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Monoamine oxidase B oxidizes a novel multikinase inhibitor KW-2449 to its iminium ion and aldehyde oxidase further converts it to the oxo-piperazine form in human

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ABSTRACT

(E)-1-{4-[2-(1H-Indazol-3-yl)vinyl]benzoyl}piperazine (KW-2449) is a novel multikinase inhibitor. During our clinical study, we found that KW-2449 is mainly metabolized to its oxo-piperazine form (M1). An inhibition study suggested that monoamine oxidase-B (MAO-B) oxidizes KW-2449 to an iminium (intermediate) and aldehyde oxidase (AO) then metabolizes the intermediate to M1. The conversion of KW-2449 to the iminium (intermediate) by MAO-B was confirmed by the formation of its cyanide adduct. This cooperative metabolic pathway by MAO-B and AO was newly identified in the metabolism of piperazine. The clearance of KW-2449 by MAO-B and AO in human was estimated based on the kinetic analysis with *in vitro-in vivo* extrapolation. The systemic clearance in human was similar to the calculated value, indicating that the extrapolation approach was applicable to KW-2449 metabolism. Finally, we found that (E)-3-amino-1-{4-[2-(1H-Indazol-3-yl)vinyl]benzoyl}-pyrrolidine (Compound A) as a stable compound against MAO-B and AO. The total body clearance of Compound A was reduced to one tenth of KW-2449, demonstrating that preventing the metabolism of MAO and AO led to more preferable pharmacokinetic profiles. As piperazine is often introduced to drug candidates to improve lipophilicity of the compound to get more hydrophilic nature, the results of this study provide useful information for future drug development.

1. Introduction

From the 1960s to the 1990s, inadequate pharmacokinetic behavior was the most significant cause of clinical attrition [1,2]. With the recognition of this problem, the pharmaceutical industry has established screening methods to assess the absorption, distribution, metabolism and excretion issues to allow the prediction of the human pharmacokinetic profiles [3]. In particular, the extensive metabolism of candidate drugs often causes low bioavailability and insufficient exposure. Various high throughput screening systems have been used to assess metabolic stability against Cytochrome P450s (CYPs) and thereby select suitable candidates in the early development stage [4]. These efforts have dramatically improved pharmacokinetic issues in drug development in the 2000s [2], however, unexpectedly large clearance by drug metabolizing enzymes remained a cause of clinical failure [5,6]. When facing unexpected drug metabolism, the identification of the metabolizing enzyme and metabolic pathway is important for the further optimization of the subsequent candidates.

(*E*)-1-{4-[2-(1*H*-Indazol-3-yl)vinyl]benzoyl}piperazine (KW-2449) is a multikinase inhibitor of FMS-like receptor tyrosine kinase (FLT3), ABL and Aurora kinase and have been investigated in the treatment of leukemia patients [7]. KW-2449 displays a potent inhibitory activity against FLT3 and showed significant dose-dependent growth inhibitory effects against various FLT3-mutated, FLT3 wild-type and imatinib-resistant leukemia cells in a non-clinical pharmacology study [8]. The administration of KW-2449 also showed transient reductions in the peripheral blast

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Abbreviation

MAO	monoamine oxidase
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
AO	aldehyde oxidase
CYP	cytochrome P450
FLT3	FMS-like receptor tyrosine kinase
IVIVE	*in vitro-in vivo* extrapolation
PK	pharmacokinetic
EDTA	ethylenediamine tetraacetic acid
NADPH	nicotinamide adenine dinucleotide phosphate
I.S.	internal standard
LC-MS/MS	liquid chromatography-tandem mass spectrometry
HPLC	high performance liquid chromatography
ESI	electroionization spray
MRM	multiple reaction monitoring
IDA	information dependent acquisition
CL_{Hint}	intrinsic hepatic clearance
CL_H	hepatic clearance
Q_H	hepatic blood flow
R_B	blood to plasma concentration ratio
CL_{Total}	total body clearance
F	bioavailability
Fa	fraction absorbed across the intestinal wall
$CL_{Extrahepatic}$	extrahepatic clearance

counts and the partial inhibition of FLT3 in a phase 1 trial; however, the development program was terminated because it failed to fully inhibit FLT3. In clinical trials, although KW-2449 was rapidly absorbed after oral administration, it was mostly converted to M1, the oxo-piperazine form of KW-2449, and the concentration of M1 was observed to be almost 10 times higher than that of KW-2449 during treatment [7]. The total body clearance of KW-2449 was 3.70 L/h/kg and much greater than the hepatic blood flow [7]. This large clearance of KW-2449 might be a cause of limited exposure to KW-2449 in the clinical trial, resulting in the insufficient inhibition of FLT3 and the clinical failure. As KW-2449 was rapidly metabolized to M1 in the clinical trials, we investigated the enzymology of the oxidation pathway of the piperazine moiety of KW-2449 to screen the successor compound with better pharmacokinetic profiles. Since KW-2449 was selected through lead optimization as a stable compound against CYP, the contribution of non-CYP enzymes to the KW-2449 metabolism was mainly explored. In the present study, we identified monoamine oxidase B (MAO-B) and aldehyde oxidase (AO) as the enzymes responsible for the formation of M1 from KW-2449. We also found that an iminium (intermediate) was present in the metabolic pathway. We additionally tried to predict the *in vivo* clearance in human using two *in vitro-in vivo* extrapolation (IVIVE) approaches, since there was no established methodology for investigating the cooperative metabolism by MAO and AO. Finally, we found (E)-3-amino-1-{4-[2-(1H-Indazol-3-yl)vinyl]benzoyl}-pyrrolidine (Compound A) as a stable compound against MAO-B and AO and demonstrated that its PK profile was superior to that of KW-2449.

2. Materials and methods

All *in vitro* experiments were performed under an insect repelling fluorescent light due to the instability of KW-2449 under the normal light conditions. The concentration described in the *in vitro* experiments refer to the final concentrations in the reaction mixture.

2.1. Chemicals

KW-2449, (E)-1-{4-[2-(1H-Indazol-3-yl)vinyl]benzoyl}piperazine-3-one (M1), (E)-4-[2-(1H-indazol-3-yl)vinyl]benzoic acid (M2), (E)-N-(2-aminoethyl)-4-[2-(1H-indazol-3-yl)vinyl]benzamide (M3), (E)-2-{4-[2-(1H-indazol-3-yl)vinyl]benzamido}acetic acid (M4) and Compound A were provided by Kyowa Hakko Kirin. ^{14}C-KW-2449 was synthesized at Amersham Biosciences (Buckinghamshire, UK). Human liver microsomes (mixture from 50 donors), human liver S9 (mixture from 50 donors), human liver cytosol (mixture from 50 donors), human liver mitochondria (mixture from 50 donors), cynomolgus liver microsomes and rat liver microsomes were purchased from Sekisui Xenotech (Kansas City, KS, USA). Human CYPs and MAOs Supersomes (recombinant CYPs and MAOs) were purchased from BD Biosciences (San Jose, CA, USA). Clorgyline hydrochloride (MAO-A inhibitor) was purchased from MP Biomedicals (Irvine, CA, USA). Pargyline hydrochloride (MAO-B inhibitor) was purchased from Sigma (St. Louis, MO, USA). Menadione (AO inhibitor) and allopurinol (xanthine oxidase inhibitor) were purchased from Nacalai Tesque (Kyoto, Japan). Sodium cyanide (NaCN, iminium ion trapping reagent) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-AO antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human hepatocytes (pooled from 3 donors) were purchased from Tissue Transformation Technologies (Edison, NJ, USA) and rat, dog and cynomolgus monkey hepatocytes were purchased from In Vitro Technologies (Baltimore, MD, USA). Human kidney and lung mitochondria were prepared in-house from the commercially available tissue blocks. Human control plasma was obtained from volunteers at our institute after obtaining their written informed consent and monkey control plasma was purchased from Hamri (Ibaraki, Japan). Water purified with the Milli-Q gradient system (Merck, Tokyo, Japan) was used. The other reagents were commercially available and of a guaranteed grade.

2.2. Animal study

All of the animal studies were performed in accordance with Standards for Proper Conduct of Animal Experiments at Kyowa Hakko Kirin Co., Ltd.

2.2.1. Pharmacokinetic study in cynomolgus monkey

KW-2449 was administered intravenously and orally to cynomolgus monkeys at a dose of 0.82 mg/kg and 100 mg/kg, respectively. Blood was drawn at 5 min to 48 h after administration. To evaluate the conversion ratio from KW-2449 to M1, M1 was administered intravenously to cynomolgus monkeys at a dose of 2 mg/kg and blood was drawn at 5 min to 24 h after administration. KW-2449 was administered intravenously to male Sprague-Dawley rat at a dose of 10 mg/kg and blood was drawn at 3 min to 48 h after administration. Another monkey study using ^{14}C-KW-2449 was also conducted to investigate the metabolic profile of KW-2449. ^{14}C-KW-2449 (8.04 MBq/kg) was administered orally to cynomolgus monkeys at a dose of 5 mg/kg and blood was drawn at 30 min after administration. Compound A was administrated intravenously to cynomolgus monkeys at 1 mg/kg and blood was drawn at 5 min to 24 h after administration. Plasma samples were

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