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Stereospecific reduction of a potent kinesin spindle protein (KSP) inhibitor in human tissues

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ABSTRACT

Compound A, 1-{(3*R*,3a*R*)-3-[3-(4-acetylpiperazin-1-yl)propyl]-7-fluoro-3-phenyl-3a,4-dihydro-3*H*-pyrazolo[5,1-*c*][1,4]benzoxazin-2-yl}ethanone, is a novel and potent inhibitor of the mitotic kinesin spindle protein. Metabolism studies with human hepatocytes showed that Compound A underwent significant ketone reduction to its biologically active metabolite M1. Here, we describe the studies that characterized the metabolic interconversion between Compound A and M1 in vitro in human tissues. LC–MS/MS analysis showed that the ketone reduction was stereospecific for M1 with no diastereomer of M1 detected in incubations with human hepatocytes. Interestingly, such stereospecific ketone reduction was not observed with Compound B, the enantiomer of Compound A. No reductive products were observed when Compound B was incubated with human hepatocytes. Studies with human liver subcellular fractions showed that Compound A was reduced to M1 primarily by human liver cytosol with little contribution from human liver microsomes and mitochondria. NADPH was the preferred cofactor for the reduction reaction. Reverse oxidation of M1 back to Compound A was also observed, preferentially in human liver cytosol with $NADP^+$ as the cofactor. The interconversion between Compound A and M1 in human liver cytosol was inhibited significantly by flufenamic acid and phenolphthalein (potent inhibitors for aldo–keto reductase 1Cs, $p < 0.05$), but not by menadione, a selective inhibitor for carbonyl reductase. In addition to the liver, S9 from human lung and kidney was also capable of catalyzing this interconversion. Collectively, the results implicated the aldo–keto reductase 1Cs as the most likely enzymes responsible for the metabolic interconversion of Compound A and its active metabolite M1.

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1. Introduction

Kinesin spindle protein (KSP), also known as *Hs* Eg5, is a member of the kinesin superfamily of molecular motors that drives the separation of centrosomes during the mitosis [1]. Inhibition of KSP prevents normal bipolar spindle formation, which leads to mitotic arrest with a characteristic monoastral phenotype and subsequently to apoptosis in transformed cells. To date, many KSP inhibitors have been synthesized and investigated for potential treatment of cancer. Among them, Compound A, 1-{(3*R*,3a*R*)-3-[3-(4-acetylpiperazin-1-yl)propyl]-7-fluoro-3-phenyl-3a,4-dihydro-3*H*-pyrazolo[5,1-*c*][1,4]benzoxazin-2-yl}ethanone (Fig. 1), is a potent and selective KSP inhibitor [2]. In vitro, Compound A exhibited excellent biochemical and cellular potencies against KSP, good aqueous solubility and limited susceptibility to P-gp-mediated efflux [2]. In vivo, Compound A demonstrated robust biomarker response (mitotic arrest as measured by phospho-histone H3) and tumor growth inhibition in a xenograft mouse model of cancer [2]. Interestingly, such potent KSP inhibition is enantioselective; Compound A is 500-fold more potent against KSP than its enantiomer, Compound B (Fig. 1).

Metabolism studies with human hepatocytes showed that Compound A underwent significant ketone reduction to its biologically active metabolite M1 (Fig. 1). The pharmacological activity of M1 was ~7-fold less potent than Compound A in vitro. Reduction of xenobiotic ketones is an important metabolic route to produce more water soluble and often less chemically reactive metabolites [3,4]. Two main protein families have been shown to be capable of catalyzing the ketone reduction, namely short-chain

Abbreviations: AKRs, aldo–keto reductases; HSD, hydroxysteroid dehydrogenase; I.S., an internal standard; KSP, kinesin spindle protein; NADPH_R, NADPH regeneration system; SDRs, short-chain dehydrogenases/reductases.

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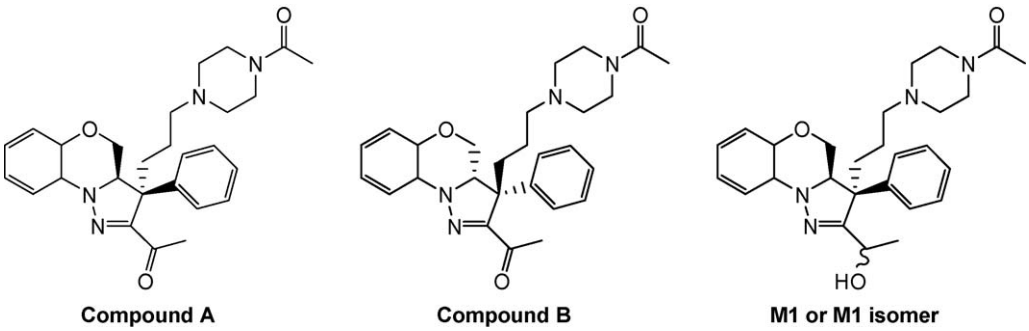

Fig. 1. Chemical structures of Compound A, Compound B and metabolite M1 or M1 isomer.

dehydrogenases/reductases (SDRs) and aldo–keto reductases (AKRs). As summarized in Table 1, the major enzymes that metabolize xenobiotic ketones in humans include carbonyl reductase, 11-β-hydroxysteroid dehydrogenase (11β-HSD), and several members of the AKR1C subfamily [5,6]. These ketone reducing enzymes are characterized by broad and overlapping substrate specificities with diverse subcellular and tissue distributions, cofactor dependences and selective inhibitor profiles [3,4]. Moreover, in contrast to carbonyl reductase, 11β-HSD and AKR1Cs are capable of catalyzing the oxidation of secondary alcohols and diols to ketones in a $NAD(P)^+$ dependent fashion [7]. Table 1 also lists the human enzymes that could potentially catalyze the oxidation of secondary alcohols to ketones, which include microsomal enzymes (e.g., cytochrome P450s and 11β-HSD) and cytosolic enzymes (e.g., AKR1Cs). In addition to their role in the xenobiotic metabolism, these ketone reducing enzymes are also involved in the metabolism of endogenous compounds, including steroid hormones, prostaglandins, bile acids and arachidonic acid.

The aim of the present work was to characterize the ketone reduction of Compound A in vitro in human tissues. As shown in Fig. 1, Compound A is an unsymmetrical ketone, which generates an additional chiral center upon reduction to alcohols. Stereochemistry of ketone reduction of Compound A was therefore assessed and compared with that of its enantiomer B. In addition, the subcellular location, tissue distribution, cofactor dependency and effects of chemical inhibitors were evaluated to determine the potential enzyme systems involved in the metabolic reduction of Compound A to its active metabolite M1. The reverse oxidative reaction from M1 back to Compound A was also evaluated. Finally, the kinetics of metabolic interconversion between Compound A and M1 was characterized in the presence of appropriate cofactors.

2. Materials and methods

2.1. Chemicals and materials

Compound A, B, M1 and M1 isomer were synthesized and purified at Merck Research Laboratories (West Point, PA, USA). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA). Pooled human tissue S9 and liver subcellular fractions (mixed gender) including microsomes, cytosol and mitochondria were purchased from XenoTech, LLC (Lenexa, KS). Fresh human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD). All the other chemical agents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Metabolism studies in human hepatocytes

Human hepatocytes (1 million viable cells/mL, total volume of 0.5 mL) were suspended in Hanks' balance salt solution with 10 mM HEPES (pH 7.4) and were incubated with 1 μM of Compound A or B at 37 °C. The incubations were terminated at 0 or 2 h by the addition of 0.5 mL of acetonitrile containing 0.4 μM of labetalol as an internal standard (I.S.). The acetonitrile extracts were analyzed by an LC–MS/MS method (see below).

2.3. Metabolism studies in human tissue subcellular fractions

All incubations were performed in triplicate or quadruplicate at 37 °C in phosphate buffer (100 mM, pH 7.4) containing 10 mM $MgCl_2$. To determine the subcellular location and cofactor dependency, Compound A or M1 (1 μM) was incubated with human tissue subcellular fractions (0.15 mg/mL) in a final volume of 0.2 mL. After a 3 min preincubation, reactions were initiated by

Table 1
Characteristics of human enzymes involved in the reduction of xenobiotic ketones to alcohols and the oxidation of secondary alcohols to ketones.

Enzymes	Reactions	Cofactor	Subcellular location	Selective chemical inhibitors
SDR family				
CBR1: carbonyl reductase	Reduction of ketones	NADPH	Cytosol	Menadione
11β-HSD[a]	Reduction of ketones	NADPH	Microsomes	Glycyrrhetinic acid
	Oxidation of secondary alcohols	$NADP^+$		
AKR family				
AKR1C1: 20α-HSD[a]	Reduction of ketones	NAD(P)H	Cytosol	Flufenamic acid, phenolphthalein
	Oxidation of secondary alcohols	$NAD(P)^+$		
AKR1C2: 3α-HSD[a] type 3	Reduction of ketones	NAD(P)H	Cytosol	Flufenamic acid, phenolphthalein
	Oxidation of secondary alcohols	$NAD(P)^+$		
AKR1C3: 3α-HSD[a] type 2; 17β-HSD[a] type 5	Reduction of ketones	NAD(P)H	Cytosol	Flufenamic acid, phenolphthalein
	Oxidation of secondary alcohols	$NAD(P)^+$		
AKR1C4: 3α-HSD[a] type 1	Reduction of ketones	NAD(P)H	Cytosol	Flufenamic acid, phenolphthalein
	Oxidation of secondary alcohols	$NAD(P)^+$		
Cytochrome P450 isozymes	Oxidation of secondary alcohols	NADPH	Microsomes	–[b]

[a] HSD: hydroxysteroid dehydrogenase.
[b] Specific chemical inhibitors for each cytochrome P450 isozyme have been recommended in FDA drug interaction guideline [27].

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