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Metabolic profiling of the anti-tumor drug regorafenib in mice

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ABSTRACT

Regorafenib is a novel tyrosine kinase inhibitor, which has been approved by the United States Food and Drug Administration for the treatment of various tumors. The purpose of the present study was to describe the metabolic map of regorafenib, and investigate its effect on liver function. Mass spectrometry-based metabolomics approach integrated with multiple mass defect filter was used to determine the metabolites of regorafenib *in vitro* incubation mixtures (human liver microsomes and mouse liver microsomes), serum, urine and feces samples from mice treated with 80 mg/kg regorafenib. Eleven metabolites including four novel metabolites were identified in the present investigation. As halogen substituted drug, reductive defluorination and oxidative dechlorination metabolites of regorafenib were firstly report in present study. By screening using recombinant cytochrome P450 s (CYPs), CYP3A4 was found to be the principal isoforms involved in regorafenib metabolism. The predication with a molecular docking model confirmed that regorafenib had potential to interact with the active sites of CYP3A4, CYP3A5 and CYP2D6. Serum chemistry analysis revealed no evidence of hepatic damage from regorafenib exposure. This study provided a global view of regorafenib metabolism and its potential side-effects.

1. Introduction

Regorafenib is a novel multiple kinase inhibitor, which targets the angiogenic (VEGFR1-3, TIE2), stromal (PDGFR-b, FGFR), and oncogenic receptor tyrosine kinases (KIT, RET, and RAF) [1]. It potently inhibits the vascular endothelial cell kinases in biochemical and cellular kinase phosphorylation process [2]. As the first line potent antitumor agent, regorafenib has been approved by the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) to treat refractory metastatic colorectal cancer [3,4], advanced metastatic gastrointestinal stromal tumors [5], and hepatocellular carcinoma [6,7]. Currently, there are few studies investigate the metabolism and disposition of regorafenib. One study reported that seven regorafenib metabolites were identified and derived from hydroxylation, *N*-demethylation and *N*-glucuronidation in healthy volunteers [8,9]. Pharmacokinetics study measured the regorafenib and two main metabolites included *N*-oxidated regorafenib and *N*-oxidated demethylated regorafenib in human plasma using LC–MS/MS [10–12]. Another clinical study found that CYP3A4 was the primary phase I metabolic enzyme for catalyzing the formation of *N*-oxidated regorafenib and *N*-oxidated demethylated regorafenib *via* oxidation reaction, and its metabolic reaction could be significantly attenuated by the potent CYP3A4 inhibitor ketoconazole [13,14]. In addition, phase II metabolic enzyme UGT1A9 was report to take part in the formation the *N*-glucuronide of regorafenib [15].

Currently, ultra-high performance liquid chromatography (UPLC)-quadrupoles time-of-flight tandem mass spectrometry (Q-TOFMS) has facilitated the high-throughput analysis of metabolites, yielding significantly improved accuracy and reliability. Therefore, UPLC-Q-TOFMS has been applied to detect the trace metabolites [16]. It has been demonstrated that metabolomics is a powerful tool for accurately screening the metabolites of drug and xenobiotica, which can eliminate the false positives and endogenous metabolites interference in complex biological sample. Generally, multiple metabolites are generated from the parent drug, and usually share the similar parent structure. Based on parent structure and substituent modifications, multiple mass defect filters (MMDF) is a useful strategy for screening the compounds with similar structure, which can be perfectly applied to filter trace homologues and analogues [17–19]. The disadvantage of MMDF is the formation of false positive result in the experiment. Thus, we proposed an approach

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by integration of metabolomics and MMDF to accurately identify drug metabolites, especially for the trace metabolites.

Although regorafenib is used clinically for anti-tumor therapy, a black box warning of regorafenib was promulgated by the US FDA to emphasize its liver risks [20]. The objective of present study was to characterize the metabolic pathways of regorafenib and its influence on liver function in mice. Molecular docking technology was also used to confirm the observations from the recombinase incubations experiment. The results indicated that CYP3A4, CYP3A5 and CYP2D6 were the major isoforms involved in regorafenib metabolism. And, regorafenib did not generate significant hepatotoxicity in mice at dose of 80 mg/kg.

2. Materials and methods

2.1. Chemicals and reagents

Regorafenib was obtained from Meilunbio company (Dalian, China). Its structure was elucidated by the comprehensive interpretation of the Electrospray Ionization-Mass Spectrum (ESI-MS) and ^{1}H-NMR (Supplementary Fig. 1A). Its purity was higher than 98% based on peak area normalizing method of UPLC-UV analysis (Supplementary Fig. 1B). Nicotinamide adenine dinucleotide phosphate (NADPH), formic acid and chlorpropamide were purchased from Sigma-Aldrich (St. Louis, U.S.A). Human liver microsomes (HLMs) and mouse liver microsomes (MLMs) were obtained from Bioreclamationivt Inc. (Hicksville, NY). Recombinant human CYPs were purchased from Xenotech, LLC (Kansas City, KS). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Micro-anticoagulant tubes (anticoagulant: EDTA dipotassium salt) were purchased from Jiangsu Xinkang Medical Instrument company (Taizhou, China).

2.2. In vitro incubation reaction to analyze the metabolic pathway of regorafenib

In vitro incubation for metabolic reaction was carried out as described previously [21]. Co-incubations experiments of regorafenib with MLMs, HLMs or each c-DNA-expressed recombinant CYPs enzyme *in vitro* were performed in 96 wells plates. The metabolic reactions were conducted in phosphate-buffered saline solution (pH = 7.4). 200 μL of final incubation system volume contained 20 mM of regorafenib, 0.5 mg/mL of MLMs, HLMs or 2 pmol/mL of each c-DNA-expressed CYPs enzyme (control, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP4A11). After pre-incubation at 37 °C for 5 min, the reactions were initiated by the addition of 20 μL freshly prepared NADPH (10 mM) into the system, and then continued for 30 min with gentle shaking. The reactions were terminated by adding 200 μL of ice cold acetonitrile. The absence of NADPH in the system was incubated separately as a control. The independent incubations were conducted parallel in triplicates. After centrifugation at 18,000 *g* for 20 min at 4 °C, a 5 μL aliquot of the supernatant was directly subjected to UPLC-ESI-QTOFMS system for metabolite analysis.

2.3. Animal experiment

The eight male C57BL/6 mice, weighting 20–22 g (6- to 7-week-old) were obtained from Hunan laboratory animal central (Changsha, China). The animals were housed on a 12 h light-dark cycle (light on from 7:00 to 19:00) at environmental temperature (22–24 °) and humidity 50–60 %, with standard laboratory water and chow provided *ad libitum*. The clinical oral dose of regorafenib is up to 160 mg once daily for adult tumor patients [22]. Here, the mice were administrated at the dose of 80 mg/kg, which was equal to four-fold of clinical dose. Eight mice were randomly divided into two groups, regorafenib group and control group (vehicle), and acclimated in metabolic cages for 24 h prior to the administration of regorafenib. The experimental groups were exposed orally to regorafenib fully suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na, 0.1 ml/20 *g* weight), and the control group administered an equal volume of 0.5% CMC-Na without fasting. The mice were housed separately in metabolic cages for 24 h after treatments. Approximately 80 μL blood samples were collected from the suborbital venous plexus of mice at 3 h and 24 h post-dosing. Each blood sample was transferred to the micro-anticoagulant tubes, and centrifuged at 2000 × *g* for 5 min. Both urinary and feces samples were harvested over 0–24 h after gavage. All biological samples were stored at −80 °C before further analysis. All experimental animal studies strictly complied with study protocols approved by the Ethics Committee of the Kunming Institute of Botany, Chinese Academy of Sciences.

2.4. Biological samples preparation

Biological samples procession was carried out as described previously [21]. In brief, plasma analysis samples were prepared *via* adding the 10 μL plasma into 190 μL 67% aqueous acetonitrile containing 5 μM chlorpropamide. Urinary samples were prepared through mixing 20 μL of urine with 180 μL of 50% aqueous acetonitrile containing 5 μM chlorpropamide. The plasma samples and urinary samples were individually vortexed for 2 min, and centrifuged at 18,000 × *g* for 20 min at 4 °C to fully precipitate protein. After centrifugation, the supernatant was transferred to clean centrifuge tube for analysis. For feces samples, 100 mg weight of feces were mixed into 2 mL 50% aqueous acetonitrile containing 5 μM chlorpropamide, and followed by homogenized by shaking for 30 min. The feces samples were centrifuged at 18,000 × *g* for 20 min to remove feces residue. Subsequently, 100 μL of each supernatant from feces homogenate was transferred to a clean centrifuge tube and mixed with 200 μL of 50% aqueous acetonitrile for second centrifugation. Each biological supernatant was transferred to an automatic sampling bottle, and 5 μL aliquot was injected for UPLC-ESI-QTOFMS system to detect the metabolites of regorafenib.

2.5. UPLC-ESI-QTOFMS analysis

In order to set up appropriate analysis system, the critical factor for chromatographic separation was considered, including the filler of column and column temperature. Regorafenib and its metabolites showed a good separation in the following analysis system. Agilent 1290 infinity UPLC system (Agilent Technologies, Santa Clara, CA) equipped with an XDB-C18 column (2.1 × 100 mm, 1.8 μm, Agilent) was used for the separation of metabolites. Based on the results of preliminary experiments, the optimized parameters of chromatography and mass spectrometry were determined as follows. The column temperature was maintained at 45 °C, and the column was eluted with a gradient elution ranging from 2% to 98% acetonitrile containing 0.1% formic acid in 16 min run. The flow rate of mobile phase was 0.3 mL/min. The Q-TOFMS was operated in positive mode with electrospray ionization (ESI^+). Agilent Tuned Liquid G1969-85000 was used for calibrating the Q-TOF until the maximum residual value less than 5. Nitrogen was used as drying gas which was set at 350 °C, 9 L/min, and the nebulizer pressure was set at 35 psi. Capillary and fragmentor voltage was set at 3.5 Kv and 135v, respectively. Centroided mass spectrums were acquired in the mass range of *m/z* 100–1000. Mass accuracy was maintained using purine ($[M+H]^+$ = *m/z* 121.050873) and HP-0921 ($[M+H]^+$ = *m/z* 922.009798) as reference. The MS/MS of regorafenib metabo-