. For each well, the firefly luciferase value was normalized to the *Renilla* luciferase value. The percentage of CAR-mediated activation (% hCAR1 activity) was calculated and reported by designating the control pcDNA3.1 (DMSO) luminescence signal as 0% and the hCAR1-WT (DMSO) signal as 100%. The Western blot on the right shows the protein levels of FLAG-CAR (WT and mutants), and the numbers (the ratio between FLAG-CAR and β -actin) below the protein bands indicate their relative intensity. The specificity of the anti-FLAG M2 antibody in detecting FLAG-CAR was demonstrated by using the pcDNA empty vector as a negative control. (B–E) Mammalian two-hybrid assays were set up in HEK293 cells transfected with expression plasmids

encoding GAL4DBD-coregulator fusion proteins, the reporter plasmid pG5luc, and VP16AD-hCAR1 fusions expressing either the WT receptor or mutant receptors (full length) involving (B) SRC-1, (C) TIF2, (D) SMRT, or (E) NCoR. Cells were treated with 0.1% DMSO (control), 0.1 μ M CITCO, or 1 μ M CINPA1 for 24 h before the luciferase activity was measured. The fold interaction represents the pG5luc reporter activity normalized to the *Renilla* luciferase internal control. Data are presented as the mean $\pm$ SD of at least three independent transfections. * $P < 0.001$ compared with DMSO treatment for each mutant/WT within the same coregulator set.

Figure 7. CINPA1 undergoes robust hydrophobic interactions with aromatic residues M168, L206, and F234. (A) HepG2 cells were transfected transiently with expression plasmids for hCAR1-WT, hCAR1-mutants, or pcDNA3.1 as a control, along with CYP2B6-luc reporter and TK-*Renilla* plasmids. Transfected cells were subsequently treated for 24 h with 0.1% DMSO, 1 μ M CINPA1, or 0.1 μ M CITCO. For each well, the firefly luciferase value was normalized to the *Renilla* luciferase value. The percentage of CAR-mediated activation was calculated and reported by designating the control pcDNA3.1 (DMSO) luminescence signal as 0% and the hCAR1-WT (DMSO) signal as 100%. The Western blot on the right shows the protein levels of FLAG-CAR (WT and mutants), and the numbers (the ratio between FLAG-CAR and β -actin) below the protein bands indicate their relative intensity. The specificity of the anti-FLAG M2 antibody in detecting FLAG-CAR was demonstrated by using the pcDNA empty vector as a negative control. (B–E) Mammalian two-hybrid assays were set up in HEK293 cells transfected with expression plasmids encoding GAL4DBD-coregulator fusion proteins, the reporter plasmid pG5luc, and VP16AD-hCAR1 fusions expressing either the WT receptor or mutant receptors (full length) involving (B) SRC-1, (C) TIF2, (D) SMRT, or

(E) NCoR. Cells were treated with 0.1% DMSO (control), 0.1 μ M CITCO, or 1 μ M CINPA1 for 24 h before the luciferase activity was measured. The fold interaction represents the pG5luc reporter activity normalized to the *Renilla* luciferase internal control. Data are presented as the mean $\pm$ SD of at least three independent transfections. * P < 0.001 compared with DMSO treatment for each mutant/WT within the same coregulator set.

Figure 8.

Residues strongly involved in maintaining the constitutive activity function of hCAR1. (A, B) HepG2 cells were transfected transiently with expression plasmids for hCAR1-WT, hCAR1-mutants, or pcDNA3.1 as a control, along with CYP2B6-luc reporter and TK-*Renilla* plasmids. Transfected cells were subsequently treated for 24 h with 0.1% DMSO, 1 μ M CINPA1, or 0.1 μ M CITCO. For each well, the firefly luciferase value was normalized to the *Renilla* luciferase value. The percentage of CAR-mediated activation was calculated and reported by designating the control pcDNA3.1 (DMSO) luminescence signals as 0% and the hCAR1-WT (DMSO) signal as 100%. The Western blot below the bar charts shows the protein levels of FLAG-CAR (WT and mutants), and the numbers (the ratio between FLAG-CAR and β -actin, multiplied by 10) below the protein bands indicate their relative intensity. The specificity of the anti-FLAG M2 antibody in detecting FLAG-CAR was demonstrated by using the pcDNA empty vector as a negative control. (C–F) Mammalian two-hybrid assays were set up in HEK293T cells transfected with expression plasmids encoding GAL4DBD-coregulator fusion proteins, the reporter plasmid pG5luc, and VP16AD-hCAR1

fusions expressing either the WT receptor or mutant receptors (full length) involving (C) SRC-1, (D) TIF2, (E) SMRT, or (F) NCoR. Cells were treated with 0.1% DMSO (control), 0.1 μ M CITCO, or 1 μ M CINPA1 for 24 h before the luciferase activity was measured. The fold interaction represents the pG5luc reporter activity normalized to the *Renilla* luciferase internal control. Data are presented as the mean $\pm$ SD of at least three independent transfections. * P < 0.001 compared with DMSO treatment for each mutant/WT within the same coregulator set.

Table 1

hCAR1 mutants. The mutated sequences are indicated in bold, and the wild-type sequences are underlined.

N165	cacttcgcagacatc <u>AA</u> Cactttcatggtactgcaagtc /HFADINTFMVLQV
N165A	cacttcgcagacatc GCC Cactttcatggtactgcaagtc /HFADIA T FMVLQV
N165D	cacttcgcagacatc GAC Cactttcatggtactgcaagtc /HFADID T FMVLQV
M168	gcagacatcaacactttc <u>ATG</u> gtactgcaagtcacatcaagtttactaag /ADINTFMVLQVIKFTK
M168A	gcagacatcaacactttc GCC gtactgcaagtcacatcaagtttactaag /ADINTF A VLQVIKFTK
V199	cagatctcccttctcaaggagcagctGTGaaatctgtcacatcgactcaat /QISLLKGAAVEICHIVLN
V199A	cagatctcccttctcaaggagcagct GCG gaaatctgtcacatcgactcaat /QISLLKGAA A EICHIVLN
H203	aaggagcagctgtggaatctgtCACatcgactcaataccactttctgt /KGAAVEICHIVLNTTFC
H203A	aaggagcagctgtggaatctgt GCC atcgactcaataccactttctgt /KGAAVEIC A IVLNTTFC
H203Y	aaggagcagctgtggaatctgt TAC atcgactcaataccactttctgt /KGAAVEIC Y IVLNTTFC
L206	gcagctgtggaatctgtcacatcgtaCTCaataccactttctgtctc /AAVEICHIVLNTTFCL
L206A	gcagctgtggaatctgtcacatcgta GCC aataccactttctgtctc /AAVEICHIV A NTTFCL
F217	actttctgtctccaaacacaaaac <u>TT</u> Cctctgcgggcctcttcgctac /TFCLQTQ N FLCGPLRY
F217A	actttctgtctccaaacacaaaac GCC ctctgcgggcctcttcgctac /TFCLQTQ N ALCGPLRY
Y224	ttcctctgcgggcctcttcgcTACacaattgaagatggagcccggtga /FLCGPLRY T IEDGARV
Y224A	ttcctctgcgggcctcttcgc GCC acaattgaagatggagcccggtga /FLCGPL R ATIEDGARV
F234	gaagatggagcccggtgtagggTTCcaggtagagtttttgagttgctc /EDGARVG F QVEFLELL
F234A	gaagatggagcccggtgtaggg GCC caggtagagtttttgagttgctc /EDGARVG A QVEFLELL
Y326	gagctccggagcattaatgaggccTACgggtaccaaaccagcacatc /ELRSINEAYGYQIQHI
Y326A	gagctccggagcattaatgaggcc GCC gggtaccaaaccagcacatc /ELRSINE A GYQIQHI
I330	aatgaggcctacgggtaccaaATCagcacatccagggcctgtctgccat /NEAYGYQ I QHIQGLSA
I330A	aatgaggcctacgggtaccaa GCC cagcacatccagggcctgtctgccat /NEAYGY Q AQHIQGLSA
L343	cagggcctgtctgccatgatgccgctgCTCaggagatctgcagc /QGLSAMPL L QEICS
L343A	cagggcctgtctgccatgatgccgctg GCC caggagatctgcagc /QGLSAMPL A QEICS