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T0901317 as an Inhibitor of Transcriptional Activation of Constitutive Androstane Receptor (CAR)

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T0901317 is a potent synthetic ligand for liver X receptor (LXR, NR1H2/3), a member of the nuclear receptor superfamily that functions as a transcription factor. However, T0901317 has been also reported to modulate the activity at least four other nuclear receptors (NRs), acting as agonists for farnesoid X receptor (FXR, NR1H4) and pregnane X receptor (PXR, NR1I2) and as antagonists for androgen receptor (AR, NR3C4) and retinoid-related orphan receptor-α (ROR-α, NR1F1). We report here that T0901317 can also function as an inhibitor for constitutive androstane receptor (CAR, NR1I3). Since CAR is a major player of xenobiotic and cholesterol metabolism in the liver, along with PXR, FXR and LXR, which are reported to be regulated by T0901317, this further complicates the interpretation of potential results with T0901317 in liver cells.

Key words : Constitutive androstane receptor (CAR), T0901317, liver X receptor (LXR), PGC-1α, antagonist

Introduction

LXRα/β (Liver X Receptor α/β, NR1H3/2) belongs to the nuclear receptor (NR) superfamily and heterodimerizes with retinoid X receptor (RXR) to regulate target genes' transcription [8,23]. LXR functions as a lipid sensor and modulates homeostasis of cholesterol by induction of genes to regulate cholesterol metabolism and hepatic lipogenesis [18]. Because of importance of cholesterol in cardiovascular disease, the LXRs and their endogenous oxysterol ligands have been extensively studied [5,8,9]. T0901317, (N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl]phenyl]-benzenesulfonamide), is well-known as a potent synthetic agonist ligand of LXR α/β [18]. However, T0901317 has been also reported to bind to other NRs and/or to activate or inhibit their transactivation activity. For example, T0901317 functions as an agonist for farnesoid X receptor (FXR, NR1H4) and activate FXR more potently than its natural ligand [7]. T0901317 also binds to and activate pregnane X receptor (PXR, NR1I2) and induces transcription of its target genes at similar potency as LXR [15]. In addition, T0901317 was recently showed to inhibit transactivation activities of androgen receptor (AR, NR3C4) and retinoid-related orphan receptor-α (ROR-α, NR1F1) by competitive inhibition of their respective ligands, dimethyl-nortestosterone or 25-hydroxycholesterol, [1,13].

Constitutive androstane receptor (CAR, NR1I3) regulates xenobiotic degradation in the liver along with pregnane X receptor (PXR, NR1I2). Although exogenously expressed CAR constitutively activates its target genes without any added ligand [3], endogenous CAR in the liver translocates from the cytoplasm to nucleus in response to direct agonist ligands such as 1, 4-bis-[2-(3,5,-dichloropyridyloxy)]benzene (TCPOBOP), and indirect activators such as phenobarbital [12].

We have found that T0901317 can also inhibit CAR transactivation, and that addition of co-activator, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) restores CAR's activity even in the presence of T0901317. Our data complement previous results on T0901317's ability to regulate the activity of other NRs and raise additional question on the interpretation of studies carried out with T0901317 in liver cells.

Materials and Methods

Cell culture, transfection and material

The human hepatoma HepG2 cell line was cultured in DMEM with 10% FBS at 37°C in CO_2 incubator. For transient transfection, HepG2 cells were seeded at $5X10^4$ cells/well

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of 48-well plates one day before transfection and transfected with DNA mixtures consisting of indicated amounts of $(NR1)_5$-TK-luc reporter [6], plasmids expressing mCAR and/or other proteins, and pRL-TK (Promega, WI, USA) using Lipofectamine2000 (Invitrogen, CA, USA). The DNA amount used for transfection was kept constant for each well. Charcoal-stripped serum was used to remove lipids which might function as ligands and to maximize ligand effect during transfection, and the specific ligand was added 6 hours after transfection. Cells were incubated for 42 hours further and harvested for luciferase assay. T0901317 and TCPOBOP were purchased from Sigma (MO, USA). Each experiment was repeated at least three times as triplicate unless otherwise indicated.

Luciferase assay

Luciferase assay was carried out with the dual luciferase assay kit (Promega, WI, USA) as previously described [10]. All data were normalized by renilla luciferase activity to compensate transfection efficiency of each sample. The results were shown as relative activities compared to the vector control. The experiments were repeated three times with triplicate and all data was combined and averaged. The result was shown as average values with standard error of the means (SEM). (**: $p \leq 0.01$, ***: $p \leq 0.001$ by ANOVA with Turkey's *post-hoc* test.).

RT-PCR

The HepG2 cells were transfected with plasmids expressing CAR and treated with 5 μM of T0901317 for 2 days, harvested and used for synthesis of cDNA for RT (reverse transcription)-PCR (polymerase chain reaction) analysis. Total RNAs were isolated from cells using Trizol (Invitrogen, CA, USA) following the manufacturer's instruction. Single stranded cDNAs were synthesized from total RNAs by SuperScript II reverse transcriptase (Invitrogen, CA, USA) and used as templates for synthesis of double stranded cDNAs using ExTaq polymerase (Takara, Japan). The PCR was carried out at 95°C for 5 min, 33 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec followed by 72°C for 10min with a CYP2B6 specific primer set (CYP2B6 sense: 5′ AGACACCATGCATGGT GCACC; CYP2B6 antisense: 5′ CCATAACAGCATCA GGAGTG) using My Cycler Thermal Cycler System (Biorad, CA, USA).

Results

T0901317 inhibits CAR's transcriptional activity

To test whether T0901317 affects CAR's transactivation activity, we transiently transfected human hepatoma HepG2 cell line with plasmids expressing murine CAR (mCAR) with $(NR1)_5$-TK-luc plasmids, luciferase reporters harboring CAR's binding sites [6]. The transfected cells were treated T0901317 and their luciferase activity was compared to cells treated with DMSO which was used to dissolve ligand. Fig. 1A showed that T0901317 moderately inhibits CAR's transactivation activity in a dose-dependent manner. Although human CAR (hCAR) and mCAR share higher sequence homology and several characteristics, hCAR was also reported to show marked pharmacological differences from mCAR [17,22]. For example, hCAR was not activated by TCPOBOP which is an agonist for mCAR [17,22]. Therefore, we tested whether T0901317, like TCPOBOP, exhibits biochemical differences between mCAR and hCAR. Fig. 1 showed that T0901317 inhibits both mCAR and hCAR although the inhibition is weaker in hCAR than in mCAR (Fig. 1A & 1B). We tested ligand activity of T0901317 using LXRα, a receptor whose ligand is T0901317. As shown in Fig. 1C, T0901317 could activate LXRα at 0.1 μM, which proved that T0901317 that we used was an active chemical.

Next, we tested dose effect of T0901317 on CAR's transactivation activity with or without 1 μM of TCPOBOP, an agonist for mCAR. Fig. 2 showed that T0901317 at 9 μM completely inhibits mCAR's activity regardless of presence of 1 μM TCPOBOP. However, 0.5 and 1 μM of T0901317 exhibited no effect on CAR's activity when 1 μM of TCBOPOP was present although they showed approximately 20% of inhibition in the absence of TCBOPOP. Generally, presence of 1 μM of TCPOBOP decreased overall inhibitory effect of T0901317, suggesting their competition for binding to mCAR (Fig. 2).

Co-expression of co-activator PGC1α abolishes effect of T0901317

CAR was reported to be activated by recruiting of co-activators such as steroid receptor coactivator-1 (SRC-1 [2]) and PGC-1α [19]. Especially, androstanol, another inhibitor of CAR, was shown to inhibit CAR's binding to coactivator SRC-1 [3]. We reasoned that, if T0901317 also inhibits CAR's binding to co-activators like androstanol, addition of co-activator might alleviate T0901317's inhibitory activity on CAR's

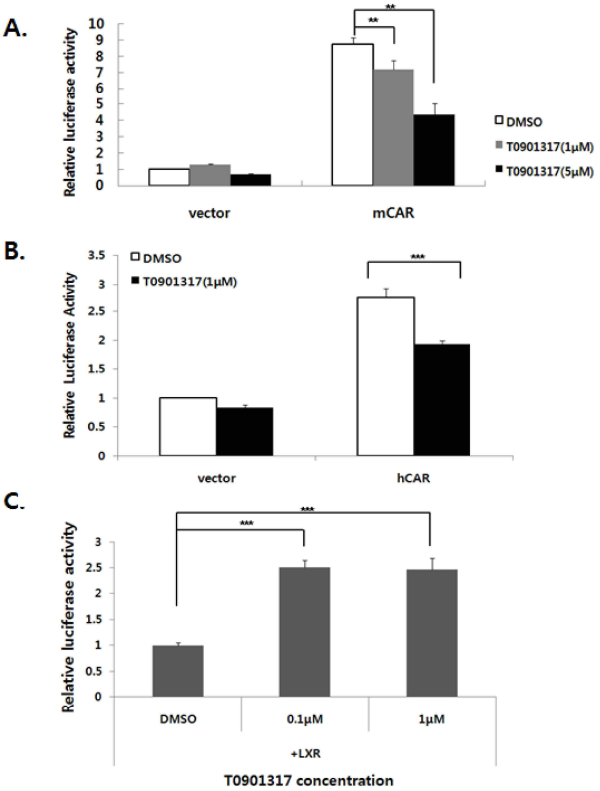

Fig. 1. T0901317 inhibits CAR's transactivation activity. (A,B) HepG2 cells were transfected with $(NR1)_5$-TK-Luc, pRL-TK and mCAR (A) or hCAR (B). T0901317 was treated at indicated concentration and DMSO was used as vehicle. (C) To prove our T0901317 is active ligand for LXR, the same HepG2 cells were transfected with $(LXRE)_3$-TK-Luc, pRL-TK and plasmids expressing LXR α and indicated concentrations of T0901317 were treated. For LXR transfection, the experiments were performed twice in triplicate and other experiments were carried out three times in triplicate. For all experiments, 100 ng of reporter, 200 ng of receptor and 25 ng of pRL-TK plasmids were used. pRL-TK was used to normalize transfection efficiency. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ for the indicated pairs.

transactivation activity. We chose PGC-1α as a co-activator to test and transiently transfected HepG2 cells with plasmids expressing PGC-1α at increasing amount in our assay system with either DMSO or T0901317.

Fig. 3 shows that transfection of plasmid expressing PGC-1α completely removed inhibitory effect on CAR's transactivation activity caused by T0901317 (Fig. 3, compare data obtained from transfections with 0 and 500 ng of PGC-1 α). As expected, co-transfection of increasing amount of PGC-1α further enhanced CAR's transactivation activity in a dose-dependent manner, in the presence of DMSO which

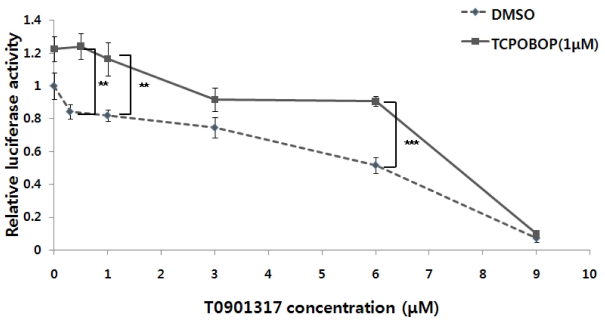

Fig. 2. Increased amounts of T0901317 exhibit progressive increase of inhibitory activity against CAR's transactivation activity. T0901317 was treated to cells transfected with mCAR, $(NR1)_5$-TK-Luc and pRL-TK from 0.5 μM to 9 μM with or without 1 μM of TCPOBOP. Luciferase assay was carried out after 48 hr incubation. Three separate experiments were done with triplicate and their average values with SEM were shown. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ for the indicated pairs.

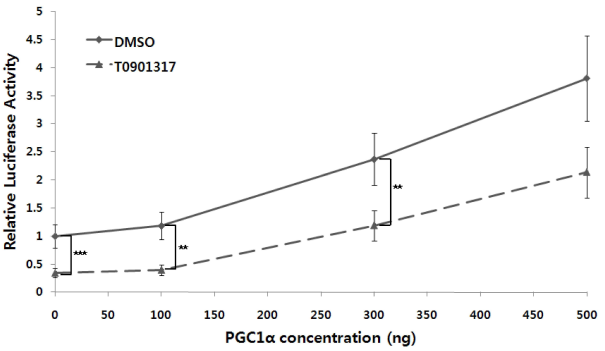

Fig. 3. Expression of PGC-1α removes inhibitory effect of T0901317 on CAR's transactivation activity. HepG2 cells seeded at 48 well plates were transfected with 75 ng of $(NR1)_5$-TK-Luc, 25 ng of pRL-TK, 100 ng of mCAR and indicated amounts of PGC-1α. DMSO or 1 μM of T0901317 was treated at 6 hr after transfection. Luciferase assay and data analysis were done as Fig. 2. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ for the indicated pairs.

was used as vehicle (Fig. 3).

Discussion

Although T0901317 has been widely used as a synthetic ligand for LXRα/β, recent studies revealed that T0901317 also functions as an agonist or antagonist for other NRs. For example, T0901317 is also reported as an agonist for farnesoid X receptor (FXR, NR1H4, [7]) and pregnane X receptor (PXR, NR1I2, [14]) and as an antagonist for androgen receptor (AR, NR3C4, [1]) and retinoid-related orphan re-

ceptor-α (ROR-α, NR1F1, [13]). With our addition of CAR in the group of NRs whose activities are affected by T0901317, most of important hepatic NRs regulating xenobiotic and cholesterol metabolism are now belong to this group. CAR, PXR, FXR and LXR are relatively close related and are known to exhibit several similar features [5]. All are mainly expressed in liver and function in metabolism of xenobiotic and cholesterol. They bind to lipid ligands, sometimes endogenous lipid, at relatively low affinity at micromolar range. In addition, they also share bile acid as activators. Bile acid was reported as a ligand for FXR [4] and PXR [20], and as an activator for CAR [16]. The addition of T0901317 as a CAR inhibitor, further complicates the interpretation of studies using T0901317. GW3965, another synthetic LXR ligand [11] shows improved specificity relative than T0901317 since it does not bind to PXR [15] and may provide a superior alternative for LXR studies.

CAR and PXR share overlapping roles in xenobiotic metabolism [17] and LXR and FXR regulates hepatic lipogenesis. All of these NRs function as lipid-sensor [5]. Therefore, information that T0901317, a well- known LXR ligand, actually functions as agonist for PXR and FXR and inhibitor for CAR requires caution in interpreting results of T0901317 studies in cells of the liver and other tissues or cells where these receptors are coexpressed.

To validate our result, we performed real time PCR analysis of CYP2B6, a typical target gene of CAR [21], using mRNAs isolated from HepG2 cells transfected with CAR and treated with 5μM of T0901317 for two days. However, we could not observe any detectable decrease in CYP2B6 mRNA level by T0901317 treatment (data not shown) although CAR over-expression itself increased CYP2B6 mRNA level. This was reasonable since CYP2B6 has been also reported to be activated by FXR and PXR, other hepatic nuclear receptors whose agonist included T0901317 [14]. Therefore, treatment of T0901317 to HepG2 cells caused to activate both FXR and PXR and, at the same time, to inactivate CAR, which might result in both activation and repression of expression of CYP2B6, respectively. The result might be no detectable decrease in CYP2B6 expression by T0901317 treatment, as we observed.

However, our assay using the reporter containing NR1 site which is a CAR binding site originated from the CYP2B6 promoter [6], clearly indicated that T0901317 functions as an antagonist for CAR.

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초록 : Constitutive androstane receptor (CAR)의 전사활성 저해제로서의 T0901317

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T0901317은 핵수용체 전사인자인 liver X receptor (LXR, NR1H2/3)의 강력한 합성 리간드이다. 그러나, T0901317은 farnesoid X receptor (FXR, NR1H4)와 pregnane X receptor (PXR, NR1I2)에 대해 작용물질(agonist)로, androgen receptor (AR, NR3C4)와 rertinoid-related orphan receptor-α (ROR-α, NR1F1)에 대해 길항제(antagonist)로 작용하여, LXR외에 적어도 다른 4종의 핵수용체에 대해 그 활성을 조절한다고 보고되었다. 우리는 T0901317이 또 다른 핵수용체인 constitutive androstane receptor (CAR, NR1I3)에 대해 저해제로 기능함을 확인하였다. CAR는 이미 T0901317에 의해 기능이 조절된다고 알려진 PXR, FXR, LXR과 더불어 간에서 생체이물과 콜레스테롤의 대사작용에 중요한 역할을 하므로 T0901317에 의해 CAR의 활성이 조절된다는 사실은, 간세포에서 T0901317을 이용한 실험결과를 해석할 때 세포 내에 이미 존재하는 이들 핵수용체 단백질의 영향을 고려하여 주의깊게 해석해야 함을 의미한다.