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Regular article

Indirubin, a component of Ban-Lan-Gen, activates *CYP3A4* gene transcription through the human pregnane X receptor

Takeshi Kumagai [a, *], Yusuke Aratsu [a, b], Ryosuke Sugawara [a], Takamitsu Sasaki [a, c], Shinichi Miyairi [d], Kiyoshi Nagata [a]

[a] Department of Environmental Health Science, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan
[b] Drug Metabolism and Pharmacokinetics Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-1 Murasaki-cho, Takatsuki, Osaka 569-1125, Japan
[c] Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[d] Laboratory of Organic Chemistry, School of Pharmacy, Nihon University, 7-7-1 Narashino, Funabashi, Chiba 274-8555, Japan

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ABSTRACT

Ban-Lan-Gen is the common name for the dried roots of indigo plants, including *Polygonum tinctorium*, *Isatis indigotica*, *Isatis tinctoria*, and *Strobilanthes cusia*. Ban-Lan-Gen is frequently used as an anti-inflammatory and an anti-viral for the treatment of hepatitis, influenza, and various types of inflammation. One of the cytochrome P450 (CYP) enzymes, CYP3A4, is responsible for the metabolism of a wide variety of xenobiotics, including an estimated 60% of all clinically used drugs. In this study, we investigated the effect of Ban-Lan-Gen on the transcriptional activation of the *CYP3A4* gene. Ban-Lan-Gen extract increased *CYP3A4* gene reporter activity in a dose-dependent manner. Indirubin, one of the biologically active ingredients in the Ban-Lan-Gen, also dose-dependently increased *CYP3A4* gene reporter activity. Expression of short hairpin RNA for the human pregnane X receptor (hPXR-shRNA) inhibited *CYP3A4* gene reporter activity, and overexpression of human PXR increased indirubin- and rifampicin-induced *CYP3A4* gene reporter activity. Furthermore, indirubin induced CYP3A4 mRNA expression in HepG2 cells. Taken together, these results indicate that indirubin, a component of Ban-Lan-Gen, activated *CYP3A4* gene transcription through the activation of the human PXR.

1. Introduction

Chinese herbal medicines are an integral part of health care in Asia, and many herbal medicines are extensively used as alternative and/or complementary therapies. However, the clinical efficacy, mechanisms of action, and safety profiles of most herbal medicines are not well characterized. Moreover, herbal medicines are often co-administered with therapeutic drugs, creating the potential for herb–drug interactions [1,2]. While the underlying mechanism of most herb–drug interactions is not known, induction and inhibition of drug-metabolizing enzymes or drug transporters have been implicated as potential mechanisms.

Ban-Lan-Gen is the common name for the dried roots of indigo plants, including *Polygonum tinctorium*, *Isatis indigotica*, *Isatis tinctoria*, and *Strobilanthes cusia*. Indigo plants have been an important source of dyes in Asia since ancient times, and today, they are commonly used as therapeutics in traditional Chinese medicine. Ban-Lan-Gen is frequently used as an anti-inflammatory and anti-viral for the treatment of hepatitis, influenza, and various kinds of inflammation [3,4]. Several biologically active ingredients have been identified in Ban-Lan-Gen, including indigoid alkaloids (*e.g.*, indigo and indirubin) and quinazolinone alkaloids (*e.g.*, tryptanthrin) [5]. In fact, indigo, indirubin, and tryptanthrin are three marker compounds found in Ban-Lan-Gen [6]. Among these marker compounds, it has been reported that indirubin causes transactivation of *cytochrome P450 1A1* (*CYP1A1*) and *CYP1A2* genes via

Abbreviations: AdCont, β-galactosidase-expressing adenovirus; AdPXR, human PXR-expressing adenovirus; AdhPXR-shRNA, AdhPXR-short hairpin RNA; AhR, aryl hydrocarbon receptor; Ct, threshold PCR cycle; CAR, constitutive androstane receptor; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; dNR1, distal nuclear receptor-binding protein 1; DMSO, dimethyl sulfoxide; D-PBS, Dulbecco's phosphate buffered saline; DR3, direct repeats spaced by 3 bases; eNR3A4, essential distal nuclear receptor-binding element; ER6, everted repeats separated by 6 bases; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBE, *Ginkgo biloba* extract; GRα, glucocorticoid receptor-α; MOI, multiplicity of infection; PAHs, polycyclic aromatic hydrocarbons; prER6, ER6 in the CYP3A4 proximal promoter; PXR, pregnane X receptor; RXRα, retinoid X receptor α; SJW, St. John's wort; $TCID_{50}$, 50% titer culture infectious dose; VDR, vitamin D receptor.

* Corresponding author.
E-mail address: ta-kuma@tohoku-pharm.ac.jp (T. Kumagai).

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the aryl hydrocarbon receptor (AhR) [7]. In addition, a content of indirubin have been reported in the roots of *I. indigotica* as main raw materials of Ban-Lan-Gen (0.224–34.4 μg/g) [5,6].

Members of the CYP supergene family of monooxygenases play an important role in detoxification by converting pollutants, plant toxins, carcinogens, and drugs to products that are then excreted in to urine or bile [8,9]. Human CYP3A4 is of particular significance in this respect, because it is involved in the metabolism of approximately two-thirds of clinically relevant drugs [10]. A number of compounds, including pesticides, herbal supplements, vitamins, and drugs, activate *CYP3A4* gene transcription both in the liver and in the small intestine [11,12]. This induction process is the molecular basis for a number of important drug interactions that occur in patients taking multiple medications.

The pregnane X receptor (PXR; NR1I2) is the principal regulator of *CYP3A4* gene expression and binds as a heterodimer with retinoid X receptor α (RXRα) to regulatory DNA sequences. These sequences include: (1) AG(G/T)TCA-like direct repeats spaced by 3 bases (DR3) located −8 kb upstream from the transcription start point and identified as distal nuclear receptor-binding element 1 (dNR1), and (2) everted repeats separated by 6 bases (ER6) located in the *CYP3A4* proximal promoter (prER6) [13]. Recently, Toriyabe et al. identified a distinct PXR response element as an essential distal nuclear receptor-binding element (eNR3A4) for *CYP3A4* gene induction [14]. PXR is activated by a number of structurally and chemically diverse ligands, such as drugs [rifampicin (RIF), clotrimazole] [15], pesticides (pyributicarb, endosulfan) [16,17], natural and synthetic steroids (dexamethasone) [15], bile acids (lithocholic acid) [18], and herbal medicines (St. John's wort [SJW], *Ginkgo biloba*, and *Sophora flavescens*) [19–21]. CYP3A4 induction by these PXR activators leads to accelerated metabolism of the activators themselves and concomitantly-administered drugs that are metabolized by CYP3A4.

Induction and inhibition of CYP3A4 by Ban-Lan-Gen are not fully understood. In this study, we investigated the effect of Ban-Lan-Gen on transcriptional activation of the *CYP3A4* gene.

2. Materials and methods

2.1. Materials

Ban-Lan-Gen (lot. 9115476) was purchased from Beijing Tong Ren Group Co., Ltd. (Beijing, China). SJW was purchased from ChromaDex (Irvine, CA). Indirubin and tryptanthrin were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). Indican, isatin, and indigo were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *Ginkgo biloba* extract (GBE) was purchased from Tama Biochemicals Co., Ltd (Tokyo, Japan). RIF was purchased from Sigma–Aldrich (St. Louis, MO). The chemicals used for this study were dissolved in dimethyl sulfoxide (DMSO). Ban-Lan-Gen, SJW, and GBE were extracted with culture medium used for the cell culture in this study, for 2 h at 37 °C. These extract solutions were centrifuged at 2,000 × *g* for 15 min at 4 °C and the supernatants were used for this study. All other reagents used were of the highest quality available. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hyclone Laboratories (Logan, UT), respectively. MEM non-essential amino acids and antibiotic-antimycotic were purchased from Invitrogen (Paisley, UK).

2.2. Cell culture

HepG2 cells were obtained from RIKEN cell bank (Tsukuba, Japan). The HepG2-derived cell line stably expressing the *CYP3A4*-luciferase reporter gene, clone 3-1-20, was maintained as reported previously [22]. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, MEM non-essential amino acids, and antibiotic-antimycotic. The cells were seeded at 3×10^4 cells per well onto 48-well tissue culture plate (BD Biosciences, Heidelberg, Germany) for luciferase gene reporter assay, and 5×10^4 cells per well onto 24-well tissue culture plate (BD Biosciences) for real-time polymerase chain reaction (PCR). After 24 h, the cell medium was changed with the extract solutions or various subjects dissolved in DMSO (final concentration, 0.1%) and cultured for 48 h.

2.3. Luciferase gene reporter assay

3-1-20 cells were washed with Dulbecco's phosphate buffered saline (D-PBS) and suspended in passive lysis buffer (Promega, Madison, WI) in a microcentrifuge tube. The cell suspension was centrifuged at 12,000 × *g* for 5 min at 4 °C, and the cell extract was used for the luciferase assay. The luciferase assay was performed using the Luciferase Assay System and a GloMax™ 96 Microplate Luminometer (Promega) according to the manufacturer's instructions. The resulting data are presented as the ratio of luminescence of treated cell samples to that of control. The luminescence of each sample was normalized by its protein concentration as determined with the Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

2.4. Reporter gene constructs and transient transfection

The luciferase reporter plasmids, pGL3-Basic and pGL4.70, were purchased from Promega. Preparation of the *CYP3A4* luciferase reporter gene constructs, including pCYP3A4-362-7.7k, pCYP3A4-362-7.7km, pCYP3A4-362m-7.7k, pCYP3A4-362m-7.7km, and pCYP3A4-362-7.7kmΔα, was described previously [14]. One day before transfection, cells were seeded in 48-well plates. Each reporter plasmid and pGL4.70 was transfected using Targefect F-1 (Targeting System, El Cajon, CA) according to the manufacturer's protocol. pGL4.70 was used for the normalization of transfection efficiency. After transfection, cells were cultured in medium in the presence of various chemicals for 48 h. Control cells were cultured with vehicle (0.1% DMSO) alone. Subsequently, the cells were harvested and suspended in passive lysis buffer (Promega). Luciferase activities were determined with Dual-Luciferase® Reporter Assay System (Promega).

2.5. Construction of recombinant adenovirus and infection

Construction of the human PXR-expressing adenovirus (AdhPXR) and AdhPXR-short hairpin RNA (AdhPXR-shRNA) was described previously [17]. Control adenovirus, a β-galactosidase-expressing adenovirus (AdCont; AxCALacZ), was provided by Dr. Izumi Saito (Tokyo University, Japan) [23]. The titer of the adenoviruses, 50% titer culture infectious dose ($TCID_{50}$), was determined as reported previously [17]. Multiplicity of infection (MOI) was calculated by dividing the $TCID_{50}$ by the number of cells. One day before transfection, cells were seeded in 24-well plates. Adenovirus infection was carried out as described previously [24].

2.6. Isolation of RNA and analysis of quantitative real-time polymerase chain reaction

Total RNA was isolated from HepG2 cells using TRI Reagent (Molecular Research Center, Inc., Montgomery, AL) according to the manufacturer's protocol. cDNA was prepared from 2.0 μg of total RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) using oligo$(dT)_{20}$ primer (Greiner Japan, Japan)