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Full Length Article

Identification of the Pig Constitutive Androstane Receptor (pgCAR) Agonist using a High-throughput Screening Assay

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Abstract

Pigs are encountering a large number of xenobiotics potentially harmful consequences, which is not only bad for animal health and pork quality but also bad for human health. Therefore, identification of the Pig Constitutive Androstane Receptor (pgCAR) agonist would be beneficial for pigs and consumer, because of its pivotal role in xenobiotics-metabolism. In this study, A stable and sensitive cell-based high throughput screening (HTS) model is conducted in a 48-well format using the human hepatoma HepG2 cells transiently transfected with pcDNA3.1-pgCAR plasmid and reporter plasmids to identify chemicals or natural compounds that would promote CAR，and calibrated with reference pgCAR agonist, phenytoin. In conclusion, three active ingredients including Vitamin C, Folate and Sitosterol, were picked out as agonists by the high-throughput screening from 13 compounds, particularly the effects of Sitosterol. © 2017 Friends Science Publishers

Keywords: pgCAR; Agonists; High-throughput screening; Xenobiotics

Introduction

Organisms encounter a large number of xenobiotics with potentially harmful consequences (Wei *et al.*, 2000). The xenobiotics including veterinary drugs, antibiotics, natural and artificial hormones, environmental contaminants and residues caused great concerns of consumers (Pompa *et al.*, 2005; Emanuela *et al*., 2011). In addition, some endogenous substances such as androstenone and skatole presented in adipose tissue of some entire males are the major causes of boar taint, (Doran *et al*., 2002; Aluwe *et al*., 2009) and metabolise in liver (Moe *et al*., 2008). Upon exposure to xenobiotics, the increasement of expression and activity of hepatic enzymes involved in the metabolism of both xenobiotics and endogenous compounds often results from the activation of "xenosensors" such as the CAR (Be´atrice *et al.*, 2013). CAR has been shown to regulate the expression of overlapping sets of genes including phase I enzymes such as CYP2B and CYP3A (Wei *et al.*, 2002).

Drug-metabolizing enzymes (DMEs) and transporters play important roles in the disposition and detoxification of these potentially harmful xenobiotics and endogenous substances (Toslon and Wang, 2010). And the nuclear receptor CAR (Constitutive Androstane Receptor) is mainly expressed in liver, where it is activated by structurally diverse ligands and then regulates cytochrome P450 and other genes whose products are involved in DMEs and transporters (Tien and Negishi, 2006; Osabe *et al*., 2009; DeKeyser and Omiecinsk, 2010). Therefore, CAR plays central role in protecting the body against xenobiotics and endogenous substances (Honkakoski and Negishi, 2000; Maglich *et al*., 2002). Moreover, research suggested that the 5-amino acid insertion that represents human CAR also has the function of ligand-activated receptor in other species' CAR, and when selecting CAR ligands can maintain signature responses in each species (Omiecinski *et al*., 2011).

Not only xenochemicals, but also endogenous substances and other naturally occurring compounds have been identified as ligands for mouse CAR and human CAR (Chang and Waxman, 2006). Among numerous of ligands, especially Phenytoin and 6-(4-chlorophenyl) imidazo [2,1-b][1,3] thiazole-5-carbald-ehyde-(3,4-dichlorobenzyl) oxime (CITCO) have been reported as CAR agonists in primary hepatocytes (Peacock, 2005; Küblbeck *et al*., 2011). Since the initial discoveries that natural products have medicinal value and serve as a platform for various drug discovery with less toxicity and drug residues (Koehn and Carter, 2005), but the potential risks related to this type of product which has been recognized as the use of herbal medicinal products (HMP) increases (Jordan *et al*., 2010). In this study, we extracted several active ingredients for detoxication from herbal medicines and discussed its regulation in liver.

In contrast with traditional method for screening drugs *in vivo*, the new optical assay methods of High-Throughput

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Screening (HTS), are synthesized using automated and combinatorial methods to screen innumerable compounds efficiently (Burbaum and Sigal, 1997). The goal of HTS assays is to provide sufficient performance to support the testing of lots of samples in a highly reproducible manner (Michael and Douglas, 2014), HTS is an early critical step to provide a rapid and accurate method in drug discovery (Malo *et al*., 2006). In the development of high throughput screening as a central model of drug discovery, fluorescence has been generally adopted as methodology which in the good graces of research fields (Fan and Wood, 2007). Thus, the screening and validation approach presented here offers a cell-based High-Throughput Screening in the use of bioluminescent assays to establish a model for screening agonists and it may be an efficient approach for rapid identification of new additives.

Transfection is a process of inserting nucleic acid into somatic cells or cell lines using various chemical or physical methods. Transfection has been popular as a non-viral delivery system. Calcium phosphate precipitation method and PEI-DNA condensation method are two most popular methods (Chahal *et al*., 2011).

For a long time, the boar is generally used the traditional way of castration to control the problem of boar taint. Researches showed that castration can not only reduce boar taint but cause animal welfare issues. It's not optimal to improve the meat production of animals by limiting slaughtering weight, proceeding genetic improvement, utilizing immunocastration. The research of endogenous mechanism and the metabolic process, will help to select the low toxicity, no-residue and more effective feed additives.

This study intends to establish a cell-based high-throughput screening model for pig CAR agonist by Dual-Luciferase® Reporter Assay System. The model is sensitive and stable to provide theoretical basis and experimental foundation for a series of agonist screening of pgCAR and gene expression. This study identified the ingredients as activators or inhibitors of pgCAR. In conclusion, the screening and validation approach presented here offers a possibility to be an efficient approach for rapid identification of new additives.

Materials and Methods

Materials

All drugs for screening were purchased from Aladdin Ltd (Shanghai, China). Cell culture medium DMEM, fetal bovine serum (FBS), PBS, trypsin (0.25%), penicillin-streptomycin, OptiMEM and pcDNA3.1 vector were purchased from GIBCO/Invitrogen (Carlsbad, CA). PGL3 plasmid, pRL plasmid and Dual-Luciferase® Reporter Assay System were ordered from Promega (Madison, WI). HepG2 cells from ATCC (Manassas, VA). *BamH I*, *Hind III* and *Bgl II* were the products of TaKaRa Biotechnology Co., Ltd. (Dalian, China). The primers and total gene sequence of $(NR1)_5$-TK were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The samples of liver were obtained from healthy boars which were fed in experimental base of Northeast Agricultural University and stored in liquid nitrogen.

Extraction, Reverse Transcription and Sequencing of pgCAR

The sus scrofa CAR sequence (gi: 83582557) was obtained from the National Center for Biotechnology Research (NCBI) website http://www.ncbi.nlm.nih.gov. Boar liver samples (100 mg) were milled in liquid nitrogen and homogenized in 1 mL RL-reagent. Total RNA was then extracted following the manufacturer's instructions. The isolated RNA was assayed by measuring the absorbance at 260 nm and purity analyzed by the 260 nm/280 nm ratio, which was always above 1.9. Then converted RNA to cDNA following the Reverse Transcription kit instructions. The RT reaction conditions: 25°C for 10 min, 37°C for 120 min, followed by 5 min incubation at 85°C. Following the reverse transcription reaction, forward primer 5'-CCC AAG CTT GCC ATG GCC AGC GGG GAA GA-3' and reverse primer 5'-CGC GGA TCC TCA GCT GCA GAT CTC CTG GA-3' were used to amplify the full encoding sequence of pgCAR using DNA polymerase, and 2 μL of the first strand cDNA as a template then following the manufacturer's instructions. The PCR conditions (94°C 5 min [94°C 30 s, 58°C 1 min, 72°C 1.5 min] ×35 cycles, 72°C 10 min). Amplified pgCAR was analyzed at 1% agarose gels in 1×TAE buffer. Then the PCR products were ligated into the pMD18-T cloning plasmid for sequencing.

Construction of Plasmids

The pgCAR sequence was inserted between the *BamH* I and *Hind* III sites of the pcDNA3.1 (+) expression vector to construct the pcDNA3.1-pgCAR expression plasmid. $(NR1)_5$-tk (thymidine kinase)-luciferase plasmid was constructed by cloning NR1 ((5'-gatcACTGTACTTT CCTGACCTTGgatc-3')$_5$) and TK promoter was inserted between *Bgl* II and *Hind* III sites of pGL3-Basic plasmid (Sueyoshi *et al*., 1999). PRL-tk plasmid was purchased from Promega.

Cell culture and Transfection

HepG2 cells were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics and grown in 25 cm^2 culture flasks to 80–90% confluency in 5% CO_2 humidified atmosphere at 37℃. The cells were trypsinized, pelleted and resuspended in DMEM/10% FBS medium without antibiotics and replated on 48-well plates at 3–8×10^4 cells/well. After 24 h, HepG2 cells were transiently transfected by polyethylenimine (PEI) (Ehrhardt *et al*., 2006) with plasmid premix [pcDNA 3.1-pgCAR plasmid,

$(NR1)_5$-tk (thymidine kinase)-luciferase plasmid and pRL-tk plasmid were premixed in a ratio of 2:1:1 (Peacock, 2005) following the manufacturer's instructions for 48-well plates and cultured in DMEM with FBS and antibiotics.

Dual Luciferase Assay

Cells were incubated for 24 h to allow for transient gene expression and then washed with 1×PBS immediately before ligand supplemented media was applied. Phenytoin was suspended and diluted to final concentration 10 µmol L^{-1}. 25 µmol L^{-1}, 50 µmol L^{-1} and 100 µmol L^{-1} using the above method (Peacock, 2005). All active ingredients [Baicalin, Liquiritin, Indigotin, Indirubin, Sitosterol, Chlorogenic acid, Matrine, Daidzein, Betaine, Oleanolic acid, Vitamin C, Vitamin E, Folate] were suspended in DMSO and diluted to final concentration 25 µmol L^{-1} in DMEM without FBS and antibiotics as the ligand supplemented media. Cells were incubated in ligand treatment media and after 24 h cells were washed with 1×PBS and lysed following the Dual-Luciferase® Reporter Assay System protocal.

Statistical Analysis

Dual-luciferase assay ligand treatments were analyzed by Duncan T-test by compare ligand treatments to the DMSO no ligand treatment.

Results

Extraction, Reverse Transcription and Sequencing of pgCAR

PgCAR was amplified by PCR from boar liver cDNA using oligonucleotide primers designed using the sus scrofa CAR sequence information from NCBI and the cutting sites of restriction enzymes, *BamH* I and *Hind* III, were added at ends of the primers. The resulting product was visualized on 1% agarose gel (Fig. 1).

The PCR products were cloned into the pMD18-T cloning vector for sequencing and the result (Fig. 2) indicates that the bases in 333 and 603 sites were different and the rest products and total length, 1047 bp, were matched with the sequence information from NCBI.

Construction of Plasmids

The pgCAR sequences were endonuclease digested by *BamH* I and *Hind* III in a double digestion reaction and visualized on 1% agarose gels and cloned into the pcDNA3.1 (+) expression vector. Then the recombinant plasmid is digested by *BamH* I and *Hind* III (Fig. 3). It showed that the recombinant plasmid was constructed successfully.

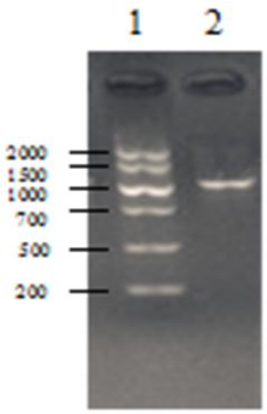

Fig. 1: PCR product of pgCAR fragenent 1: 2000 marker; 2: PCR product of pgCAR

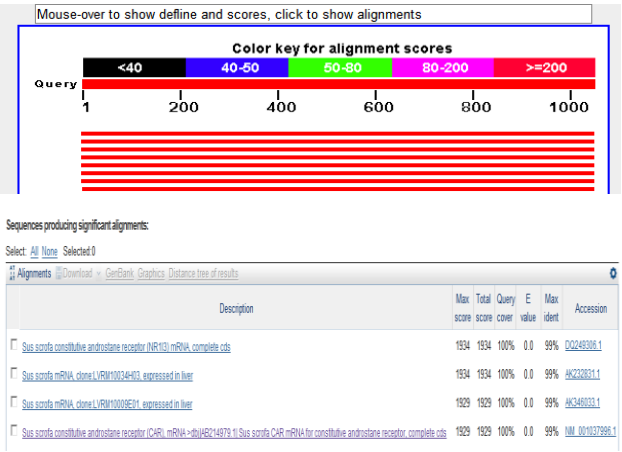

Fig. 2: The result of sequencing (blastn) the bases in 336 and 606 sites were different

Transfection

We used the non-modified polyethylenimine (PEI) reagent which is a cheap and effective transfection reagent to optimize transfection protocols for HepG2 cell-lines, and transiently transfected a number of cell-lines up to 70–80%.

Dual Luciferase Assay

Dose response of pgCAR activator phenytoin in HepG2: A dose response curve was generated to determine the proper concentration range that induces pgCAR reporter gene activation by Phenytoin using the dual-luciferase reporter assay. The results showed that the reporter gene was activated at the 10–100 µmol L^{-1}, with the 25 µmol L^{-1} dose resulting in the highest activation of 1.38 fold above the control (Fig. 4).

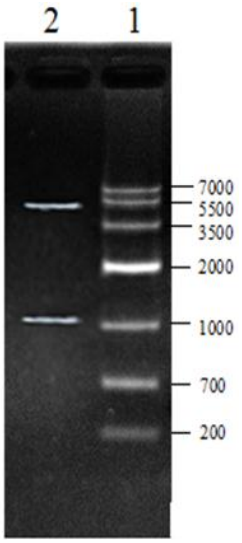

Fig. 3: Restriction enzymatic analysis of recombinant vector pcDNA-CAR 1: Marker 7000; 2: pcDNA3.1-CAR was digested by Hind III /BamH I

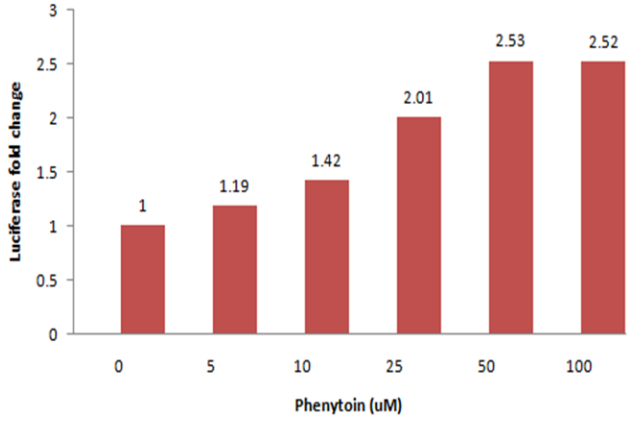

Fig. 4: Dose response of phenytoin ligand treatments on pgCAR Dual Luciferase Assay in HepG2 cells

Effects of the Active Ingredients of Herbal Medicines on pgCAR

The results of the pgCAR dual-luciferase assay showed at Fig. 5, effects of 13 active ingredients of herbal medicines were tested at 25 μmol L^{-1}. Many of the compounds could cause significant reductions in pgCAR, this situation is exemplified by treatment with these ligands, Baicalin, Liquiritin, Indigotin, Daidzein, Betaine and Matrine, which were shown to give activities 88, 75, 99, 75, 90 and 90%, respectively compared to no-ligand DMSO control. And the two ligands, Liquiritin and Daidzein that significantly inhibited pgCAR gene activation (P<0.05). While pgCAR was activated by the other seven compounds, Indirubin, Chlorogenic acid, Vitamin C, Folate, Sitosterol, Oleanolic acid and Vitamin E,

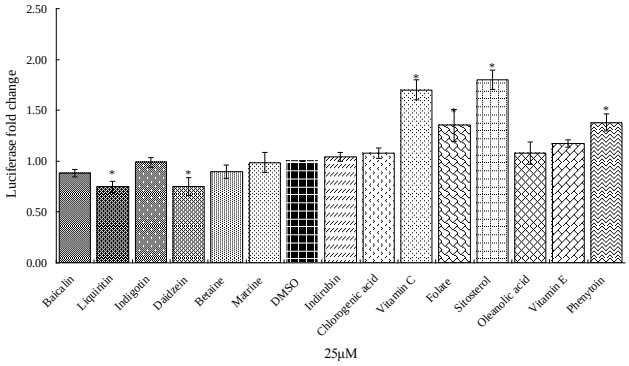

Fig. 5: Dose response of ligands treatments on pgCAR Dual luciferase Assay in HepG2 cells

which showed that reporter gene expression was activated above the DMSO control by 1.04, 1.04, 1.70, 1.35, 1.80, 1.08 and 1.17 fold respectively. Especially, Vitamin C, Folate and Sitosterol had significantly difference compared to the DMSO control (P<0.05).

Discussion

The rest products code for the same 348 amino acids. It may be the interspecific difference, but it has no effect on this experiment. This result indicated the correct pig gene was isolated using this approach.

From the Fig. 4, we can see that pgCAR is activated by Phenytoin and the dose response experiment was conducted to determine at what concentration phenytoin could significantly activate the pgCAR receptor in HepG2 cells. The date indicated that significant activations were starting at the 25 μmol L^{-1} dose. This is the reason why we chose other compounds at the 25 μmol L^{-1} dose on HepG2 cells. The activation was lower at 100 μmol L^{-1}, which may be caused by the drug toxicity for cells or the dose response range, which was probably too high to see intermediate responses. In addition, Phenytoin is believed to activate CAR indirectly since it does not bind directly to the ligand binding domain of CAR (Wang *et al*., 2004).

For the pgCAR dual-luciferase assay, NR1 as the CAR responsive element was cloned upstream of the firefly luciferase reporter gene to pick up the ligands which regulate pgCAR causing altered reporter gene expression. In this experiment, to ensure equal ligand treatment, the activities were shown by firefly luciferase activities standardized against Renilla luciferase activities for the internal control pRL-tk plasmid and the HepG2 cell line was used because it lacks the nuclear receptor CAR but expresses its dimerization partner RXR, which is involved in recognition of the responsive element in the regulatory regions of genes (Peacock, 2005).

Natural products are popular with the characteristic of wide range of sources, less toxicity and very little residues (Koehn and Carter, 2005). In this study, we extracted

several active ingredients from herbal medicines for detoxication and founding new feed additives.

Conclusion

This study was successfully identified ligands that activate or repress pgCAR and found that Vitamin C, Folate and Sitosterol significantly activated the expression of pgCAR gene. This will be a new discovery for feed additive.

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