Published in final edited form as:

Methods Mol Biol. 2016 ; 1473: 33–42. doi:10.1007/978-1-4939-6346-1_4.

Quantitative High-throughput Luciferase Screening in Identifying CAR Modulators

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Summary

The constitutive androstane receptor (CAR, NR1I3) is responsible for the transcription of multiple drug metabolizing enzymes and transporters. There are two possible methods of activation for CAR, direct ligand binding and a ligand-independent method, which makes this a unique nuclear receptor. Both of these mechanisms require translocation of CAR from the cytoplasm into the nucleus. Interestingly, CAR is constitutively active in immortalized cell lines due to the basal nuclear location of this receptor. This creates an important challenge in most in vitro assay models because immortalized cells cannot be used without inhibiting the basal activity. In this book chapter, we go into detail of how to perform quantitative high-throughput screens to identify hCAR1 modulators through the employment of a double stable cell line. Using this line, we are able to identify activators, as well as deactivators, of the challenging nuclear receptor, CAR.

Keywords

Constitutive Androstane Receptor (CAR); Cytochrome P450 2B6 (CYP2B6); Luciferase; Quantitative high-throughput screening (qHTS)

1. Introduction

The constitutive androstane receptor (CAR, NR1I3), is a well-known transcription factor found mainly in the liver and intestine which modulates the expression of drug metabolism genes, such as oxidation and conjugation enzymes, while also regulating certain transporters [1,2]. Through this gene regulation, CAR has the potential to play a major role in drug-drug interactions. Drugs which activate CAR, for example, can induce cytochrome P450 (CYP) 2B6 and CYP3A4 which in turn will increase the clearance of any drug metabolized by these two enzymes. Alternately, when CAR is deactivated and CYP2B6 and CYP3A4 protein expression is inhibited, a previously prescribed drug can become toxic to the body due to the decreased metabolism. Recently, it has also been implicated that CAR plays a pivotal role in energy metabolism [3,4]. Therefore, it is not only important to identify compounds causing potential drug-drug interactions, but also drugs which can have a therapeutic effect through hCAR activation.

One of the unique features of CAR is the differing initial localization found when comparing human primary hepatocytes (HPH) and immortalized cell lines. This nuclear receptor has basal localization in the cytoplasm until activation occurs. Once stimulated, CAR will translocate into the nucleus and begin the activation process. However, in immortalized cell lines, CAR constantly resides inside the nucleus without stimulation. When performing in vitro assays, this nuclear localization results in high constitutive activity and difficulty to increase the signal further even after xenobiotic stimulation [5–7]. Because of the high cost and low availability of HPH, it is not an ideal option to use them in high-throughput screenings (HTS). Using immortalized cells is an excellent choice when working with HTS because of their easy culturing and relatively cheap characteristics.

Due to the basal activity of hCAR in immortalized cells as stated previously, a low concentration of a known deactivator can be co-treated with a test compound to decrease the constitutive activity and allow for the prediction of an activator. The most well-known deactivators of CAR are 1-(2-chlorophenylmethylpropyl)-3-isoquinoline-carboxamide (PK11195), meclizine, and clotrimazole [8–10]. However, along with deactivating CAR, meclizine has inconsistently been reported as a hCAR inverse agonist, a mCAR agonist, and also to have no effect on hCAR [8,11]. There is also conflicting clotrimazole data. This drug's deactivation effects came into question when confirmation could not be completed along with varying results in different cell lines [12,13]. Deactivation of hCAR by PK11195 has not been called into question; however, it has also been shown to be an activator of PXR in HPH. This activation of PXR overrides its deactivation of CAR in HPH, proving PK11195 to be a viable option of hCAR deactivation only in immortalized cell lines [9].

The luciferase reporter gene assay is a common technique utilized to determine modulation of receptors [14,15]. A basic way to perform this assay is to transfect an expression plasmid containing the nuclear receptor along with a vector containing the promoter region of its target gene with a downstream luciferase reporter into a cell line of choice. However, when performing a HTS using 1536-well plates, the amount of cells per well is an important aspect of each experiment. Because transfection rates are not 100%, it is difficult to use transient transfection in a HTS. Therefore, stably transfecting the cells is an important first step in this protocol.

There are significant issues to overcome when identifying hCAR modulators using in vitro methods. This newly adopted quantitative HTS approach overcomes many of the difficulties residing throughout the luciferase assay [16]. However, there are still limitations for this HTS. For instance, this assay is more likely to identify direct activators than indirect activators. Further studies should be completed to confirm actual hCAR modulation. Here, we outline step-by-step instructions to generate this HepG2-CYP2B6-hCAR stable cell line alongside the quantitative HTS luciferase method.

2. Materials

2.1 Equipment

1. GLOMAX® 20/20 Single-tube Luminometer (Promega) for the luciferase assay.

2. CO₂ incubator MCO-17AIC for all cell work.
3. 1536-well plates for the high-throughput screen.
4. Multidrop Combi (Thermo Fisher Scientific Inc) for plating cells in the large screen.
5. Pintool station (Kalypsys) to transfer compounds into the 1536-well assay plates.
6. Bioraptr Flying Reagent Dispenser workstation (Beckman Coulter) to add activator/deactivator into the assay plates.
7. ViewLux plate reader (PerkinElmer) to read the luminescent and fluorescent signals created by the ONE-Glo and CellTiter Fluor reagents (Promega).

2.2 Reagents and Solutions

1. Collagen solution: Using distilled water, prepare 130 µg/mL MCDI (N-Cyclohexyl-N-(2-morpholinoethyl)carbodiimide metho-p- toluenesulfonate) solution. Make a 100 µg/mL solution of collagen type I from rat tail in MCDI solution.
2. Seeding medium for HepG2 cells: 500 mL DMEM, 50 mL fetal bovine serum (FBS), 5 mL penicillin-streptomycin.
3. Transfection medium for HepG2 cells: 500 mL DMEM, 50 mL FBS.
4. Culture medium for the HepG2-CYP2B6-hCAR cells: DMEM, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.6–1 mg/mL geneticin, 10 µg/mL blasticidin.
5. CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime).
6. PK11195 (1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide).
7. Opti-MEM® I reduced-serum medium.
8. pEF6/V5-hCAR expression plasmid.
9. pGL4.17[luc2/Neo]-CYP2B6-2.2 kb construct containing both the PBREM and XREM.
10. ONE-Glo luciferase reagent (Promega) to create a luminescent signal during the assay.
11. CellTiter-Fluor reagent (Promega) used for determining the viability of cells.

3. Methods

3.1 Generation of hCAR-CYP2B6-HepG2 Cell Line

1. Pre-coated collagen dishes are used to plate the HepG2 cells so that a clear monolayer occurs. Table 1 describes the volume of cold collagen solution which

should be added to each dish and well or collagen-coated flasks and dishes may be purchased. Once the solution is in the dish/flask/plate, incubate at 37°C with 5% CO₂ overnight. The next day, take the items out of the incubator and remove the liquid. Add PBS to each well and seal each lid with parafilm. Once wrapped, store the items in 4°C until ready for using (*see* Note 1).

2. Warm the seeding medium to 37°C in a water bath.
3. Plate cells in a pre-coated 6-well plate at a seeding density of 5×10^5 cells/well using the pre-warmed medium. Incubate cells for 5 hours, or until attached, at 37°C and 5% CO₂.
4. Check confluency of the cells; they should be about 70 – 90% confluent to proceed to the next step.
5. Use Table 2 to combine the proper amounts of each reagent to the wells of plated HepG2 cells. Let the mix sit for 5 to 25 minutes at room temperature before adding to the well.
6. Replace the seeding medium in the well with 1.75 mL of transfection medium.
7. Add 250 µL of the transfection mix into each well and incubate at 37°C and 5% CO₂ overnight.
8. Trypsinize two of the wells and reseed cells into about ten 10 cm² dishes using the culture medium for HepG2-CYP2B6-hCAR cells for selection. The confluency should be around 10 – 20% to allow optimal space for colony growth.
9. Change the medium, including blasticidin and geneticin, on every dish every 3–4 days. Allow cells to grow for about 2 weeks until the colonies can be seen with the naked eye.
10. Identify the single colonies on each dish (about 5–10 colonies) and isolate them using a cloning cylinder. Trypsinize each colony by adding 100 µL of trypsin inside the cylinder and allow it to sit for about 2 min.
11. Acquire two pre-coated 48-well plates (*see* Note 2) and add 500 µL of culture medium into each well of plate 1.
12. Gently pipette the trypsin inside each cylinder and pipette all liquid from inside one cylinder into one of the wells with medium on plate 1. Mix the well by gently pipetting up and down and transfer 250 µL of the contents into the corresponding empty well in plate 2.
13. Repeat steps 10 – 12 until each colony has been plated in corresponding wells for both plates.

¹Once coated, the plates can be kept in 4°C up to 2 months.

²Use plate 1 to perform the luciferase assay to determine if the cells have both hCAR1 and CYP2B6 transfected properly into them and use plate 2 to keep colony growing.

14. Treat every well on plate 1 with 1 μ M CITCO and incubate at 37°C/5% CO₂ for 24 h. Incubate plate 2 with culture medium only until the luciferase assay on plate 1 is complete.
15. After plate 1 is treated for 24 h, rinse each well with PBS and add 75 μ L lysis buffer to create the cell lysate (*see* Note 3).
16. Combine 25 μ L of firefly from a Dual-Luciferase[®] Assay System Kit (Promega) and 15 μ L of the lysate in a 1.5 mL tube and read the luminescence value using a GLOMAX luminometer.
17. Once the data is collected, keep any colonies, in plate 2, which have a value above 1000 so that further tests can be completed.
18. Once plate 2 is confluent, transfer the cells which have a luminescence value above 1000 to a 6-well plate and allow the colony to grow until confluent using culture medium.
19. For each colony, using culture medium, plate 6 wells of a pre-coated 48-well plate at a concentration of 5×10^4 cells/well, and keep the rest of the cells from that colony in one well of a 6-well plate to allow the colony to grow. Incubate these plates at 37°C and 5% CO₂.
20. Once the cells have attached to the bottom of the plate, treat 3 of the wells with 0.1% DMSO and the other 3 wells with 1 μ M CITCO in culture medium. Allow the cells to incubate at 37°C/5% CO₂ for 24 h.
21. Repeat steps 19 and 20 for all colonies to be analyzed.
22. Calculate the average of the three DMSO treated wells and divide each CITCO value by this average. Complete this calculation for each colony separately. Then, determine the average of these three newly calculated numbers to identify the fold induction for each colony. The colony with the highest fold induction and smallest standard deviation was chosen to use.
23. The selected colony should be kept growing in culture medium in a collagen-coated flask. This newly generated double stable cell line should be used to perform the quantitative high-throughput screen in the next assay.

3.2. Assay Optimization in a 1536-Well Plate Format

1. Using culture medium, plate the double stable HepG2-CYP2B6-CAR cells into a 1536-well plate at a density of 2,500 cells/well in 4 μ L using the Multidrop Combi.
2. Allow plates to incubate at 37°C/5% CO₂ for 4–5 h or until the cells have attached to the bottom.

³The experiment can either proceed immediately to the next step or the entire plate with lysate can be stored in a –20°C freezer until the assay is ready to be completed.

3. Create a positive control plate. For the agonist mode, the first column should be 16 duplicate concentrations of a dose response curve of CITCO. The starting concentration is 20 mM (final assay concentration of 77 μ M) with a 1:2 serial dilution. The second column should have the top 16 wells be CITCO at a single dose of 13 mM (final assay concentration of 50 μ M), while the bottom 16 wells are 10.4 mM (final assay concentration of 40 μ M) CITCO. The third column should have the top 16 wells contain DMSO and the bottom 16 wells comprise of 20 mM (final assay concentration of 77 μ M) tetraoctylammonium bromide for cytotoxicity control. The fourth column should be comprised solely of DMSO. For the antagonist mode, the first column should be 16 duplicate concentrations of a dose response curve of PK11195. The starting concentration is 20 mM (creating a final concentration of 77 μ M) with a 1:2 serial dilution. The second column should have the top 16 wells be PK11195 at a single dose of 7.8 mM (final assay concentration of 30 μ M), while the bottom 16 wells are 5.2 mM (final assay concentration of 20 μ M) PK11195. The third column should have the top 16 wells contain DMSO and the bottom 16 wells comprise of 20 mM (final assay concentration of 77 μ M) tetraoctylammonium bromide. The fourth column should be comprised solely of DMSO.
4. Create a plate with only DMSO in columns 5–48.
5. Once the cells have attached, use the Pintool station to transfer 23 nL of the positive control or DMSO into the assay plates.
6. Use PK11195 to reduce the basal level of CAR expression and test several concentrations of PK11195 (final 0, 0.5, 0.75, 1, and 1.5 μ M) for agonist mode in every well. For the antagonist mode, test several CITCO concentrations (final 0, 25, 50, and 100 nM) in every well.
7. Incubate the assay plates at 37°C/5% CO₂ for 23 h.
8. Add 1 μ L of Cell-Titer Fluor to each well and incubate at 37°C/5% CO₂ for 1 h.
9. Read the fluorescence of each plate by using a ViewLux plate reader.
10. Immediately following the fluorescence reading, add 4 μ L of ONE-Glo reagent and allow the plates to incubate at room temperature for 30 minutes.
11. Read the luminescence of each plate by using a ViewLux plate reader.
12. Use the following calculations to acquire the CV, S/B ratio, and z-factors for each plate.

$$CV = (\text{Standard deviation of replicates} / \text{mean of replicates}) * 100\%$$

$$Z \text{ factor} = 1 - 3 * [(\text{st. dev. Positive control} + \text{st. dev. Negative control}) / (\text{mean positive control} - \text{mean negative control})]$$

$$S/B = \text{signal} / \text{background}$$

To determine the optimal concentration to use, look at every factor (Table 3) as well as the graphical representation of the concentration curves, as shown in Fig 1.

3.3. Quantitative High-throughput Screen

1. Create new positive control plates using the same methods as in the previous section.
2. Acquire compound plates, where the test compounds are in columns 5 – 48. Each compound has fifteen 2.236-fold serial dilutions so that half maximal activation (EC_{50} s) and half-maximal inhibition (IC_{50} s) can be obtained.
3. Add activator or deactivator to each plate as optimized in Subheading 3.2 (*see* Note 4).
4. Perform qHTS as previously determined to be the optimal assay condition in Subheading 3.2.
5. Normalize the agonist mode plates to 57 μ M CITCO equaling 100%, and 38 μ M PK11195 equaling 100% for the antagonist mode plates. DMSO should also be set to equal 0% activity. The EC_{50} s, IC_{50} s, and efficacy (maximal response value) for each compound will be calculated by in house data pipeline (*see* Chapter 12 in this book).

References

1. Honkakoski P, Zelko I, Sueyoshi T, Negishi M. The Nuclear Orphan Receptor CAR-Retinoid X Receptor Heterodimer Activates the Phenobarbital-Responsive Enhancer Module of the CYP2B Gene. *Molecular and Cellular Biology*. 1998; 18(10):5652–5658. [PubMed: 9742082]
2. Wang H, LeCluyse E. Role of Orphan Nuclear Receptors in the Regulation of Drug-Metabolising Enzymes. *Clin Pharmacokinet*. 2003; 42(15):1331–1357. DOI: 10.2165/00003088-200342150-00003 [PubMed: 14674787]
3. Lynch C, Pan Y, Li L, Heyward S, Moeller T, Swaan PW, Wang H. Activation of the constitutive androstane receptor inhibits gluconeogenesis without affecting lipogenesis or fatty acid synthesis in human hepatocytes. *Toxicology and Applied Pharmacology*. 2014; 279(1):33–42. <http://dx.doi.org/10.1016/j.taap.2014.05.009>. [PubMed: 24878338]
4. Dong B, Saha PK, Huang W, Chen W, Abu-Elheiga LA, Wakil SJ, Stevens RD, Ilkayeva O, Newgard CB, Chan L, Moore DD. Activation of nuclear receptor CAR ameliorates diabetes and fatty liver disease. 2009; 106doi: 10.1073/pnas.0909731106
5. Chen T, Tompkins LM, Li L, Li H, Kim G, Zheng Y, Wang H. A single amino acid controls the functional switch of human constitutive androstane receptor (CAR) 1 to the xenobiotic-sensitive splicing variant CAR3. *The Journal of pharmacology and experimental therapeutics*. 2010; 332(1): 106–115. [PubMed: 19820207]
6. Li H, Chen T, Cottrell J, Wang H. Nuclear Translocation of Adenoviral-Enhanced Yellow Fluorescent Protein-Tagged-Human Constitutive Androstane Receptor (hCAR): A Novel Tool for Screening hCAR Activators in Human Primary Hepatocytes. 2009; 37doi: 10.1124/dmd.108.026005
7. Zelko I, Sueyoshi T, Kawamoto T, Moore R, Negishi M. The Peptide Near the C Terminus Regulates Receptor CAR Nuclear Translocation Induced by Xenochemicals in Mouse Liver. *Molecular and Cellular Biology*. 2001; 21(8):2838–2846. DOI: 10.1128/mcb.21.8.2838-2846.2001 [PubMed: 11283262]
8. Huang W, Zhang J, Wei P, Schrader WT, Moore DD. Meclizine Is an Agonist Ligand for Mouse Constitutive Androstane Receptor (CAR) and an Inverse Agonist for Human CAR. *Molecular Endocrinology*. 2004; 18(10):2402–2408. DOI: 10.1210/me.2004-0046 [PubMed: 15272053]

⁴For example, in the agonist mode, add 1 μ L of 4.5 μ M PK11195, diluted in culture medium, to make a final concentration of 0.75 μ M PK11195 inside each well using the Bioraptr. For the antagonist mode, add 1 μ L of 300 nM CITCO, diluted in culture medium, to make a final concentration of 50 nM CITCO inside each well also using the Bioraptr.

9. Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M, Wang H. The Peripheral Benzodiazepine Receptor Ligand 1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide Is a Novel Antagonist of Human Constitutive Androstane Receptor. 2008; 74doi: 10.1124/mol.108.046656
10. Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, Kliewer SA. Orphan Nuclear Receptors Constitutive Androstane Receptor and Pregnane X Receptor Share Xenobiotic and Steroid Ligands. *Journal of Biological Chemistry*. 2000; 275(20):15122–15127. DOI: 10.1074/jbc.M001215200 [PubMed: 10748001]
11. Lau AJ, Yang G, Rajaraman G, Baucom CC, Chang TKH. Differential Effect of Meclizine on the Activity of Human Pregnane X Receptor and Constitutive Androstane Receptor. *Journal of Pharmacology and Experimental Therapeutics*. 2011; 336(3):816–826. DOI: 10.1124/jpet.110.175927 [PubMed: 21131266]
12. Mäkinen J, Frank C, Jyrkkärinne J, Gynther J, Carlberg C, Honkakoski P. Modulation of Mouse and Human Phenobarbital-Responsive Enhancer Module by Nuclear Receptors. *Mol Pharmacol*. 2002; 62(2):366–378. DOI: 10.1124/mol.62.2.366 [PubMed: 12130690]
13. Toell A, Kröncke K-D, Kleinert H, Carlberg C. Orphan nuclear receptor binding site in the human inducible nitric oxide synthase promoter mediates responsiveness to steroid and xenobiotic ligands. *Journal of Cellular Biochemistry*. 2002; 85(1):72–82. DOI: 10.1002/jcb.10104 [PubMed: 11891851]
14. Freitas J, Miller N, Mengeling BJ, Xia M, Huang R, Houck K, Rietjens IMCM, Furlow JD, Murk AJ. Identification of Thyroid Hormone Receptor Active Compounds Using a Quantitative High-Throughput Screening Platform. *Current Chemical Genomics and Translational Medicine*. 2014; 8:36–46. DOI: 10.2174/2213988501408010036 [PubMed: 24772387]
15. Shukla SJ, Sakamuru S, Huang R, Moeller TA, Shinn P, VanLeer D, Auld DS, Austin CP, Xia M. Identification of Clinically Used Drugs That Activate Pregnane X Receptors. *Drug Metabolism and Disposition*. 2011; 39(1):151–159. DOI: 10.1124/dmd.110.035105 [PubMed: 20966043]
16. Lynch C, Zhao J, Huang R, Xiao J, Li L, Heyward S, Xia M, Wang H. Quantitative High-Throughput Identification of Drugs as Modulators of Human Constitutive Androstane Receptor. *Sci Rep*. 2015; 5 <http://www.nature.com/srep/2015/150520/srep10405/abs/srep10405.html#supplementary-information>. doi: 10.1038/srep10405

Figure 1.
 Concentration curves of hCAR1 optimization. A double stable HepG2.CYP2B6.CAR cell line was used to perform a luciferase assay in agonist and antagonist mode. Varying

concentrations of PK11195 and CITCO were used to identify the EC₅₀ of CITCO and PK11195 respectively for the agonist mode (A) and antagonist mode (B).

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Table 1

Preparation of collagen-coated plates.

	Collagen solution (mL)	1× PBS (mL)
10 cm ² Dish	10	10
6-well plate	2	2
48-well plate	0.25	0.25

Table 2

Transfection Mix.

6-well plate (μL/well)	
Opti-MEM®	227
CYP2B6 (100 ng/μL)	12
hCAR1 (100 ng/μL)	6
Lipofectamine® 2000	5
250 μL/well	

Table 3

Optimization Factors.

	Agonist Mode					Antagonist Mode				
	0 μ M	0.5 μ M	0.75 μ M	1 μ M	1.5 μ M	0 μ M	25 nM	50 nM	100 nM	
S/B	2.14	3.81	4.30	5.10	5.87	7.18	8.00	7.68	7.92	
CV (%)	5.04	5.53	10.00	7.43	10.06	6.42	6.13	2.96	6.29	
Z factor	0.53	0.60	0.64	0.73	0.54	0.78	0.83	0.93	0.90	