

Nigramide C is a natural agonist of human pregnane X receptor

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ABBREVIATIONS:

CAR, constitutive androstane receptor; CYP3A4, cytochrome P450 3A4; HPH, human primary hepatocyte; NigC, nigramide C; PXR, pregnan X receptor; SRC-1, steroid receptor

co-activator 1; XREM, xenobiotic responsive enhancer module

ABSTRACT

Pregnan X receptor (PXR) is known as a xenosensor, playing a key role in response to xenochemical stimuli. Activation of PXR enhanced expression of various drug-metabolizing enzymes and transporters such as CYP3A4. During a screening of a natural compounds library for novel ligands of human xenosensing receptors by the mammalian 1-hybrid assay, two cyclohexene-type amide alkaloids were isolated, with nigramide C (NigC) showing the most potent activation of human PXR (hPXR). NigC-mediated hPXR activation was enhanced by overexpression of steroid receptor co-activator 1 (SRC1), peroxisome proliferator-activated receptor gamma, co-activator 1 alpha, and protein arginine methyltransferase 1. A direct interaction between the ligand binding domain of hPXR and the receptor interaction domain of SRC1 was observed. NigC induced the expression of endogenous cytochrome P450 3A4 mRNA and protein in both cultured hepatoma cells and primary hepatocytes. However, in primary hepatocytes, the relative agonist activity of NigC was not as potent as that of rifampicin, probably because of lower metabolic stability of NigC in these cells. In conclusion, NigC was found to be an effective agonist of hPXR. NigC is useful tool for investigation of hPXR function.

Introduction

Pregnane X receptor [PXR, also known as steroid X receptor (SXR)], a member of the nuclear receptor superfamily predominantly expressed in the liver and intestine, is known as a xenosensor, playing a key role in response to xenochemical stimuli. Upon activation, PXR forms a heterodimer with retinoid X receptor alpha (RXR α) and recruits co-activators instead of co-repressors, resulting in the enhanced expression of various drug-metabolizing enzymes and transporters. These include both cytochrome P450 3A4 (CYP3A4) and multidrug resistance protein 1 (MDR1), which contribute to the metabolism of more than half of the prescribed medicines (Chibueze et al., 2011; Chen et al. 2012). The target genes of PXR have specific responsive elements in their promoter regions. In the *CYP3A4* proximal promoter region, an everted repeat of half hexamers separated by six nucleotides (ER6) was identified as a proximal PXR responsive element (prER6; -169/-152) (Blumberg et al., 1998). Furthermore, the distal enhancer module in the *CYP3A4* promoter (xenobiotic responsive enhancer module, XREM; -7.8/-7.2 kb) contains two additional PXR responsive elements, direct repeat 3 (DR3) nuclear receptor-binding element 1 (dNR1, -7733/-7719) (Goodwin et al., 1999), and DR4-type PXR responsive element (eNR3A4, -7618/-7558) (Toriyabe et al., 2009).

Recently, PXR has received renewed attention as a molecular target for the treatment of

inflammatory bowel disease (Shah et al., 2007; Cheng et al., 2012), dyslipidemia (Gao and Xie, 2012), and cholestatic liver disease (Kakizaki et al., 2011). For example, recent clinical trials with the antibiotic rifaximin, a PXR agonist in the gastrointestinal system, revealed its potential therapeutic value in the treatment of intestinal inflammation in humans (Cheng et al., 2012) .

A large number of chemicals have been reported to activate PXR. These compounds have large chemical structural diversity, including steroids, xenobiotics, and natural products such as clinical drugs and medicinal herb extracts. However, most of the known PXR agonists are also ligands of the closely related xenosensing nuclear receptor, constitutive androstane receptor (CAR) and our knowledge on selective PXR agonists are quite limited. Only a few of compounds, exemplified by rifampicin and ginkgolides A-C (Lau et al. 2012), have been known as selective PXR agonists but not that of CAR.

Pepper, the fruits of *Piper nigrum* L. (Piperaceae), is one of the most well-known spices in the world. The biological activities of pepper, such as stimulation of the central nervous system, analgesic, and antipyretic activities were reported due to its major constituents: ilepcimide (**10**) and piperine (**11**) (Wei et al. 2004a). The edible roots of *P. nigrum* were traditionally used in soup to promote sleep in Hainan island, where is the largest region for planting of *P. nigrum* in China. Phytochemical investigations of the roots of *P. nigrum* disclosed cyclohexane-type amide alkaloids as the typical constituents, but their biological activity are yet unclear (Wei et

al. 2004b).

During our investigation of discovery of novel ligands of nuclear receptors, in the present study, we reported the identification of nigramide C (NigC), a naturally occurring cyclohexane-type amide alkaloid from the root of *Piper nigrum*, as a novel PXR agonist. The role of NigC in the transcriptional activity of PXR was examined.

Materials and Methods

Chemicals

The cyclohexene-type amide alkaloids (**1–9**, **12**, and **13**) and related monomeric alkaloids ilepcimide (**10**) and piperine (**11**) used in the present study are natural compounds (**1** or NigC, **3** or nigramide J, **5**, **6**, **8**, **10**, and **12**) isolated from the roots of *Piper nigrum*, or semi-synthetic compounds (**2**, **4**, **7**, **9**, **11**, and **13**) (Wei et al., 2004a, 2004b, 2005). The purity of these compounds (> 98%) was determined by high-performance liquid chromatography and NMR analyses.

Plasmid construction

The expression plasmids for hPXR/ligand binding domain (LBD) fused with GAL4/DNA-binding domain (DBD) or VP16 were constructed using pcDNA5/GAL4 and pcDNA5/VP16, respectively. A segment of the receptor interaction domain (RID) of SRC1 (Leo et al 2000) (SRC1/RID, 569–781) was cloned into pcDNA5-GAL4/DBD. The expression plasmids for hCAR/ligand binding domain (LBD) fused with GAL4/DNA-binding domain (DBD) was described previously (Kanno et al., 2010). The preparation of the XREM-driven luciferase reporter plasmid (XREM-luc) has been described elsewhere (Kanno et al., 2010). The genes encoding the full-length human SRC1, SRC2, peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1), and protein arginine methyltransferase 1

(PRMT1) were amplified from cDNA of HepG2 cells and inserted in-frame into pcDNA5/TO expression plasmids with N-terminal myc tags.

Cell culture

The stable hPXR-expressing HepG2 cell line (Hep-hPXR) was established by pCMV3tag6-hPXR transfection, followed by serial hygromycin selections. HepG2 and Hep-hPXR cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and penicillin-streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Luciferase reporter analysis

Using PEI Max reagent (Polysciences Inc., Warrington, PA.), cells were transfected with the appropriate expression and reporter plasmids, as well as pGL4.74 (hRluc/TK; Promega, Madison, WI) as an internal standard. After overnight incubation, the cells were treated with individual test compounds for 24 h. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities were normalized against those of Renilla luciferase.

Quantitative reverse-transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from whole cell lysates using ISOGEN II (Nippon Gene Co. , Toyama,

Japan), and cDNA was synthesized using a ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative RT-PCR (qRT-PCR) was conducted (40 cycles of 98 °C for 5 s, 55 °C for 20 s and 72 °C for 30 s) using the THUNDERBIRD™ SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol and the 7500 fast system SDS software (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Specific primers were used to target *CYP3A4* (5' -CCAAGCTATGCTCTTCACCG-3' and 5' -TCAGGCTCCACTTACGGTGC-3') and *β-actin* (5'-TCCTCCTGAGCGCAAGTACTC-3' and 5'-CTGCTTGCTGATCCACATCTG-3') (Kanno et al., 2012).

Mammalian 2-hybrid assay

HepG2 cells were transfected with the pG5luc reporter plasmid, pGL4.74 and expression vectors for VP16-hPXR/LBD and GAL4-SRC1/RID using the PEI Max reagent. On the following day, the cells were treated with NigC or solvent (0.1% DMSO) for 24 h. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System.

Western blot analysis

HepG2 cells were harvested and lysed in SDS sample buffer. The whole-cell lysates were resolved by SDS-PAGE and immunoblotting was performed using anti-CYP3A4 (1:1000 dilution; Cell Signaling Technology Inc., Beverly, MA) and anti-Tubulin (1:20000 dilution; MBL, Nagoya, Japan) as primary antibodies. A horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution; Cell Signaling Technology Inc.) was used as secondary antibodies. Band

intensity was analyzed using Image Quant Software (Molecular Dynamics, Sunnyvale, CA).

Human primary hepatocytes and treatment

Human primary hepatocytes (HPHs) (HPH#1, Male, BIOPREDIC International, Rennes, France; HPH#2, Female, In Vitro ADMET Laboratories, Inc., Columbia, MD, USA) were plated and cultured according to the manufacturers' protocols. Cells were treated with each compound for 24 h. The mRNA expression level was measured by the above-mentioned qRT-PCR.

Statistical analysis

Statistically significant differences were determined using one-way analysis of variance followed by Tukey's multiple comparison test as the *post hoc* test, and differences were considered statistically significant at $P < 0.05$.

Results

Screening for human PXR agonists using the natural compounds isolated from the root of *Piper nigrum*

During the screening of a natural compounds library for novel ligands of human PXR (hPXR) by the mammalian 1-hybrid assay using GAL4-hPXR/LBD and pG5-luc in HepG2 cells, the cyclohexene-type amide alkaloid, nigramide C (NigC), showed potent activation of hPXR.

NigC belongs to a class of dimeric amide alkaloids possessing a cyclohexene ring (Fig. 1). To clarify the structure-activity relationship as an agonist of hPXR, the effects of some structurally related compounds on the hPXR-dependent transcription were evaluated. HepG2 cells transfected with GAL4-hPXR/LBD and pG5luc were treated with individual test compounds at either 10 μ M (Fig. 2A) or 1 μ M (Fig. 2B). NigC (**1**) showed the most potent activity, followed by compounds **2** and NigJ (**3**). However, two biosynthetic precursors of the nigramide compounds, ilepcimide (**10**) and piperine (**11**), did not show a statistically significant effect.

Nigramide C is an agonist of PXR but not CAR

To verify the effect of NigC on the hPXR-dependent transactivation of a reporter gene, HepG2 cells transfected with GAL4-hPXR/LBD and pG5luc were treated with increasing concentrations of NigC (0.1–25 μ M) for 24 h. A dose-dependent increase in the luciferase

activity was observed with NigC in the concentration range of 1–25 μ M (Fig. 3A). Effective activation comparable to that of 10 μ M Rif was observed with NigC at 1 μ M or higher. We further examined the effect of NigC on hPXR-dependent transcriptional activation using the XREM-luc reporter assay. HepG2 cells transfected with XREM-luc and hPXR expression vector were treated with increasing concentrations of NigC (0.1–25 μ M) for 24 h. Similarly to the results presented in Fig. 2, a dose-dependent increase in the luciferase activity was observed with NigC in the concentration range of 1–25 μ M (Fig. 3B). This activation was more efficient than the positive control (10 μ M Rif). To test whether NigC affects hCAR-dependent transcriptional activation, we conducted the luciferase reporter assay using GAL4-hCAR/LBD. NigC at concentrations of up to 10 μ M had no effect on the basal transactivity of hCAR (supplemental figure and our previously report, Kanno et al., 2014). Furthermore, we investigated whether NigC could reverse inverse agonist-mediated suppression. Although PK11195-suppressed transactivity of CAR was reversed by CITCO, CAR agonist, CAR activity was not affected by NigC (Fig.3C). These results suggest that NigC is not inverse agonist and agonist of CAR.

Nigramide C enhances the interaction between PXR and co-activators

When activated by an agonist, PXR recruits various co-activators. To analyze the effect of various co-activators on the NigC-mediated activation of PXR, HepG2 cells were

cotransfected with XREM-luc, hPXR expression vector, and the expression vectors for the individual co-activators, SRC1 (Watkins et al., 2003), SRC2 (Sugatani et al., 2005), PGC1 α (Bhalla et al., 2004), and PRMT1 (Xie et al., 2009). Similarly to Rif, NigC-mediated hPXR activation was enhanced by the overexpression of all co-activators tested (Fig. 4A).

Furthermore, to see whether NigC promotes direct protein-protein interaction between the co-activators and hPXR, a mammalian 2-hybrid assay was carried out in HepG2 cells transfected with pG5luc, VP16-hPXR/LBD, or VP16, and GAL4-SRC1/RID. The interaction of hPXR with SRC1/RID was enhanced by NigC to a similar level as that by Rif (Fig. 4B).

Nigramide C induces CYP3A4 mRNA and protein expression in HepG2-derived cells stably overexpressing PXR (HepG2-hPXR) and in human primary hepatocytes

We examined whether NigC could induce the expression of endogenous CYP3A4 mRNA and protein in Hep-hPXR cells, in which hPXR was stably overexpressed. Hep-hPXR cells were treated with NigC (1 or 10 μ M) or Rif (10 μ M) for 24 h (mRNA) or 48 h (protein). As shown in Fig. 5A and B, similarly to Rif, NigC enhanced the expression of CYP3A4 mRNA and protein at both 1 and 10 μ M compared with the solvent control (0.1% DMSO). Finally, we tested whether NigC could promote hPXR-dependent CYP3A4 mRNA induction in two different lots of human primary hepatocytes (HPH#1 and #2). As shown in Fig. 6, Rif enhanced CYP3A4 mRNA expression by 14.6- and 11.3-fold in HPH#1 and HPH#2, respectively, compared with

the solvent control in the corresponding HPH lot. Conversely, NigC promoted a relatively lower induction of *CYP3A4* mRNA; 4.4- and 3.2-fold compared with the control in HPH#1 at 1 and 10 μ M, respectively, and 5.4-fold in HPH#2 at 10 μ M.

Discussion

PXR is a key regulator of drug metabolism in response to xenobiotics. Among compound **1** (NigC) and its structurally related compounds (Fig. 1), the most effective hPXR agonist activity was observed by NigC, which was followed by compounds **2** and **3** (NigJ) (Fig. 2). The 3,4-methylenedioxyphenyl moiety at C-1 seems to be a requisite for potent agonist activity with insertion of an olefin group greatly decreasing the activity (**1** vs. **6** and **2** vs. **7**). Compared with the aliphatic chain, the 3,4-methylenedioxyphenyl moiety is preferable for the agonist activity, which is evidenced by comparing compounds **1** and **3** (Fig. 2B). Altering the olefin position in the cyclohexene ring may contribute to the PXR agonist activity, which is demonstrated by comparing compounds **1** and **2** (Fig. 2B), and **6** and **7** (Fig. 2A).

The spiciness of black pepper is mainly caused by piperine (**11**) and structurally related compounds. While these compounds function as insecticides in the original plant (Siddiqui et al., 2005), in mammalian systems they function as inhibitors of various xenobiotic metabolizing enzymes such as CYP3A4 and transporters represented by ATP-binding cassette sub-family B member 1 (ABCB1; also known as P-glycoprotein or MDR1) upon acute exposure. Although up-regulation of PXR target genes, such as CYP3A4 and MDR1, was observed after long-term exposure to **11** (Wang et al., 2013), we did not observe a significant activation of hPXR by the monomer-type compounds **10** and **11** (Fig. 2A).

NigC markedly enhanced the luciferase reporter activity measured using GAL4-hPXR/LBD in

HepG2 cells (Fig. 3A). Furthermore, NigC induced the XREM-luc reporter activity in hPXR-transfected HepG2 cells (Fig. 3B). These observations suggest that NigC transactivates the XREM-enhancer of *CYP3A4* via interaction with hPXR/LBD to a similar extent as does Rif, a well-known agonist of hPXR (Goodwin et al., 1999; Bertilsson et al., 1998). Generally, agonist binding causes conformational changes in the LBD of nuclear receptors, leading to recruitment of co-activators in place of co-repressors. We demonstrated that overexpression of known PXR co-activators enhanced NigC-mediated hPXR transactivation (Fig. 4A). Additionally, our results obtained by a mammalian 2-hybrid assay demonstrated that NigC-mediated hPXR activation enhanced the recruitment of SRC1 (Fig. 4B).

In most cases, agonists of hPXR are known to act as ligands of hCAR. For example, clotrimazole, T0901317, and PK11195 are not only agonists of hPXR but also inverse agonists of hCAR (Moore et al., 2000; Li et al., 2008; Kanno et al., 2013). In the current study, the basal transcriptional activity of ligand-free hCAR was not affected by NigC at concentrations of up to 10 μ M, although a little but significant suppression was observed at 25 μ M (Fig. 3C and supplemental Figure). In contrast, the basal activity of hCAR was significantly suppressed in the presence of 10 μ M PK11195. These observations indicate that NigC is not ligand of hCAR. It is notable that compound **3** (NigJ), which differ its structure from **1** by only C-6 aliphatic moiety, have been identified as a potent inverse agonist of hCAR (Kanno et al.,

2014).

CYP3A4 plays a major role in the metabolism of a wide range of clinically used pharmaceutical drugs and xenobiotics. Because PXR is the dominant activator of *CYP3A4* transcription, we further investigated whether NigC could induce CYP3A4 expression. Although NigC increased the expression of endogenous *CYP3A4* mRNA in Hep-hPXR cells and was as potent as Rif (Fig. 5), only a modest induction was observed in human primary hepatocytes compared with Rif (Fig. 6). The difference in the induction level of the endogenous gene by NigC between Hep-hPXR cells and human primary hepatocytes remains to be solved in future studies. However, it is possible that this difference stems from different metabolic stability of NigC in different cell types, as in the case of buprenorphine, which showed potent induction of CYP3A4 in PXR-transfected HepG2 cells but not in human primary hepatocytes, because of different metabolic stabilities (Li et al., 2010). Taking account of the contents of NigC in the roots of *P. nigrum* (0.0015%) (Wei et al. 2004b), the intake amount of NigC by human would be very low (<0.5 mg/day). But the interaction of NigC and other drugs would be taken into consideration when it was used at a high concentration. In summary, NigC was found to be an effective agonist of hPXR, as demonstrated by binding to the LBD of hPXR and recruitment of various nuclear receptor co-activators. The induction of a typical hPXR target gene (*CYP3A4*) was proven in both human hepatoma cells and human primary hepatocytes. The marked difference in the effect on the basal activity of hCAR

between the structurally related NigC and NigJ could provide a clue to how the binding of CAR co-factors to helix12 is regulated. Nig C having totally different chemical structures with current known hPXR agonist, which maybe serve as a possible chemical probe for PXR research. The specificity of NigC as an hPXR agonist and the putative susceptibility to metabolic degradation suggest that NigC could also be a potential therapeutic candidate for intestinal inflammation in humans.

Authorship Contributions

Participated in research design: Yuichiro Kanno and Wei Li

Conducted experiments: Yuichiro Kanno, Tomofumi Yatsu and Wei Li

Contributed new reagents or analytic tools: Yuichiro Kanno and Wei Li

Performed data analysis: Yuichiro Kanno, Wei Li, Kazuo Koike and Yoshio Inouye

Wrote or contributed to the writing of the manuscript: Yuichiro Kanno, Wei Li Kazuo Koike and

Yoshio Inouye

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Figure legends for Figures

Fig. 1. Chemical structure of NigC and the related compounds.

Fig. 2. Activation of hPXR by NigC and the related compounds.

HepG2 cells were transfected with the pG5luc reporter plasmid (0.1 μ g) together with the GAL4/DBD-hPXR/LBD (0.05 μ g) and pGL4.74 (0.01 μ g) plasmids. Cells were treated with the individual test compounds at 10 μ M (A) or 1 μ M (B), 10 μ M Rif as a positive control, or solvent (0.1% DMSO). After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Results are shown as averages of fold activation over the solvent control after Renilla normalization of four individual transfection experiments. (mean \pm S.D., n = 4).

Fig. 3. Effect of NigC on the transcriptional activity of hPXR and hCAR.

(A) HepG2 cells were transfected with the pG5luc reporter plasmid (0.1 μ g) together with the expression vector for GAL4/DBD-hPXR/LBD (0.05 μ g) and pGL4.74 (0.01 μ g). (B) HepG2 cells were cotransfected with XREM-luc reporter plasmid (0.1 μ g), hPXR expression vector (0.05 μ g), and pGL4.74 (0.01 μ g). (C) HepG2 cells were cotransfected with pG5luc (0.1 μ g), GAL4/DBD-hCAR/LBD (0.05 μ g), and pGL4.74 (0.01 μ g). Cells were treated with increasing

concentrations of NigC (0.1–25 μ M), Rif (10 μ M), PK11195 (10 μ M) as another positive control, or solvent (0.1% DMSO). After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Results are shown as averages of fold activation over the solvent control after Renilla normalization of four individual transfection experiments. (mean \pm S.D., n = 4).

Fig. 4. Effect of NigC on the interaction between hPXR and its co-factors.

(A) HepG2 cells were cotransfected with XREM-luc (0.1 μ g), hPXR (0.05 μ g), individual cofactor expression plasmids (0.05 μ g), and pGL4.74 (0.01 μ g). (B) HepG2 cells were cotransfected with pG5luc (0.1 μ g), the expression vectors for VP16-hPXR LBD and GAL4-SRC-1/RID, and pGL4.74 (0.01 μ g). After 24 h, cells were treated with NigC (10 μ M), Rif (10 μ M), or solvent (0.1% DMSO). Luciferase activity was measured 24 h post-treatment. Results are shown as averages of fold activation over the solvent control after Renilla normalization of four individual transfection experiments. (mean \pm S.D., n = 4). ; ** P < 0.01.

Fig. 5. Effects of NigC on the expression of an endogenous hPXR target gene in Hep-hPXR cells.

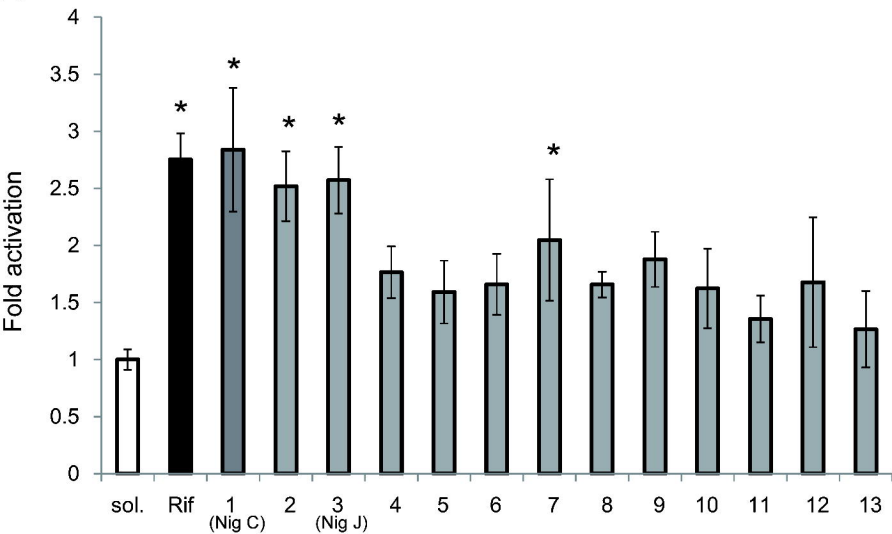
HepTR-hPXR cells were treated with NigC (1 or 10 μ M), Rif (10 μ M), or solvent (0.1% DMSO).

After 24 h, cells were harvested and the mRNA level of *CYP3A4* (A) was measured by qRT-PCR. Results normalized against β -Actin mRNA levels are expressed as averages of fold activation over the solvent control of four individual experiments (mean \pm S.D., n = 4); **P* < 0.05; ***P* < 0.01. (B) For *CYP3A4* protein analysis, cells were treated for 48 h. Whole cell lysates were separated by SDS-PAGE, and proteins were detected by immunoblotting using antibodies against *CYP3A4* and tubulin, which was used as a loading control.

Fig. 6. Effects of NigC on the expression of an hPXR target gene in human primary hepatocytes.

Human primary hepatocytes (HPH) were treated with NigC (1 or 10 μ M), Rif (10 μ M) or solvent (0.1% DMSO). After 24 h, cells were harvested and the mRNA levels of *CYP3A4* were measured by qRT-PCR. The results normalized against β -*actin* mRNA levels are presented as fold expression over the solvent control (mean \pm S.D., n = 4).

A



B

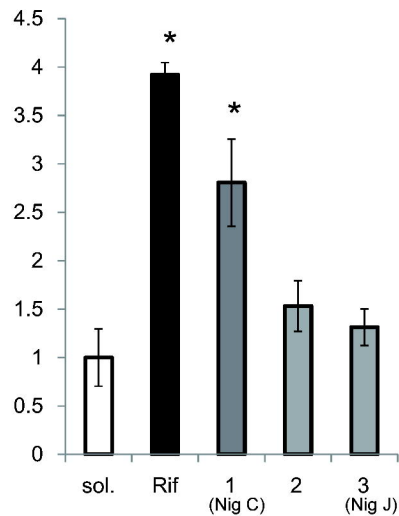
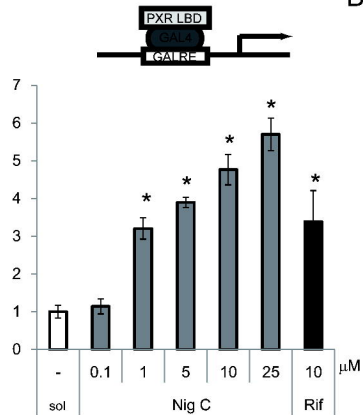


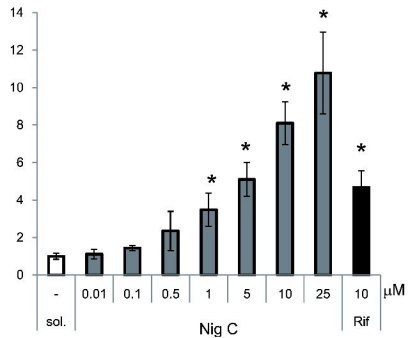
Figure 2

A

Fold activation



B



C

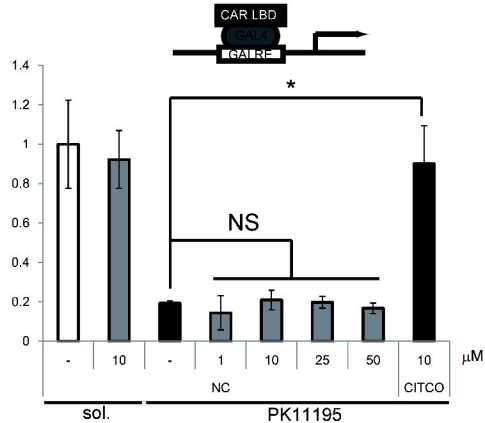
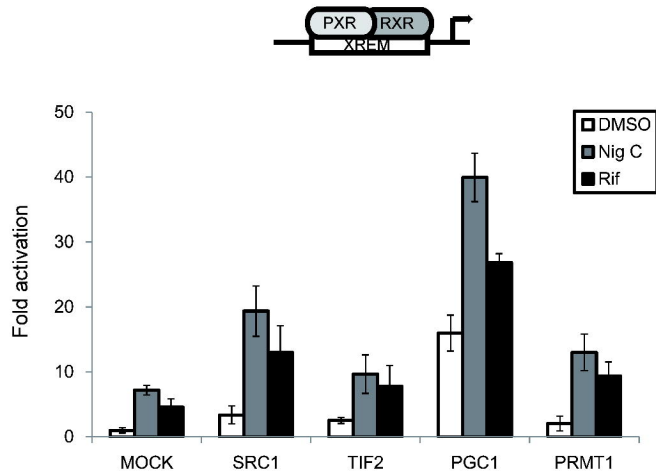


Figure 3

A



B

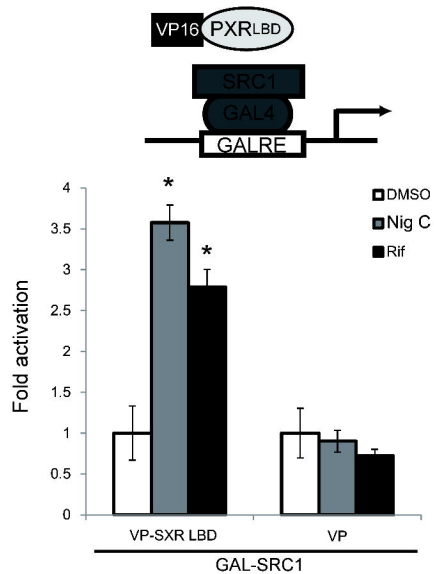
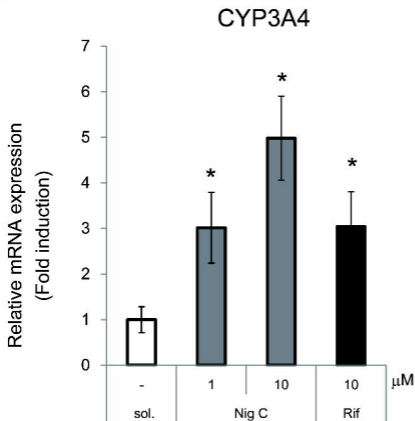


Figure 4

A



B

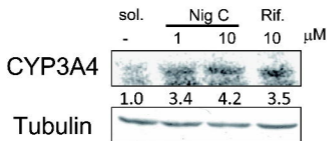
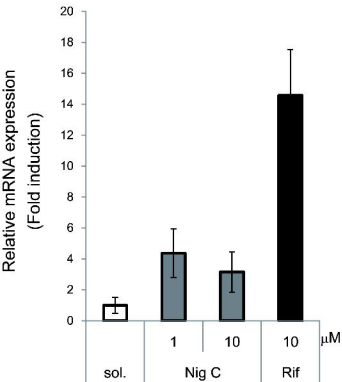


Figure 5

CYP3A4

HPH #1



HPH #2

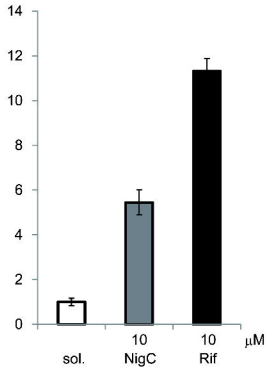


Figure 6

Nigramide C is a natural agonist of human pregnane X receptor

Yuichiro Kanno, Tomofumi Yatsu, Wei Li, Kazuo Koike, and Yoshio Inouye

Drug Metabolism and Disposition

supplemental Figure

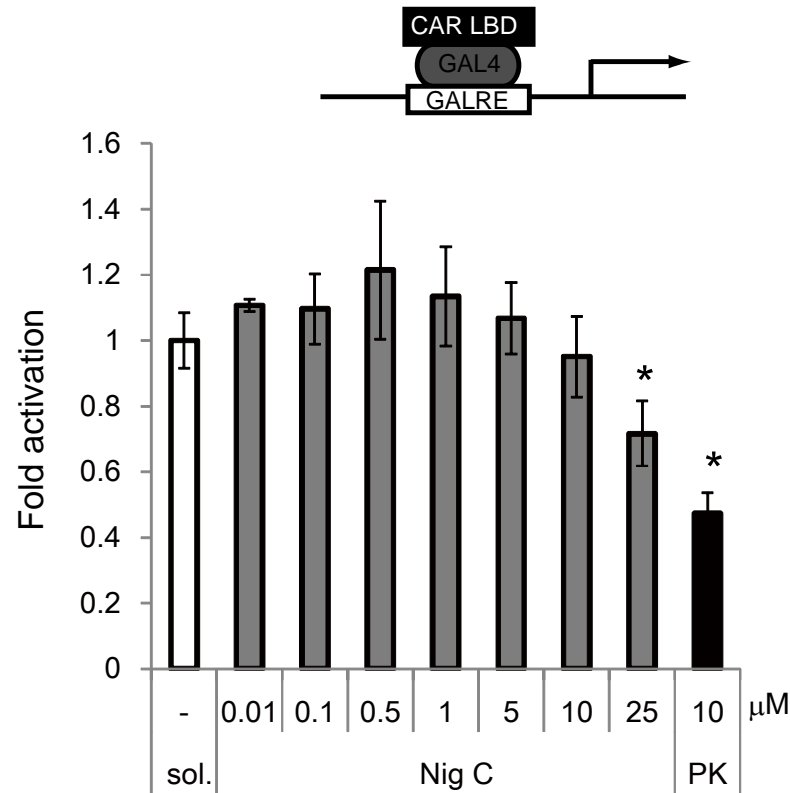


Figure S1. HepG2 cells were cotransfected with pG5luc (0.1 μg), GAL4/DBD-hCAR/LBD (0.05 μg), and pGL4.74 (0.01 μg). Cells were treated with increasing concentrations of NigC (0.1–25 μM), PK11195 (10 μM) as positive control, or solvent (0.1% DMSO). After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Results are shown as averages of fold activation over the solvent control after Renilla normalization of four individual transfection experiments. (mean ± S.D., n = 4).