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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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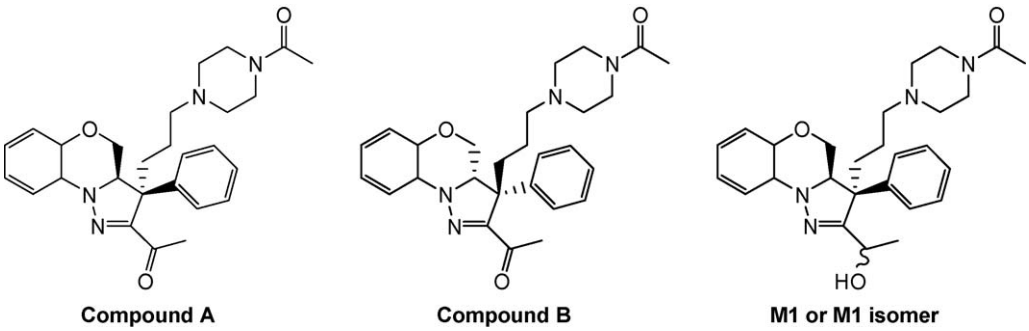

Fig. 1. Chemical structures of Compound A, Compound B and metabolite M1 or M1 isomer.

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].

the addition of the cofactor and were terminated after 20 min of incubations at 37 °C by the addition of 200 μL of acetonitrile containing 0.4 μM of labetalol (I.S.). The resultant supernatant following centrifugation was subjected to LC–MS/MS analysis. Five different cofactors have been investigated in the present study, 1 mM NAD^+, 1 mM $NADP^+$, 1 mM NADH, 1 mM NADPH and NADPH regeneration system (NADPH_R: 1 mM NADPH, 20 mM DL-isocitric acid and 0.4 units/per incubation isocitric dehydrogenase). Control experiments were conducted by excluding either the cofactor or the protein from the incubation mixtures.

Investigation of time dependent metabolism of Compound A or M1 (1 μM) in human liver cytosol (0.15 mg/mL) was carried out with the corresponding preferred cofactor for varying periods of incubation time up to 60 min. Kinetic studies were conducted using 0.1–40 μM of Compound A or M1 with human liver cytosol (0.15 mg/mL) in the presence of the appropriate cofactors under the optimized incubation conditions, which were chosen to ensure the reaction velocities were linear with incubation time and protein concentrations.

Inhibition studies with various chemical inhibitors (5 or 20 μM) were performed with Compound A or M1 (1 μM) and human liver cytosol (0.15 mg/mL) in the presence of the appropriate cofactors. The inhibitors were dissolved in 50% (v/v) acetonitrile aqueous stock solutions (50-fold). Control incubations contained the same concentration of acetonitrile without inhibitor (1%, v/v, final concentration).

2.4. Sample analysis

The separation of M1, M1 isomer, Compound A, and I.S. (labetalol) was accomplished on an Inertsil® ODS-3 column (2.1 mm × 50 mm, 5 μm, Varian, Inc., Palo Alto, CA) using PE 200 binary pumps (Perkin-Elmer Life and Analytical Sciences, Inc., Wellesley, MA). Solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in acetonitrile. The mobile phase was delivered at a flow rate of 0.5 mL/min with a linear increase of solvent B from 5% to 50% over a period of 3.5 min and from 50% to 95% over 0.1 min. This gradient was held for 0.9 min before returning to 5% over 0.1 min. Equilibration was allowed for an additional 1.4 min, giving a total chromatographic run time of 6.0 min. The analytes were detected by a PE Sciex API 3000 tandem mass spectrometer with a Sciex turbo ionspray interface (Applied Biosystems, Foster City, CA). Selected reaction monitoring experiments in the positive ionization mode were performed using a dwell time of 150 ms per transition to detect ion pairs at m/z 479/167 (Compound A or B), 481/463 (M1 or M1 isomer) and 329/162 (labetalol, I.S.). Standard curves were prepared by plotting the analyte/I.S. peak area ratios against the concentrations of the analyte spiked in tissue subcellular incubation mixtures. For each analyte, standard curves showed satisfactory linearity, accuracy and precision (<20% coefficient of variation).

2.5. Data analysis

Estimates of apparent K_m and V_{max} were obtained by fitting the untransformed data to Michaelis–Menten kinetics using GraphPad Prism 4.0 (San Diego, CA). This program was also used for graphing the enzyme kinetic figures. The in vitro intrinsic clearance (CL_{int}) was determined by V_{max}/K_m ratio. Statistical comparison of K_m and V_{max} under various conditions was conducted by two-sample t-test. The p-values were adjusted by Bonferroni's criterion [8]. For all other analysis, comparisons were conducted by ANOVA and post hoc tests for multiple testing adjustments [8].

3. Results

3.1. Stereospecific reduction of Compound A in vitro in human hepatocytes

A robust LC–MS/MS method has been developed to separate M1 from its stereoisomer (M1 isomer, Fig. 2A). No attempt has been made to separate Compound A from its enantiomer B, since synthetic standards of both compounds have been confirmed to be enantiomerically pure by a chiral column [2]. It is also unlikely, based on their chemical structures, for chiral interconversion between Compound A and B to occur chemically and enzymatically under the current experimental conditions (Fig. 1). When Compound A was incubated with human hepatocytes, only M1 was observed with no M1 isomer detected by LC–MS/MS (Fig. 2B). In contrast, Compound B, the enantiomer of Compound A, which also contains a prochiral ketone group (Fig. 1), did not undergo ketone reduction at all. No peaks corresponding to 481/463 ion transition were detected when Compound B was incubated with human hepatocytes (Fig. 2C).

3.2. Characterization of ketone reduction of Compound A in vitro in human liver subcellular fractions

Fig. 3 shows the cofactor dependence of ketone reduction of Compound A in vitro in human liver S9. No reductive metabolite (M1) was formed when Compound A was incubated with NAD^+, $NADP^+$, or buffer control (no cofactor, Fig. 3A). Both NADH and NADPH were able to serve as electron donors for ketone reduction of Compound A. The formation rate of M1, however, was ~10-fold higher when NADPH was used as the cofactor compared to NADH ($p < 0.05$). NADPH_R appeared to produce slightly greater amount of reductive metabolite (M1) than NADPH alone, but the difference between the two was not statistically significant (Fig. 3A). Reverse oxidation of M1 back to Compound A was also observed (Fig. 3B). Either NAD^+ or $NADP^+$ was able to serve as the cofactor for the oxidative reaction of M1 to Compound A. $NADP^+$ was slightly preferred over NAD^+ in human liver S9 (~2-fold, $p < 0.05$, Fig. 3B).

Among all the human liver subcellular fractions, cytosol exhibited the highest formation rate of M1 on a per-milligram-of-protein basis ($p < 0.05$, Fig. 4A). Very little M1 was formed when Compound A was incubated with human liver microsomes and mitochondria in the presence of NADPH_R (Fig. 4A). Consistent with the reduction of Compound A to M1, M1 was oxidized back to Compound A primarily by human liver cytosol with little

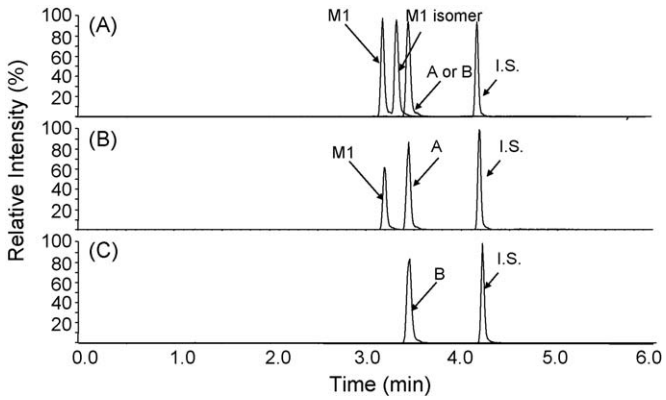

Fig. 2. Representative LC–MS/MS profiles of metabolism of Compound A or B in human hepatocytes with selective ion monitoring at m/z 479/167 (Compound A or B), 481/463 (M1 or M1 isomer) and 392/162 (labetalol, I.S.). (A) Synthetic standards; (B) incubation of Compound A with human hepatocytes; (C) incubation of Compound B with human hepatocytes.

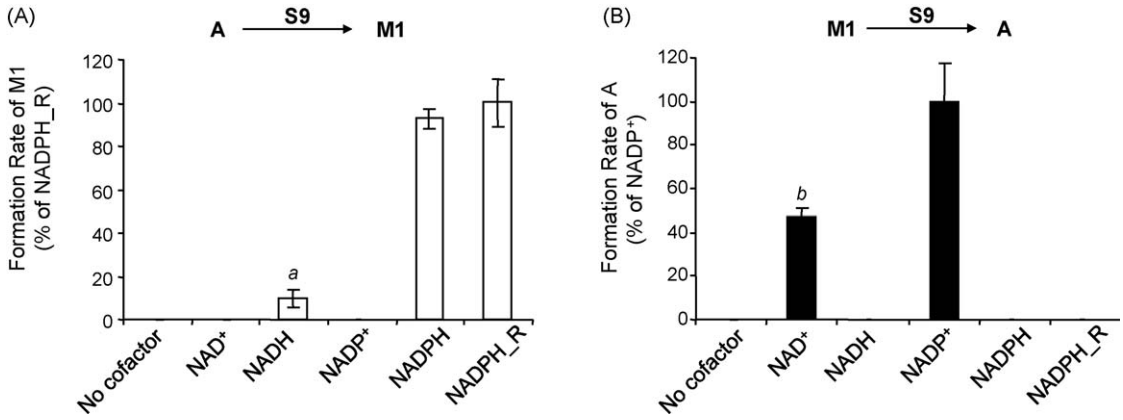

Fig. 3. Cofactor dependence of ketone reduction of Compound A to M1 (A) and oxidation of M1 back to Compound A (B) in vitro in human liver S9. Values represent means ± SD ($n = 4$). [a]Significantly different from NADPH in incubations with human liver S9 ($p < 0.05$); [b]significantly different from $NADP^+$ in incubations with human liver S9 ($p < 0.05$).

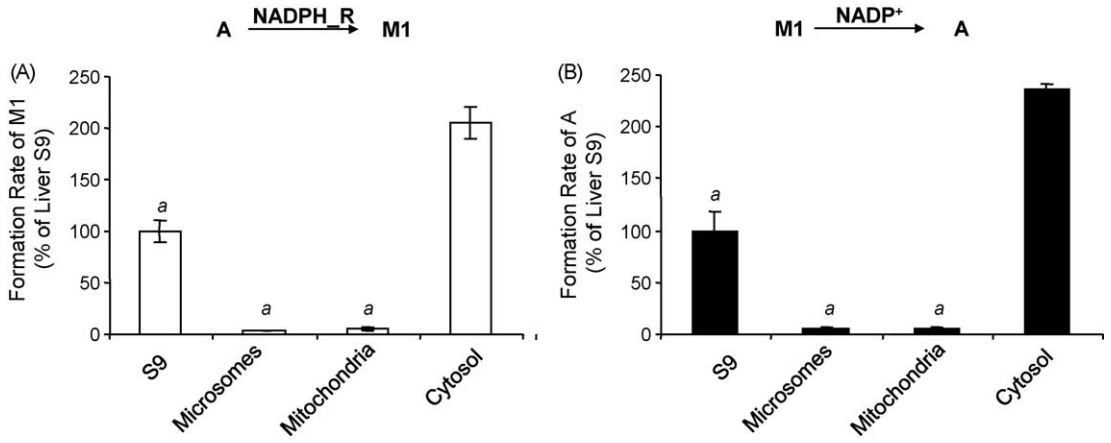

Fig. 4. Subcellular location of ketone reduction of Compound A to M1 (A) and oxidation of M1 back to Compound A (B) in vitro in human liver subcellular fractions in the presence of the corresponding preferred cofactor. Values represent means ± SD ($n = 4$). [a]Significantly different from human liver cytosol ($p < 0.05$).

contribution from human liver microsomes and mitochondria ($p < 0.05$, Fig. 4B).

Time dependent interconversion between Compound A and M1 was characterized in human liver cytosol. In the presence of NADPH_R, formation of M1 was readily apparent, and increased with incubation time (Fig. 5A). At the end of 1-h incubation, ~50% of Compound A was converted to M1. In contrast, the extent of metabolism was much less for the reverse reaction; ~16% of M1 was oxidized back to Compound A over the same 1-h incubation (Fig. 5B). The sum of M1 and its metabolite A was slightly lower than 100% of initial concentration at the measured time points (Fig. 5B). The small difference (7–13%) from 100% of initial concentration is likely due to experimental and analytical variation. Consistent with this, we have not observed any

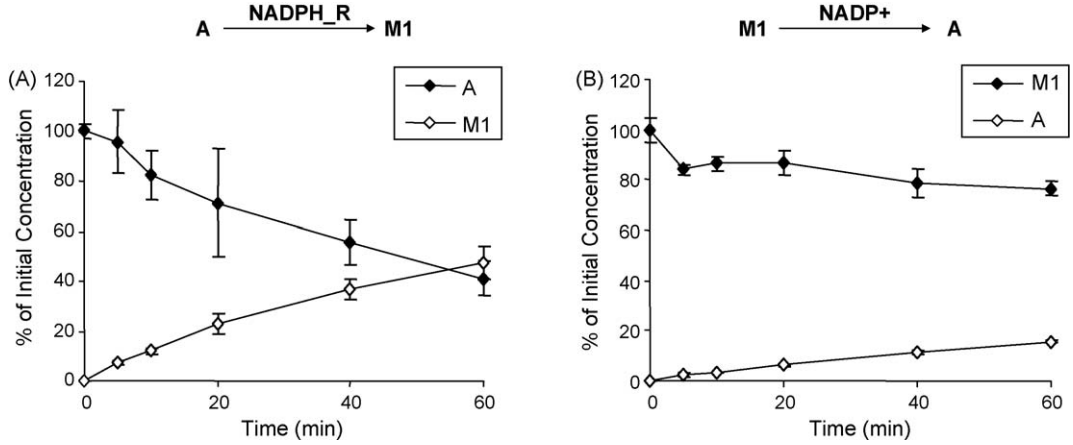

Fig. 5. Time dependent reduction of Compound A to M1 (A) and oxidation of M1 to Compound A (B) in vitro in human liver cytosol. Values represent means ± SD ($n = 4$).

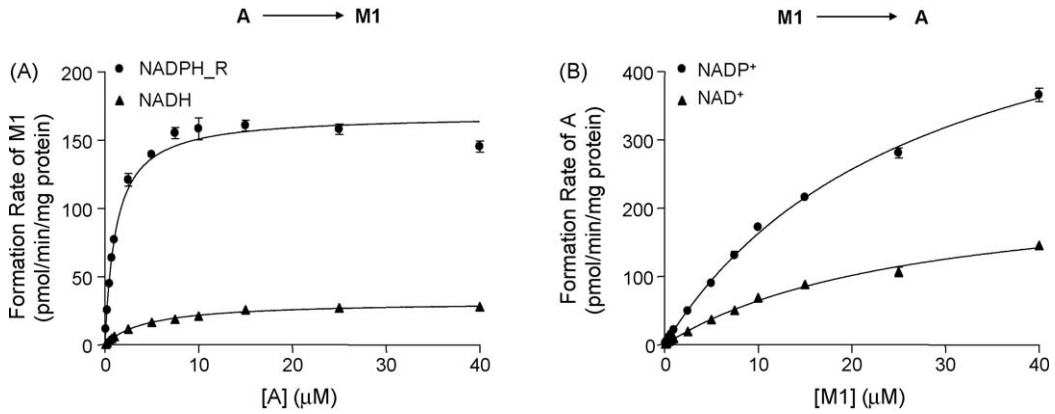

Fig. 6. Kinetics of ketone reduction of Compound A to M1 in the presence of NADPH_R or NADH (A) and oxidation of M1 back to Compound A in the presence of $NADP^+$ or NAD^+ (B) in human liver cytosol. Values represent means ± SD ($n = 4$).

Table 2
Kinetic parameters of metabolic interconversion between Compound A and M1 in human liver cytosol.

Reaction	Cofactor	K_m (μM)	V_{max} (pmol/min/mg)	CL_{int} (μL/min/mg)
A → M1	NADPH_R	1.2 ± 0.1[a]	160 ± 3[a]	140
	NADH	4.6 ± 0.3	32 ± 1	6.9
M1 → A	$NADP^+$	27 ± 2	610 ± 20[b]	23
	NAD^+	27 ± 3	240 ± 10	8.8

Data are expressed as are Mean ± SD ($n = 4$).
[a] Significantly different from NADH using two-sample t-test ($p < 0.05$).
[b] Significantly different from NAD^+ using two-sample t-test ($p < 0.05$).

additional peak during the MS analysis, indicating that there is no alternative metabolic pathway in incubations of M1 with human liver cytosol in the presence of $NADP^+$.

The kinetics of metabolic interconversion between Compound A and M1 in human liver cytosol followed apparent hyperbolic kinetics over a substrate concentration range of 0.1–40 μM (Fig. 6). The derived kinetic parameters (K_m, V_{max} and CL_{int} defined as V_{max}/K_m) are listed in Table 2. In the presence of NADPH_R, reduction of Compound A to M1 was characterized by a high affinity and relatively low capacity reaction as indicated by the low K_m of 1.2 μM and V_{max} of 160 pmol/min/mg protein (Fig. 6A). On the contrary, the oxidation of M1 back to Compound A in the presence of $NADP^+$ was described as a low affinity (K_m of 27 μM) and high capacity (V_{max} of 610 pmol/min/mg protein) reaction (Fig. 6B). The overall CL_{int} of the reductive reaction using NADPH_R was 6-fold higher than that of oxidative reaction using $NADP^+$ (Table 2).

Compared to that with NADPH_R, kinetic studies with NADH revealed an increase in K_m (4.6 μM, $p < 0.05$) and a decrease in V_{max} (32 pmol/min/mg protein, $p < 0.05$) for the reduction of Compound A to M1 (Fig. 6A and Table 2). The apparent CL_{int} was 20-fold higher when NADPH_R was used as the cofactor compared to NADH (Table 2), indicating that NADPH_R is the preferred cofactor for the reduction of Compound A to M1 in vitro. Such cofactor preference, however, was significantly less for the reverse reaction. CL_{int} of oxidation of M1 back to Compound A was only 2.6-fold higher for $NADP^+$ than that for NAD^+ (Table 2).

3.3. Tissue distribution

As illustrated in Fig. 7A, in addition to the liver, S9 from human lung and kidney was able to catalyze the reduction of Compound A to M1 in the presence of NADPH_R. Reverse oxidation of M1 back to Compound A was also observed in the above three human tissues in the presence of $NADP^+$(Fig. 7B). Among the various human tissues studied, the kidney exhibited the highest capacity to catalyze the metabolic interconversion between Compound A and

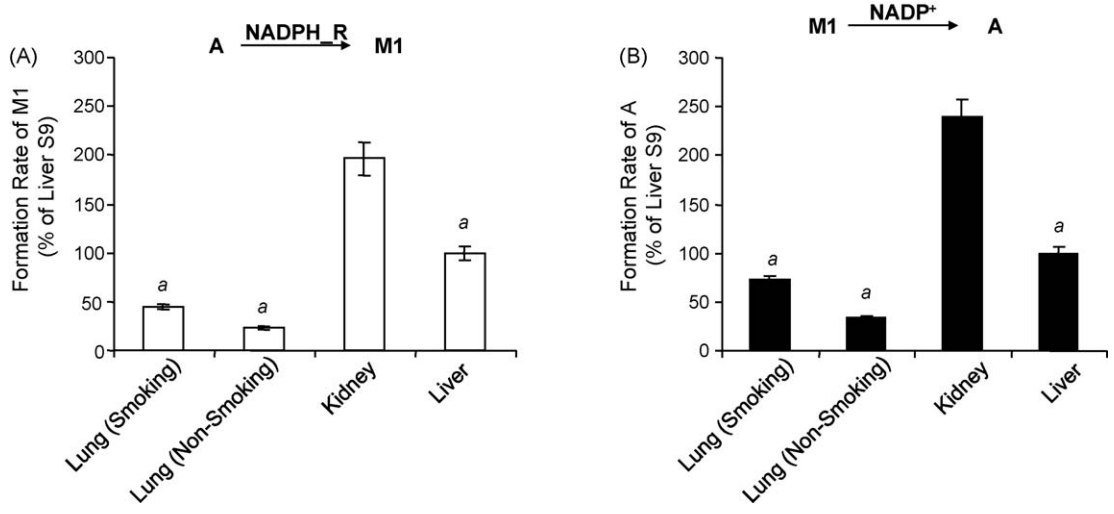

Fig. 7. Metabolic interconversion between Compound A and M1 in human tissue S9. (A) Ketone reduction of Compound A to M1 in the presence of NADPH_R; (B) oxidation of M1 back to Compound A in the presence of $NADP^+$. Values represent means ± SD ($n = 3$). [a]Significantly different from human kidney S9 ($p < 0.05$).

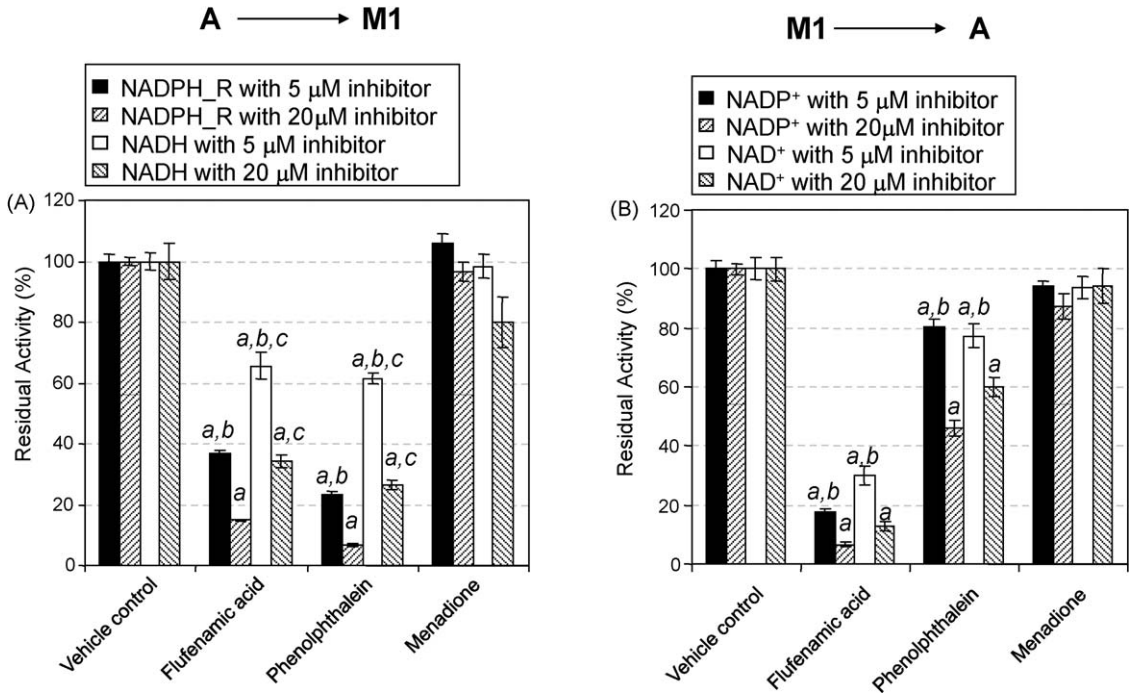

Fig. 8. Effects of inhibitors selective for different ketone reducing enzymes on the reduction of Compound A to M1 (A) and the oxidation of M1 to Compound A (B) in human liver cytosol. Values represent means ± SD ($n = 4$). [a]Significantly different from the vehicle control in the presence of the same cofactor ($p < 0.05$); [b]significantly different from 20 μM of the same inhibitor in the presence of the same cofactor ($p < 0.05$); [c]significantly different from NADPH_R in the presence of the same concentration of the inhibitor ($p < 0.05$).

M1 on a per-milligram-of-S9 protein basis ($p < 0.05$, Fig. 7). Pooled lung S9 from non-smoking individuals ($n = 5$) appeared to have slightly lower reaction rate (~2-fold) than that from smoking individuals ($n = 5$, Fig. 7), however, the difference between the two subpopulations was not statistically significant when multiple comparisons were conducted by ANOVA and post hoc tests.

3.4. Inhibition studies

Selective inhibitors (5 and 20 μM) were evaluated to identify the potential enzyme(s) responsible for ketone reduction of Compound A to M1 in human liver cytosol in the presence of NADPH_R or NADH. The addition of flufenamic acid and phenolphthalein, the diagnostic inhibitors of AKR1Cs [2,9], resulted in a dose-dependent inhibition of M1 formation ($p < 0.05$, Fig. 8A). In the presence of NADPH_R, M1 formation was strongly inhibited by 85% and 93% by 20 μM of flufenamic acid and phenolphthalein, respectively (Fig. 8A). The extent of inhibition by flufenamic acid and phenolphthalein was slightly less when NADH was used as the cofactor as compared to the corresponding NADPH_R ($p < 0.05$, Fig. 8A). Menadione, a potent inhibitor of carbonyl reductase [3,10], did not significantly inhibit the M1 formation at a concentration of 20 μM. Similar inhibitory profiles were also observed for the reverse reaction. Oxidation of M1 to Compound A was significantly inhibited by flufenamic acid and phenolphthalein ($p < 0.05$), but not by menadione (Fig. 8B). It was noted that inhibitory profiles of flufenamic acid and phenolphthalein against the two reactions were different. Phenolphthalein strongly inhibited the reduction of Compound A to M1 (~93% of inhibition at 20 μM in the presence of NADPH_R), but only moderately inhibited the reverse oxidative reaction (~54% of inhibition at 20 μM in the presence of $NADP^+$, Fig. 8). In contrast, flufenamic acid appeared to inhibit oxidative reaction to a greater extent as compared with reductive reaction (93% and 85% of inhibition at 20 μM for oxidative and reductive reactions in the presence of $NADP^+$ or NADPH_R, respectively).

4. Discussion

As efforts in drug design strive to reduce or remove cytochrome P450 mediated metabolic liability, increasing numbers of drug candidates were found to be cleared by other mechanisms [11], such as ketone reduction. In contrast to the cytochrome P450s, ketone reduction is much less well characterized. The goal of the present study is to use Compound A as a model substrate to characterize the ketone reduction pathway in vitro in human tissues. Its potential impact on the development of a drug candidate was also discussed.

As shown in Fig. 2, ketone reduction of Compound A is stereospecific for M1; only M1 was formed with no M1 isomer detected in vitro in human tissues. It is worth noting that the absolute configuration of M1 was not determined experimentally in the present study, and that according to Baumann and Prelog's empirical formula [12] and a number of examples in the literature [3,13,14], M1 possibly takes the *S*-configuration. Importantly, lack of the absolute configuration of M1 should not impact the result interpretation and conclusion since the synthetic standards for M1 and its isomer were available and our HPLC method was able to separate the two from each other (Fig. 2). In addition to product stereospecificity, we found that ketone reduction was also stereospecific for the substrate. Ketone reduction prefers Compound A, the *RR*-isomer, as a substrate. Its *SS*-enantiomer, Compound B, did not undergo appreciative reductive reaction under the same incubation condition (Fig. 2). Compared to product stereospecificity, there are fewer published examples of substrate stereospecificity for ketone reduction. One classic example is the reduction of warfarin that appeared to have a preference for the *R*-enantiomer in human tissues [13].

Reduction of Compound A to M1 prefers NADPH as a cofactor. NADH, on the other hand, was also able to serve as the electron donor for the reductive reaction, but using NADH the substrate affinity and capacity were much lower compared to the reaction kinetics using NADPH_R ($p < 0.05$, Table 2). As a result, the CL_{int} of

the reduction of Compound A is ~20-fold higher when NADPH_R was used as a cofactor than that of NADH. In comparison to the reductive reaction, the oxidative reaction exhibited much less cofactor preference with CL_{int} ratio of 2.6 between $NADP^+$ and NAD^+ (Table 2). For the oxidative reaction, the cofactors appeared to have little impact on the affinity of the enzyme(s) to the substrate (the same K_m), but significantly impacted the enzymatic capacity of the reaction (V_{max}, $p < 0.05$). It is possible that a single enzyme (e.g., an isozyme from AKR1C subfamily) mediated the metabolic interconversion between Compound A and M1 in the presence of various cofactors (Table 1). However, our current data could not rule out the possibility that multiple enzymes might be involved in the two reactions with one or more enzymes preferring one reaction or cofactor over the other.

Consistent with tissue distribution reported in the literature [15], ketone reductase activity of Compound A was evident in all three human tissues studied: the S9 fractions of the lung, kidney and liver. Among all the human tissue S9 studied, the kidney S9 exhibited the highest capacity to catalyze the metabolic interconversion between Compound A and M1 on a per-milligram-of-protein basis with ~2-fold and 4–8-fold higher activity than that for the liver and lung, respectively ($p < 0.05$, Fig. 7). However, the liver may still contribute more to the overall reduction of Compound A to M1 as compared to the kidney and lung, considering that the liver weight is ~6-fold and 1.8-fold higher than that of kidney and lung, respectively [16]. The reverse reaction was also observed in the above three human tissues in a $NADP^+$ dependent manner. The rank order of the oxidative activity was the same as the reductive reaction: kidney > liver > smoking lung > non-smoking lung (Fig. 7B). Pooled lung S9 from smoking individuals ($n = 5$) appeared to have slightly higher reaction rate (~2-fold) than that from non-smoking individuals ($n = 5$, Fig. 7). The difference between the two was statistically significant only when a single comparison between smoking and non-smoking lung S9 was made (t-test, $p < 0.05$). Although a definitive conclusion could not be made due to limited number of sample size, it remains possible that smoking induces the ketone reducing enzymes in the lung. It has been documented that one or more ketone reductase(s) were induced by cigarette smoke constituents, such as polycyclic aromatic hydrocarbons, in vitro in human hepatoma (HepG2) cells [17].

Study with human liver subcellular fractions showed that reduction of Compound A to M1 was mediated predominately by cytosolic enzymes with little contribution from microsomal and mitochondrial fractions (Fig. 4). This observation rules out the possible involvement of 11-β-HSD, a microsomal reductase, suggesting that carbonyl reductase and AKR1Cs (AKR1C1–1C4) are likely the possible candidates in mediating the reduction reaction (Table 1). Our inhibition study showed that 20 μM of menadione did not significantly inhibit the M1 formation (Fig. 8A). As a potent inhibitor of carbonyl reductase with K_i of 5.6 μM [18], menadione would be expected to exhibit significant inhibition at a concentration of 20 μM if carbonyl reductase contributes significantly to the reduction of Compound A to M1. The fact that ketone reduction was inhibited significantly by flufenamic acid and phenolphthalein rather than menadione indicated that ARK1Cs were the most likely enzymes responsible for the reduction of Compound A to M1. Due to lack of specific inhibitors for AKR1C isozymes, the relative contribution of each AKR1C isozyme was not evaluated.

The reverse reaction, oxidation of M1 back to Compound A, also occurred in a cofactor dependent fashion. The enzyme system(s) involved in the oxidative reaction were determined to be cytosolic and $NAD(P)^+$ dependent (Figs. 3B and 4B). The lack of human liver microsomes-mediated oxidation suggested little contribution of cytochrome P450s and 11-β-HSD (Table 1). Significant inhibition of the oxidative reaction by flufenamic acid and phenolphthalein, but not by menadione supported the involvement of AKR1Cs in the oxidation of M1. It is interesting that flufenamic acid and phenolphthalein behaved differently in the inhibition of the reductive and oxidative reactions. Flufenamic acid appeared to inhibit the oxidative reaction to a greater extent than the reductive reaction (Fig. 8). Conversely, the extent of inhibition of the oxidative reaction by phenolphthalein was much less than that of the reductive reaction (Fig. 8). Due to lack of commercially available AKR1C isozymes and isoform selective inhibitors, the potential involvement of each specific AKR1C isozyme in the reductive and oxidative reactions could not be evaluated. It is possible that certain AKR 1C isozyme(s) preferentially catalyze the reductive reaction, while the other isozyme(s) preferentially catalyze the oxidative reaction. In addition, it has been shown that phenolphthalein differentially inhibited four AKR1C isozymes with the highest affinity for AKR1C4 [9,19]. On the other hand, flufenamic acid was shown to be a potent inhibitor of AKR1C1–1C3 and a weak inhibitor of AKR1C4 [9,19]. Moreover, their inhibitory potency on the four AKR 1C isozymes was only evaluated for the reductive reaction. The inhibitory effect and their relative potency of the two agents on the oxidative reaction have not been systemically evaluated. Therefore, further investigation on the relative selectivity of flufenamic acid and phenolphthalein on the inhibition of AKR1C isozymes and the potential involvement of AKR 1C isoenzymes (e.g., AKR 1C1–1C4) in the metabolic interconversion between Compound A and M1 will be valuable to better understand this observation. The present study highlights that the tools to study the involvement of AKR 1C1 through 1C4 in drug metabolism are insufficient to understand isoform specificity. Since interaction with these isoforms may have different implications, widely available recombinant proteins and selective inhibitors for use in cytosolic preparations would be highly desired.

In summary, Compound A undergoes ketone reduction to M1 in vitro in human tissues. This reaction is stereospecific for the substrate as well as for the product formation. Several unique features of ketone reduction of Compound A to M1, namely cofactor dependence, subcellular preference, reversibility and wide tissue distribution, have been revealed. AKR1Cs have been identified as the major enzymes for the metabolic interconversion of Compound A and its active metabolite M1.

The unique characteristics of ketone reduction of Compound A may potentially impact its clinical development. The reversibility, multiple tissue involvement and potential species difference (data not shown) would pose a challenge in the human PK prediction of Compound A, as a preclinical candidate. On the other hand, a drug candidate like Compound A that undergoes significant ketone reduction may have less drug interaction potential. The abundance and common substrate specificity of ketone reducing enzymes suggest that if one pathway is inhibited, alternative pathways may contribute to the elimination. Since Compound A is an anticancer drug candidate, ketone reduction to a less active metabolite in tumor tissues may also impact its clinical study design and efficacy data interpretation. It has been documented that carbonyl reductase activity was significantly elevated in tumor tissues as compared to the corresponding normal tissues [20]. Over-expression of AKR1C3 was also reported in steroid hormone dependent cancer tissues like prostate and breast tumors [21,22]. Several anticancer drugs, such as daunorubicin [23,24], doxorubicin and oracin [25,26], have been shown to undergo significant reduction to their inactive reductive metabolites in cancer cell lines, which in turn induced the ketone reducing enzymes upon exposure of the cell lines to these anticancer drugs [23,24,26]. Considering that M1 is ~7-fold less potent than Compound A in vitro, it remains to be determined whether over-expression of ketone reducing enzyme(s) in tumor tissues could impact clinical outcome of Compound A.

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References

[1] Cox CD, Breslin MJ, Mariano BJ, Coleman PJ, Buser CA, Walsh ES, et al. Kinesin spindle protein (KSP) inhibitors. Part 1: the discovery of 3,5-diaryl-4,5-dihydropyrazoles as potent and selective inhibitors of the mitotic kinesin KSP. Bioorg Med Chem Lett 2005;15:2041–5.
[2] Garbaccio RM, Tasber ES, Neilson LA, Coleman PJ, Fraley ME, Olson C, et al. Kinesin spindle protein (KSP) inhibitors. Part 7: design and synthesis of 3,3-disubstituted dihydropyrazolobenzoxazines as potent inhibitors of the mitotic kinesin KSP. Bioorg Med Chem Lett 2007;17:5671–6.
[3] Rosemond MJC, Walsh JS. Human carbonyl reduction pathways and a strategy for their study in vitro. Drug Metab Rev 2004;24(36):335–61.
[4] Matsunaga T, Shintani S, Hara A. Multiplicity of mammalian reductases for xenobiotic carbonyl compounds. Drug Metab Pharmacokinet 2006;21:1–18.
[5] Oppermann U. Carbonyl reductases: the complex relationships of mammalian carbonyl- and quinine-reducing enzymes and their role in physiology. Ann Rev Pharmacol Toxicol 2007;47:293–322.
[6] Jin Y, Penning TM. Aldo–keto reductases and bioactivation/detoxication. Annu Rev Pharmacol Toxicol 2007;47:263–92.
[7] Atalla A, Breyer-Pfaff U, Maser E. Purification and characterization of oxidoreductases catalyzing carbonyl reduction of the tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in human liver cytosol. Xenobiotica 2000;30:755–69.
[8] Scheffe H. The analysis of variance. New York: John Wiley & Sons; 1999.
[9] Rosemond MJC, St. John-William L, Yamaguchi T, Fujishita T, Walsh JS. Enzymology of a carbonyl reduction clearance for the HIV integrase inhibitor, S-1360: role of human liver cytosolic aldo–keto reductases. Chem Biol Interact 2004;147:129–39.
[10] Maser E, Stinner B, Atalla A. Carbonyl reduction of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by cytosolic enzymes in human liver and lung. Cancer Lett 2000;148:135–44.
[11] Obach RS. Potent inhibition of human liver aldehyde oxidase by raloxifene. Drug Metab Dispos 2004;32:89–97.
[12] Baumann P, Prelog V. Reakinonen mit Mikro organismen. 5. mitteilung. Die Stereospezifische reduction von stereomeren dekalindeionen-(1-4). Helv Chim Acta 1958;41:2362–79.
[13] Hermans JJR, Thijssen HHW. The in vitro ketone reduction of warfarin and analogues. Biochem Pharmacol 1989;38:3365–70.
[14] Eyles DW, Pond SM. Stereospecific reduction of haloperidol in human tissues. Biochem Pharmacol 1992;44:867–71.
[15] Hoffmann F, Maser E. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. Drug Metab Rev 2007;39:87–144.
[16] Davis B, Morris T. Physiological parameters in laboratory animals and humans. Pharm Res 1993;7:1903–5.
[17] Burczynski ME, Lin H, Penning TM. Isoform-specific induction of a human aldo-reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. Cancer Res 1999;59:607–14.
[18] Porter SJ, Somogyi AA, White JM. Kinetics and inhibition of the formation of 6β-naltrexol from naltrexone in human liver cytosol. Br J Clin Pharmacol 2000;50:465–71.
[19] Steckelbroeck S, Oyesanmi B, Jin Y, Lee SH, Kloosterboer HJ, Penning TM. Tibolone metabolism in human liver is catalyzed by 3α/3β-hydroxysteroid dehydrogenase activities of the four isoforms of the aldo–keto reductase (AKR)1C subfamily. J Pharmacol Exp Ther 2006;316:1300–9.
[20] Lopez de Cerain A, Marin A, Idoate MA, Tunon MT, Bello J. Carbonyl reductase and NADPH cytochrome P450 reductase activities in human tumoral versus normal tissues. Eur J Cancer 1999;35:320–4.
[21] Lin H, Steckelbroeck S, Fung K, Jones AN, Penning TM. Characterization of a monoclonocal antibody for human aldo–keto reductase AKR1C3 (type 2 3α hydroxysteroid dehydrogenase/type 5 17β-hydroxysteroid dehydrogenase); immunohistochemical detection in breast and prostate. Steroids 2004; 69:795–801.
[22] Fung K, Samara ENS, Wong C, Metwalli A, Krlin R, Bane B, et al. Increased expression of type 2 3α-hydroxysteroid dehydrogenase (AKR1C3) and its relationship with androgen receptor in prostate carcinoma. Endocrine Relat Cancer 2006;13:169–80.
[23] Soldan M, Netter KJ, Maser E. Induction of daunorubicin carbonyl reducing enzymes by daunorubicin in sensitive and resistant pancreas carcinoma cells. Biochem Pharmacol 1996;51:117–23.
[24] Ax W, Soldan M, Koch L, Maser E. Development of daunorubicin resistance in tumor cells by induction of carbonyl reduction. Biochem Pharmacol 2000;59:293–300.
[25] Novotna R, Wsol V, Xiong G, Maser E. Inactivation of the anticancer drugs doxorubicin and oracin by aldo–keto reductase (AKR) 1C3. Toxicol Lett 2008; 181:1–6.
[26] Gavelova M, Hladikova J, Vildova L, Novotna R, Vondracek J, Krcmar P, et al. Reduction of doxorubicin and oracin and induction of carbonyl reductase in human breast carcinoma MCF-7 cells. Chem Biol Interact 2008;176:9–18.
[27] Food and Drug Administration. Guidance for industry: drug interaction studies—study design, data analysis, and implication for dosing and labeling; 2006.