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TSU-16, (Z)-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone, is a potent activator of aryl hydrocarbon receptor and increases *CYP1A1* and *CYP1A2* expression in human hepatocytes

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ABSTRACT

(Z)-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone (TSU-16), is a potent anti-angiogenic agent that inhibits the tyrosine kinase of vascular endothelial growth factor receptor-2. In clinical trials with daily or twice weekly intravenous administration of TSU-16, its increased clearance was observed. To understand the mechanism underlying this observation, we have investigated the TSU-16-mediated regulation of cytochrome P450 expression. In human hepatocytes, TSU-16 increased mRNA levels of *CYP1A1* and *CYP1A2*, but not *CYP2B6* and *CYP3A4*. The extent of increase and profiles of the time-dependent changes in *CYP1A1* and *CYP1A2* mRNA levels after TSU-16 treatment were similar to those after treatment with 3-methylcholanthrene (3MC), a well-known activator of the aryl hydrocarbon receptor (AhR). In reporter assays using a plasmid construct that contained the human *CYP1A1* 5′-flanking region including the region crucial for the AhR-dependent transcription of both human *CYP1A1* and *CYP1A2*, TSU-16 treatment increased reporter activities to an extent similar to that obtained with 3MC. Treatment of HepG2 cells and human hepatocytes with AhR-targeting siRNA suppressed the increase in both mRNA levels and CYP1A activities after treatment with TSU-16 as well as after that with omeprazole or 3MC. TSU-16 also time-dependently reduced cellular AhR protein levels in HepG2 cells to a similar extent with 3MC treatment. Furthermore, we demonstrated that unlabeled TSU-16 and 3MC but not omeprazole completely inhibited the specific binding of [^{3}H]-3MC to mouse Hepa1c1c7 cytosol, suggesting TSU-16 as an AhR ligand. In conclusion, our present results suggest that TSU-16 binds to and activates AhR to enhance the expression of both human *CYP1A1* and *CYP1A2*. Because TSU-16 is metabolized mainly by CYP1A2, its increased clearance after repeated dosing may be attributed to the enhanced expression of hepatic *CYP1A2*.

1. Introduction

Vascular endothelial growth factors (VEGFs) are crucial regulators of vascular development during embryogenesis (vasculogenesis) as well as blood-vessel formation (angiogenesis) in humans. VEGFs bind to 3 receptor tyrosine kinases, i.e., VEGF receptor-1 (VEGFR-1), VEGFR-2, and VEGFR-3. VEGFR-1, also known as Flt-1, and VEGFR-2, also known as KDR/Flk-1, are expressed in endothelial cells, and VEGFR-3, also known as Flt-4, is expressed primarily in lymphatic vessels. Among them, VEGFR-2 is exclusively expressed in vascular endothelial cells. Because the VEGFR-2 system is a dominant signal transduction pathway in regulating tumor angiogenesis [1], the inhibition of VEGFR-2 function is sufficient to prevent tumor growth in experimental models [2].

TSU-16, (Z)-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone, was developed as a selective and potent small-molecule inhibitor of the VEGFR-2 tyrosine kinase with the inhibition con-

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; TSU-16, (Z)-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone; CYP, cytochrome P450; AhR, aryl hydrocarbon receptor; OMP, omeprazole; 3MC, 3-methylcholanthrene; KHB, Krebs–Henseleit buffer; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HHM, Human Hepatocyte Serum-Free Medium; RT-PCR, reverse transcription-polymerase chain reaction; BSA, bovine serum albumin; EROD, ethoxyresorufin-*O*-deethylase; XRE, xenobiotic-responsive element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PD98059, 2-(29-amino-39-methoxyphenyl)-oxanaphthalen-4-one; U0126, 1, 4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one.

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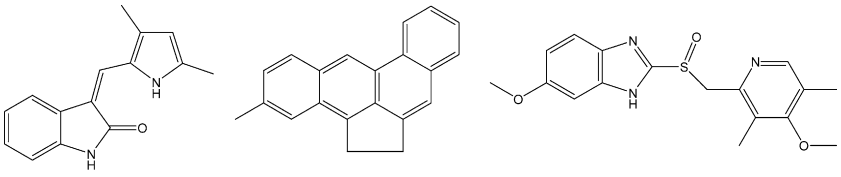

Fig. 1. Chemical structures of the compounds used in this study.

stant (*Ki*) of 0.16 μM [3]. In clinical trials, the TSU-16 plasma concentration decreased after repeated oral or intravenous administration, suggesting enhanced clearance of the inhibitor [4,5]. The extent of increase in the clearance after daily oral dosing of TSU-16 was greater than that after daily intravenous dosing [4,5]. These results suggest that TSU-16 is a high hepatic clearance drug and that the enhanced clearance is a consequence of the accelerated metabolism of TSU-16. This kind of increased clearance after repeated dosing could be problematic, because it leads to a loss of efficacy and dose adjustment is often required during the treatment.

Drug-metabolizing enzymes, especially cytochrome P450s (CYPs), play main roles in drug clearance. CYPs metabolize a number of drugs primarily in the liver and their expression levels are increased upon the administration of various drugs. Because it has been reported that TSU-16 is mainly metabolized by CYP1A2 in human liver microsomes [6], the drug may increase hepatic CYP1A2 levels after repeated dosing. In the present study, we have investigated the effects of TSU-16 treatment on the expression of human CYPs to understand the molecular mechanism for the increased clearance of TSU-16 after the administration of repeated dosing. We here demonstrate that TSU-16 is a potent agonist of the aryl hydrocarbon receptor (AhR) and increases CYP1A1 and CYP1A2 levels in human hepatocytes.

2. Material and methods

2.1. Reagents

TSU-16 (Fig. 1) was synthesized by SUGEN Inc. (South San Francisco, CA). Insulin, hydrocortisone, 7-ethoxyresorufin, resorufin, omeprazole (OMP), 3-methylcholanthrene (3MC), and Krebs–Henseleit buffer (KHB) were purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), William's E medium, penicillin–streptomycin solution, and fetal bovine serum (FBS) were purchased from Invitrogen Corp. (Carlsbad, CA). Human Hepatocyte Serum-Free Medium (HHM) was purchased from TOYOBO (Tokyo, Japan). FuGENE 6 and HiperFect were purchased from Roche Diagnostics (Basel, Switzerland) and QIAGEN (Valencia, CA), respectively. [^{3}H]-3MC (10.7 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Oligonucleotide primers and TaqMan probes were synthesized by Operon (Tokyo, Japan).

2.2. Hepatocyte cultures

Cryopreserved human hepatocytes (HH 382: white, female, 19 years old; HH 393: white, female, 30 years old, and HH 426: white, female, 51 years old) purchased from XenoTech (Lenexa, KS) were thawed by using a Hepatocytes Isolation Kit (XenoTech) according to the manufacturer's instruction. The hepatocytes were suspended in William's E medium supplemented with 10 μg/ml insulin, 1 μM hydrocortisone, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) FBS. Final cell viability prior to plating was determined by the Trypan Blue exclusion method. Suspended cells were seeded into collagen-coated 24-well plates (BD Biosciences, Heidelberg, Germany) at a cell density of 2.0×10^5/0.5 ml/well. After the cultures had been incubated for 3–5 h at 37 °C in an atmosphere of 5% CO_2/95% air, the medium was replaced with fresh HHM supplemented with 50 μg/ml Matrigel (BD Biosciences). The medium was changed daily thereafter. Forty-eight hours after plating, the cells were treated with TSU-16 (1 and 10 μM), 3MC (0.5 and 5 μM), OMP (1 and 10 μM) or vehicle (DMSO) for 6, 12, 24, 48, or 72 h. After the chemical exposure, CYP1A activity and CYP mRNA levels (only 48-h exposure for *CYP2B6* and *CYP3A4* mRNAs) were determined.

2.3. Extraction of protein and total RNA from cells

Total protein and total RNA were extracted from hepatocytes and HepG2 cells by using a Protein and RNA Extraction Kit for mammalian cells (TAKARA Bio, Otsu, Japan) or RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Protein concentrations were determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instruction.

2.4. Determination of mRNA levels

Quantitative analyses of mRNA levels were performed with a QuantiTect Probe RT-PCR Kit (QIAGEN) for *CYP1A1*, *CYP1A2*, *CYP3A4* and AhR mRNAs or QuantiTect SYBR Green RT-PCR Kit (QIAGEN) for *CYP2B6* mRNA by using the primers and probes shown in Table 1 and an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes were designed by using Primer Express software (Applied Biosystems) from human mRNA sequence. The mRNA levels were normalized against those of *β-actin* (*ACTB*), which were determined with TaqMan Endogenous Control Human ACTB (Applied Biosystems). To determine the EC_{50} values for the increase in *CYP1A1* and *CYP1A2* mRNA levels in HepG2 cells, the data from concentration–response curves were fitted to a three-parameter sigmoid (Hill) model, according to the following equation: $y = (E_{max} \times y^{\gamma})/(EC_{50}{}^{\gamma} + x^{\gamma})$, where E_{max} is the maximum induction and EC_{50} is the concentration giving 50% of E_{max}. The curve-fitting was carried out using SigmaPlot 2001 (Systat Software, Chicago, IL).

2.5. CYP1A1 reporter construct

A 1.5-kb fragment of the human *CYP1A1* 5′-flanking region (−1360 to +146) was amplified by polymerase chain reaction (PCR) using a pair of primers 5′-ACGCGTCGTAACGATGTTAGC-3′ and 5′-CTCGAGGGTACTGAGCTGAGCT-3′. The resulting 1.5-kb fragment was subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and was then inserted into the MluI and XhoI sites of the pGL3-basic vector (Promega). The plasmid was designated pGL3-1A1. Correct orientation of the insert was confirmed by DNA sequence analysis.

2.6. Reporter gene assay

HepG2 cells (American Type Culture Collection, Manassas, VA) were seeded into 48-well plates (BD Biosciences) containing DMEM