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Retrorsine, but not monocrotaline, is a mechanism-based inactivator of P450 3A4

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ABSTRACT

Retrorsine (RTS) and monocrotaline (MCT) cause severe toxicities via P450-mediated metabolic activation. The screening of mechanism-based inhibitors showed RTS inactivated 3A4 in the presence of NADPH. Unlike RTS, MCT failed to inhibit P450 3A4 and other enzymes tested. Further studies showed the loss of P450 3A4 activity occurred in a time- and concentration-dependent way, which was not recovered after dialysis. Dextromethorphan, a P450 3A4 substrate, protected the enzyme from the inactivation. Exogenous nucleophile glutathione (GSH) and reactive oxygen species scavengers catalase and superoxide dismutase did not protect P450 3A4 from the inactivation. GSH trapping experiments showed both P450 3A4 and 2C19 converted RTS and MCT to the corresponding electrophilic metabolites which could be trapped by GSH to form 7-GSH-DHP conjugate. We conclude that RTS and MCT are metabolically activated by P450 3A4 and 2C19, and that RTS, but not MCT, is a mechanism-based inactivator of P450 3A4.

1. Introduction

Retrorsine (RTS) and monocrotaline (MCT) are two retronecine-type pyrrolizidine alkaloids (PAs), present in *Senecio* and *Crotalaria* species, respectively (Fig. 1). RTS is a 12-membered macrocyclic diester PA with an α,β-unsaturated double bond linked to the ester group at the C-7 position of the necine base, which exhibits hepatotoxicity [1,2], whereas MCT, an 11-membered macrocyclic diester PA without α,β-unsaturated double bond linked to the ester, predominantly induces pneumotoxicity [3,4]. The major routes of human exposure to PAs are accidental intake of PA-contaminated foods and intentional ingestion of PA-containing herbal tea, herbal medicine, and dietary supplements [5,6]. Previous studies have implied that metabolic activation of PAs is associated with their toxicities [7,8]. Retronecine-type PAs are metabolically converted to electrophilic dehydrogenated metabolites by cytochromes P450, and the resulting pyrrole metabolites have two electrophilic sites at C-7 and C-9 positions that are readily attacked by nucleophilic groups in DNA and proteins to form 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP) modified DNA or protein adducts, and interstrand DNA–DNA and DNA–protein crosslinking, possibly leading to various toxicities [9,10]. Several DHP-derived DNA adducts have been observed in *in vitro* experiments when incubating RTS or MCT with rat liver microsomes in the presence of calf thymus DNA, and were proposed to be biomarkers of tumorigenicity induced by retronecine-type PAs [11,12]. Moreover, the target proteins of MCT in pulmonary artery endothelial cells have been identified and were considered to be associated with MCT-induced pulmonary hypertension [13,14].

Given the reported alkylation of DNA and proteins, we speculated the reactive metabolites of PAs might bind covalently to the nucleophilic amino acids located in active sites of P450s, acting as suicide or mechanism-based inactivators (Fig. 1). Currently, information regarding the interaction of PAs with cytochromes P450 is limited. It has been reported that the metabolic activation of retronecine-type PAs is mainly catalyzed by P450 3A and 2B isoforms [8,10]. However, the specific P450 isoenzymes participating in RTS metabolic activation have not been identified, though one or more isoenzymes were suggested to be involved in RTS metabolism [15]. MCT is mainly metabolized by P450 3A [16] and was found to inhibit the liver drug-metabolizing enzymes in rats [17], but the inhibitory effect on the individual P450 isoenzymes remains unknown.

In the present studies, we examined the effects of RTS and MCT metabolism on the enzymatic activities of eight P450 isoenzymes, including P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and

Abbreviations: DHP, 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine; 7,9-diGSH-DHP, 7,9-diglutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine; GSH, glutathione; 7-GSH-DHP, 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizidine; K_I, a concentration of inactivator required for half-maximal inactivation; k_{inact}, a maximal rate constant for inactivation; k_{obs}, initial rate constants for inactivation; MRM, multiple reaction monitoring; P450, cytochrome P450; PA, pyrrolizidine alkaloid; SIM, selected ion monitoring.

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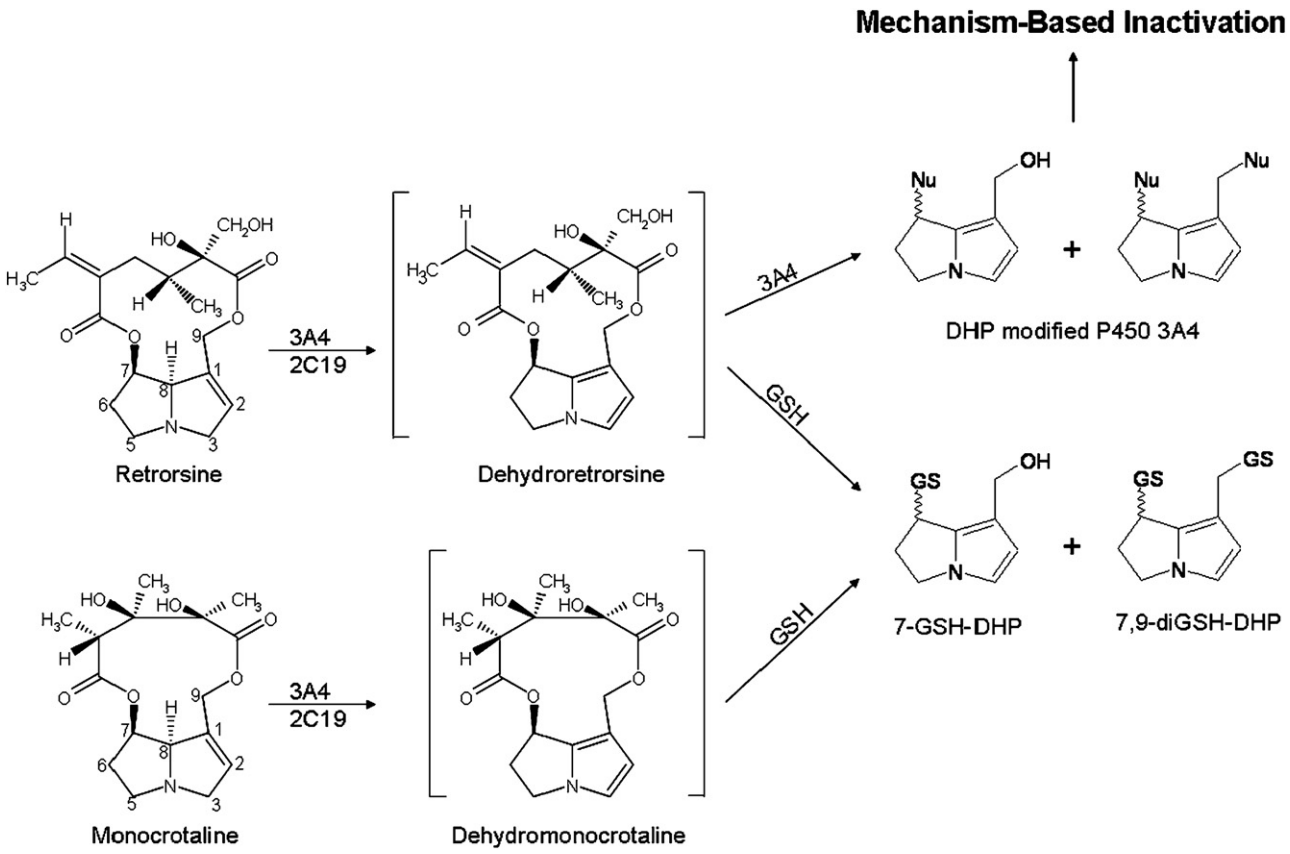


Fig. 1. Mechanisms for the metabolic activation of RTS and MCT.

3A4. Then, the kinetic characteristics of mechanism-based inactivation of P450 3A4 by RTS were studied. Additionally, the P450 isoenzymes involved in metabolic activation of RTS and MCT were also investigated by GSH trapping experiments.

2. Materials and methods

2.1. Chemicals

Retrorsine (RTS), monocrotaline (MCT), testosterone, phenacetin, coumarin, tolbutamide, dextromethorphan, 4-nitrophenol, acetaminophen, nirvanol, 7-hydroxycoumarin, 4-hydroxytolbutamide, 4′-hydroxymephenytoin, dextrorphan, 4-nitrocatechol, 2-fluoro-4-nitrophenol, (+)-camptothecin, L-glutathione reduced (GSH), NADPH, trifluoroacetic acid, o-chloranil, sodium borohydride, ammonium acetate, and HPLC-grade formic acid were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI). 6β-Hydroxytestosterone and 11β-hydroxytestosterone were from Steraloids Inc. (Pawling, NY) and Ikapharm (Ramat-Gan, Israel), respectively. *S*-Mephenytoin was purchased from BD Biosciences (St. Louis, MO). HPLC-grade methanol, ethanol, and acetonitrile (ACN), and Tris, DMSO, chloroform, anhydrous Na_2SO_4, and potassium hydroxide (KOH) were from EMD Biosciences (La Jolla, CA). Human P450 Supersomes™ enzymes (P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6. 2E1, and 3A4) were purchased from BD Biosciences (St. Louis, MO). Catalase from bovine liver and superoxide dismutase from bovine liver were obtained from Sigma Chemical Co. (Milwaukee, WI).

2.2. Synthesis

Dehydromonocrotaline was synthesized according to a procedure described previously [18]. In brief, MCT (0.06 mmol) was dissolved in chloroform (5.0 mL), and a solution of o-chloranil (0.10 mmol) in chloroform (5.0 mL) was added at room temperature. After 5 min, the mixture was shaken vigorously with a cooled solution (1.0 mL) containing KOH (70%) and sodium borohydride (2%) for 10–15 s. The organic phase was separated, immediately dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give the nearly pure dehydromonocrotaline, which was further purified by recrystallization from the mixture of benzene and petroleum ether. Glutathione conjugates 7-GSH-DHP and 7,9-diGSH-DHP were synthesized from dehydromonocrotaline. Dehydromonocrotaline (0.03 mmol) dissolved in DMSO (100 μL) was added into a solution of glutathione (0.045 mmol) in water (4.0 mL), and the mixture was stirred overnight at room temperature. The resulting GSH conjugates were purified by HPLC. 7-GSH-DHP: ESI-MS *m/z* 443.4 ($[M+H]^+$, 100), 367.3 (75). 7,9-diGSH-DHP: ESI-MS *m/z* 732.1 ($[M+H]^+$, 100), 424.8 (25).

2.3. Mechanism-based inhibitor screening

The primary reaction mixtures contained 0.1 μM individual P450s (100 pmol), 3.2 mM $MgCl_2$, and 100 μM RTS or MCT in 0.1 M Tris–HCl or potassium phosphate. Control incubation was conducted in the absence of RTS and MCT. The organic solvent was typically methanol at a final concentration of 0.5% (v/v). After equilibration of the reaction mixtures at room temperature for 3 min, the reactions were initiated by the addition of NADPH to a final concentration of 0.45 mM, and the mixtures were incubated at 37 °C for 0, 5, and 30 min. At indicated time points, aliquots of the primary reaction mixtures were transferred at a dilution of 1:5 to a secondary reaction mixture containing the corresponding probe substrate (Table 1), 0.45 mM NADPH, and 3.2 mM $MgCl_2$ in 0.1 M Tris–HCl (pH 7.4). The secondary incubation mixtures were further incubated at 37 °C for various time periods (20 min for P450s

Table 1
Substrates and products involved in mechanism-based inactivation screening studies.

Isoenzyme	Probe substrate	Concentration (μM)	Metabolite	Detection
CYP 1A2	Phenacetin	200[a]	Acetaminophen	254 nm[b]
CYP 2A6	Coumarin	400[a]	7-Hydroxycoumarin	*m/z* 163 → 107[c]
CYP 2B6	*s*-Mephenytoin	100 [26]	Nirvanol	*m/z* 205[c]
CYP 2C9	Tolbutamide	100 [27]	4-Hydroxytolbutamide	*m/z* 287[c]
CYP 2C19	*s*-Mephenytoin	100[a]	4-Hydroxymephenytoin	*m/z* 235[c]
CYP 2D6	Dextromethorphan	10 [28]	Dextrorphan	*m/z* 258[c]
CYP 2E1	4-Nitrophenol	500[a]	4-Nitrocatechol	350 nm[b]
CYP 3A4	Testosterone	200[a]	6β-Hydroxyltestosterone	*m/z* 305[c]

[a] The concentrations were determined according to the protocols provided by the manufacturer.
[b] Products were monitored by HPLC/UV.
[c] Products were monitored by LC/MS.

1A2, 2A6, 2B6, 2C9, 2D6, and 3A4; 35 min for 2C19 and 2E1), followed by addition of equal volume ice-cold ethanol containing selective internal standards (1.0 μg/mL camptothecin for 1A2, 2A6, 2B6, 2D6, 2C9, and 2C19; 1.5 μg/mL 11-hydroxytestosterone for 3A4; and 10 μg/mL 2-fluoro-4-nitrophenol for 2E1). Samples were centrifuged at 14,000 rpm for 20 min, and the supernatants were analyzed directly by LC/MS or HPLC/UV. The residual enzyme activities were determined by monitoring the amounts of the corresponding products formed in the secondary incubation mixtures.

2.4. Time-, concentration-, and NADPH-dependent inactivation of P450 3A4 by RTS

The primary incubation mixtures contained 0.1 μM P450 3A4 (100 pmol) and RTS at various concentrations (0, 5, 10, 25, 50, 100, and 200 μM) in 0.1 M potassium phosphate buffer (pH 7.4). To determine the dependency of NADPH in enzyme inactivation, 100 μM RTS and P450 3A4 were incubated in the absence of NADPH as negative control. The reactions were initiated by the addition of NADPH, and aliquots of the primary mixtures (100 μL) containing 10 pmol of P450 3A4 were transferred at 0, 4, 8, and 12 min to the secondary incubation mixtures containing 200 μM testosterone and 0.45 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4) to a total reaction volume of 0.5 mL. After 20 min of incubation, the reaction was stopped by adding 0.5 mL ice-cold ethanol containing 11-hydroxytestosterone (1.5 μg/mL), followed by centrifugation at 14,000 rpm for 20 min. The supernatants were collected and analyzed by LC/MS as described below.

2.5. Substrate protection

Substrate protection from RTS-induced inactivation of P450 3A4 was determined by including 200 μM dextromethorphan together with 100 μM RTS in the primary reaction mixture. The reaction was initiated with NADPH, and aliquots of the mixtures (100 μL) containing 10 pmol P450 3A4 were transferred at 0, 4, and 12 min time points to the secondary incubation mixture for the determination of testosterone 6β-hydroxylase activities of P450 3A4. Control incubations lacking both RTS and dextromethorphan, incubations only containing dextromethorphan, and incubations with RTS alone were also performed in parallel.

2.6. Effects of GSH, catalase, and superoxide dismutase on the inactivation

GSH (2 mM) was included in the primary reaction mixture together with 0.1 μM P450 3A4 (100 pmol), 100 μM RTS, and 0.45 mM NADPH. Aliquots were removed for determining the remaining enzyme activities. In control samples, an equal volume of phosphate buffer was added in place of GSH. In a separate study, P450 3A4 was incubated with RTS and NADPH in the presence or absence of a mixture of catalase and superoxide dismutase (each 800 unit/mL). The residual activities were monitored as described below.

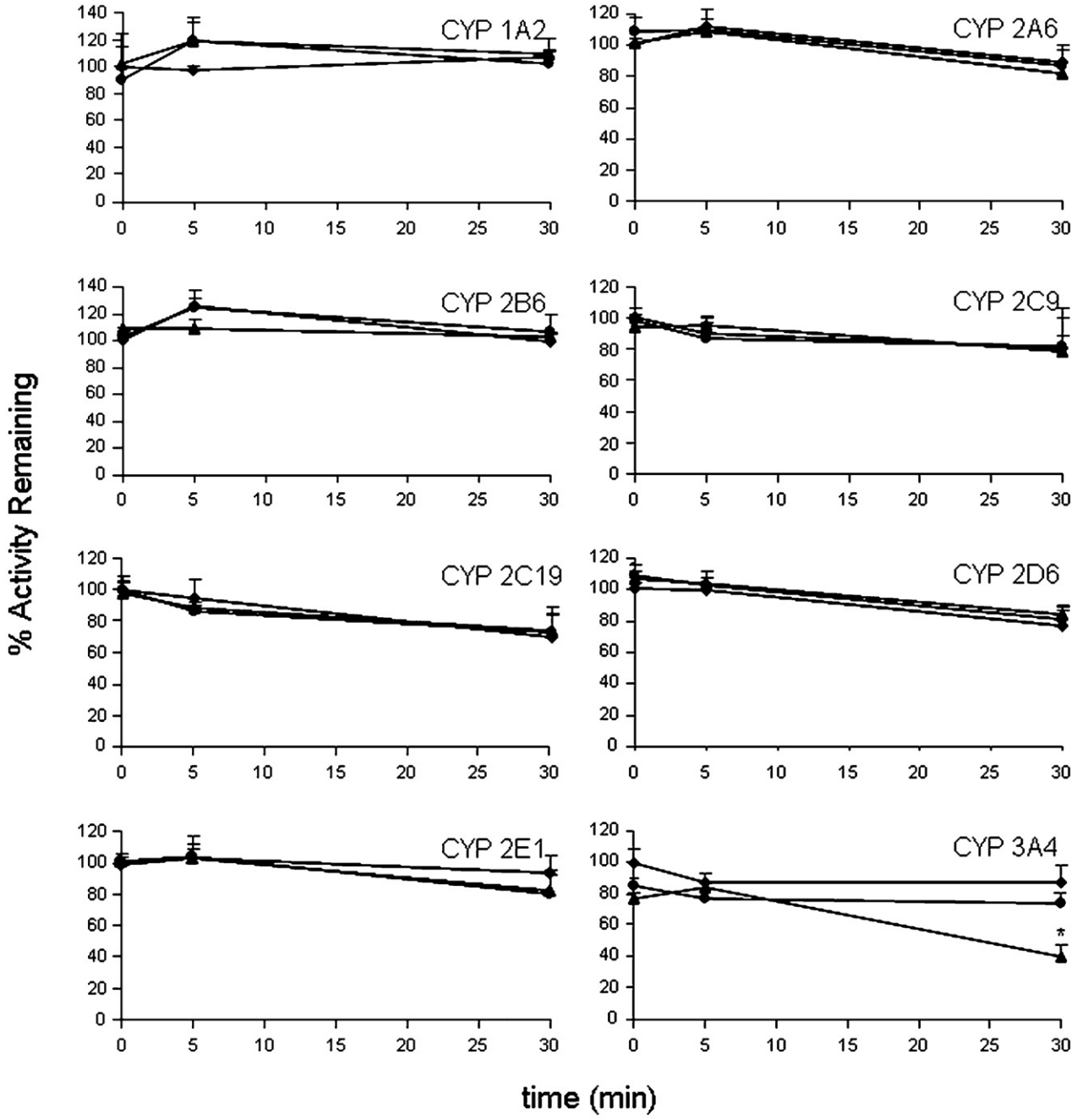
2.7. Irreversibility of inactivation

Primary incubations containing 200 μM RTS were performed in the presence of NADPH at 37 °C. The control incubations lacked RTS. Aliquots of the mixtures were withdrawn at 0 and 25 min for enzymatic activity determination (non-dialyzed sample). At 25 min time point, aliquots of the control and inactivated samples containing 100 pmol P450 3A4 were transferred respectively to Slide-A-Lyzer membranes (molecular weight cut off 3500 Da; Pierce, Rockford, IL) and dialyzed at 4 °C for 4 h against 2 L potassium phosphate buffer (0.1 M, pH 7.4) supplemented with 0.5 mM EDTA. The dialyzed samples were brought to room temperature and added to the secondary incubation mixture for the determination of the residual enzyme activities. The remaining activities of the dialyzed and non-dialyzed samples were measured as described below.

2.8. Enzymatic reaction product analysis

The resulting metabolites of each probe substrates (except acetaminophen and 4-nitrocatechol) were analyzed by LC/MS. The LC/MS system consists of Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) coupled to an API 2000™ triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA). The chromatographic separations were carried out on an Alltima HP C18 column (100 × 2.1 mm i.d., 3 μm, Alltech Associates Inc., Deerfield, IL). For 7-hydroxycoumarin, 4-hydroxytolbutamide, 4′-hydroxymephenytoin, demethylmephenytoin, nirvanol and dextrorphan, a linear 2.0 min-gradient from 90:10 to 10:90 (water:ACN supplemented with 0.1% formic acid) was applied. 6β-Hydroxytestosterone and 11β-hydroxytestosterone were separated with a solvent system consisting of solvent A (0.1% formic acid in water) and solvent B (50% ACN, 45% methanol, and 5% water supplemented with 0.1% formic acid) using linear gradients from 30% B to 80% B over 5 min. Quantification was performed using either multiple reaction monitoring (MRM) or selected ion monitoring (SIM) in positive-ion electrospray ionization mode (Table 1).

Acetaminophen and 4-nitrocatechol were monitored by a Shimadzu LC-10Avp series HPLC system (Kyoto, Japan), consisting of LC-10ADvp pump, DGU-14A degasser, SIL-10ADvp auto-injector, CTO-10Avp column oven, and SPD-M10Avp diode array UV detector. The chromatographic separation was also performed on the Alltima HP C18 column with the mobile phase composed of a mixture of water and methanol. The mobile phase for acetaminophen analysis included 10 mM ammonium acetate, and a gradient elution started at 40% methanol and graded to 100% methanol in 9 min. The UV detector wavelength was set at 254 nm. For analysis of 4-nitrocatechol, the mobile phase was supplemented with



The rates of the secondary reactions at time zero.

Isoenzyme	Vehicle	MCT	RTS	Isoenzyme	Vehicle	MCT	RTS
	(pmol/min/pmol P450)				(pmol/min/pmol P450)		
1A2	23.4	21.5	23.1	2C19	67.6	67.0	66.2
2A6	11.7	12.8	11.9	2D6	5.51	5.94	5.71
2B6	5.60	5.76	5.11	2E1	9.84	10.7	11.2
2C9	19.7	20.3	18.7	3A4	10.3	8.78	8.07

Fig. 2. The remaining enzyme activities of individual P450s after exposure to vehicle (♦), 100 μM MCT (●), or 100 μM RTS (▲) in the presence of NADPH for 0, 5, and 30 min. Each data represents the mean ± SD of duplicate incubations in two separate experiments. $^{*}p < 0.05$.

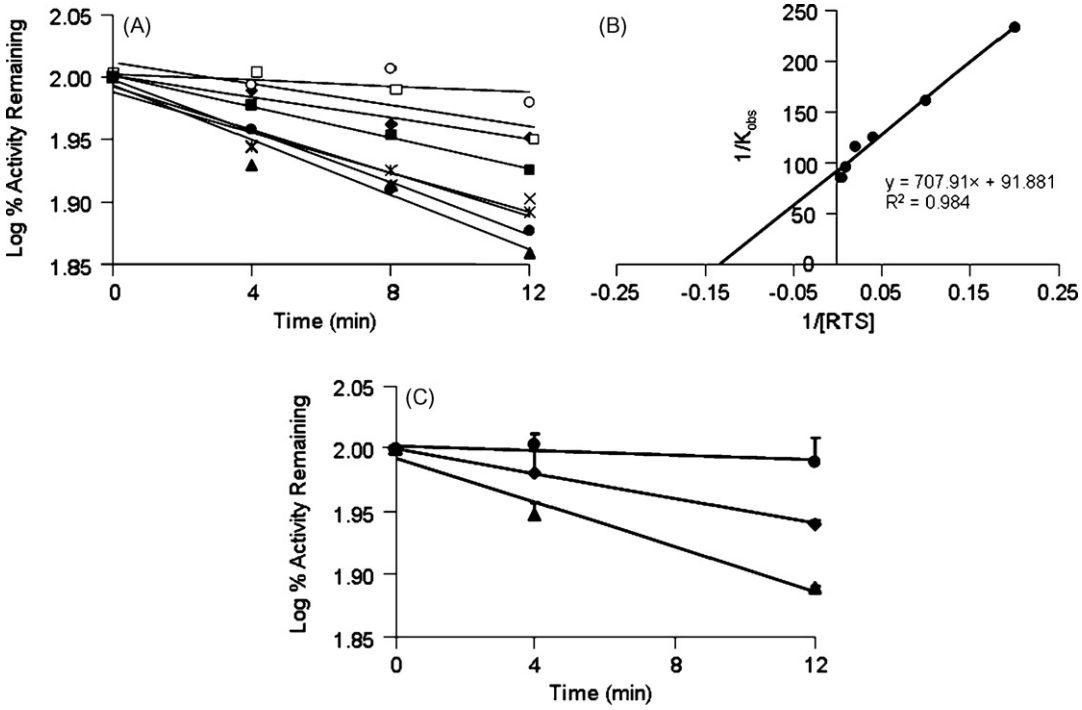


Fig. 3. (A) Time, concentration and NADPH-dependent inactivation of P450 3A4 by RTS. P450 3A4 was incubated with 0 μM (○), 5 (♦), 10 (■), 25 (×), 50 (●), 100 (✱), and 200 μM (▲) RTS, respectively, in the presence of NADPH or incubated with 100 μM RTS without NADPH (□) at 37 °C for 0, 4, 8 and 12 min. Aliquots of incubation mixtures were transferred to the secondary incubation mixtures for the determination of residual enzymatic activity. Each data represents the average of four separate experiments. (B) Double reciprocal plot of the rates of inactivation as a function of RTS concentrations. (C) Substrate protection against inactivation of P450 3A4 by RTS. P450 3A4 was incubated with vehicle (●) and 100 μM RTS in the presence (♦) or absence (▲) of 200 μM dextromethorphan. Each data represents the mean ± SD of duplicate incubations in two separate experiments.

0.1% trifluoroacetic acid, and a linear 7-min gradient from 50% to 80% methanol was applied. The UV detector wavelength was set at 350 nm.

2.9. GSH conjugate trapping

RTS (200 μM) or MCT (200 μM), and 5 mM GSH were incubated with 0.1 μM individual P450 enzymes, including 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, or 3A4 (50 pmol for each) in the presence or absence of 1.0 mM NADPH at 37 °C for 60 min. GSH conjugates 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizidine (7-GSH-DHP) and 7,9-diglutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (7,9-diGSH-DHP) were detected by LC/MS [19–21]. For analysis of GSH-DHP, the mobile phase consisted of water and ACN supplemented with 0.1% trifluoroacetic acid. The gradient was maintained at 10% ACN for 0.5 min, followed by a linear increase to 90% ACN in 5 min and then kept at 90% ACN for 5.5 min. Then the column was equilibrated at 10% ACN for 7 min. Substrates and GSH conjugates were monitored in positive SIM mode, including MCT (*m*/*z* 326), RTS (*m*/*z* 352), GSH-DHP (*m*/*z* 443), and diGSH-DHP (*m*/*z* 732). The spray voltage was set at 5 kV; source temperature set at 350 °C; and the dwell time for each ion was 200 ms.

3. Results

3.1. Mechanism-based inhibitor screening

One of the objectives of the present study was to examine whether RTS and MCT produced mechanism-based inactivation of those human cytochromes P450 mainly responsible for drug metabolism. The study was initiated by examination of the remaining activities of P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 at 0, 5, and 30 min after exposure to 100 μM RTS or MCT in the presence of NADPH. The plots of percentage of remaining enzyme activities versus preincubation time are shown in Fig. 2. RTS resulted in a loss of about 30% P450 3A4 enzyme activity after 30 min of incubation, whereas MCT failed to inactivate 3A4. In the screening of the other enzymes, including P450 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, and 2E1, no such enzyme inactivation observed in P450 3A4 incubations with RTS was found in comparison with the corresponding spontaneous enzyme activity loss after exposure to vehicle.

3.2. Time-, concentration-, and NADPH-dependent inactivation of P450 3A4 by RTS

The remaining testosterone hydroxylase activities of P450 3A4 were measured and a semilogarithmic plot of percent remaining activity versus preincubation time was performed (Fig. 3A). P450 3A4 retained the catalytic activity in control incubations (without RTS) and incubations with RTS lacking NADPH over 12-min incubation, whereas, in the presence of NADPH, RTS produced a time course inhibition of P450 3A4 activity. Since there was no significant loss of P450 3A4 activity in control incubations over 12 min of incubation, the remaining enzymatic activity of the control incubations at time 0 was normalized to 100%. When P450 3A4 was preincubated with various concentrations of RTS (5–200 μM) in the presence of NADPH, the loss of enzyme activity increased progressively with increasing concentrations of RTS, and the enzyme inactivation followed pseudo first-order kinetics. The initial rate constants for inactivation (k_{obs}) were determined by linear regression analysis of the time course data, and a double reciprocal plot of the values for k_{obs} and the RTS concentrations was performed to give a maximal rate constant for inactivation (k_{inact}) of 0.025 min^{-1} and a concentration of inactivator required for half-maximal inactivation (K_I) of 7.7 μM (Fig. 3B). Therefore, the inactivation efficiency represented by k_{inact}/K_I was 3.25 min^{-1} mM^{-1}.

3.3. Substrate protection

An alternate substrate, dextromethorphan [22], was included in the primary incubations along with RTS at a molar ratio of 2:1 (dextromethorphan:RTS). The remaining P450 3A4 activity was monitored and normalized to control activity at time 0. After correcting for the inhibitory effect of dextromethorphan on testosterone 6β-hydroxylation (56.8 ± 3.4%), the remaining enzyme activity in samples coincubated with RTS and dextromethorphan was 94.8 ± 0.2% at 12 min, higher than samples incubated in the presence of RTS (77.7 ± 4.1%), indicating that dextromethorphan reduced the rate of P450 3A4 inactivation produced by RTS (Fig. 3C).

3.4. Effects of GSH and catalase/superoxide dismutase

After 4 and 12 min of preincubation with 100 μM RTS and NADPH, the remaining P450 3A4 activity were 88.6 ± 1.4% and 76.8 ± 5.1%, respectively. The inclusion of 2 mM GSH, an electrophile trapping agent, in the primary reaction mixture produced a 5% increase in the remaining enzyme activities (92.9 ± 2.7% at 4 min, and 81.6 ± 3.6% at 12 min). In addition, a mixture of catalase and superoxide dismutase, scavengers of reactive oxygen species, had no protective effect against the inactivation of P450 3A4 by RTS, and the remaining enzyme activities were 87.1 ± 5.0% and 78.3 ± 5.9% at 4 and 12 min respectively.

Table 2
Irreversibility of the inactivation of P450 3A4 by RTS.

Preincubation		Pre-dialysis		Post-dialysis
		0 min	25 min	25 min
% Remaining activity	Vehicle	100 ± 2.7	92.6 ± 5.7	50.2 ± 4.0
	RTS	96.7 ± 4.4	68.1 ± 3.9	38.6 ± 2.6
% of Control		96.7	73.5	76.9

Data are mean ± SD (n = 3).

3.5. Irreversibility of inactivation

P450 3A4 was incubated with RTS (200 μM) in the presence of NADPH, followed by 4-h dialysis. The testosterone 6β-hydroxylation activities of control samples and those pretreated with RTS were determined before and after dialysis, and normalized to control activity at time 0 (Table 2). The percent remaining P450 3A4 activities measured after dialysis were corrected by the volume change due to dialysis. No recovery in activity was observed for inactivated samples.

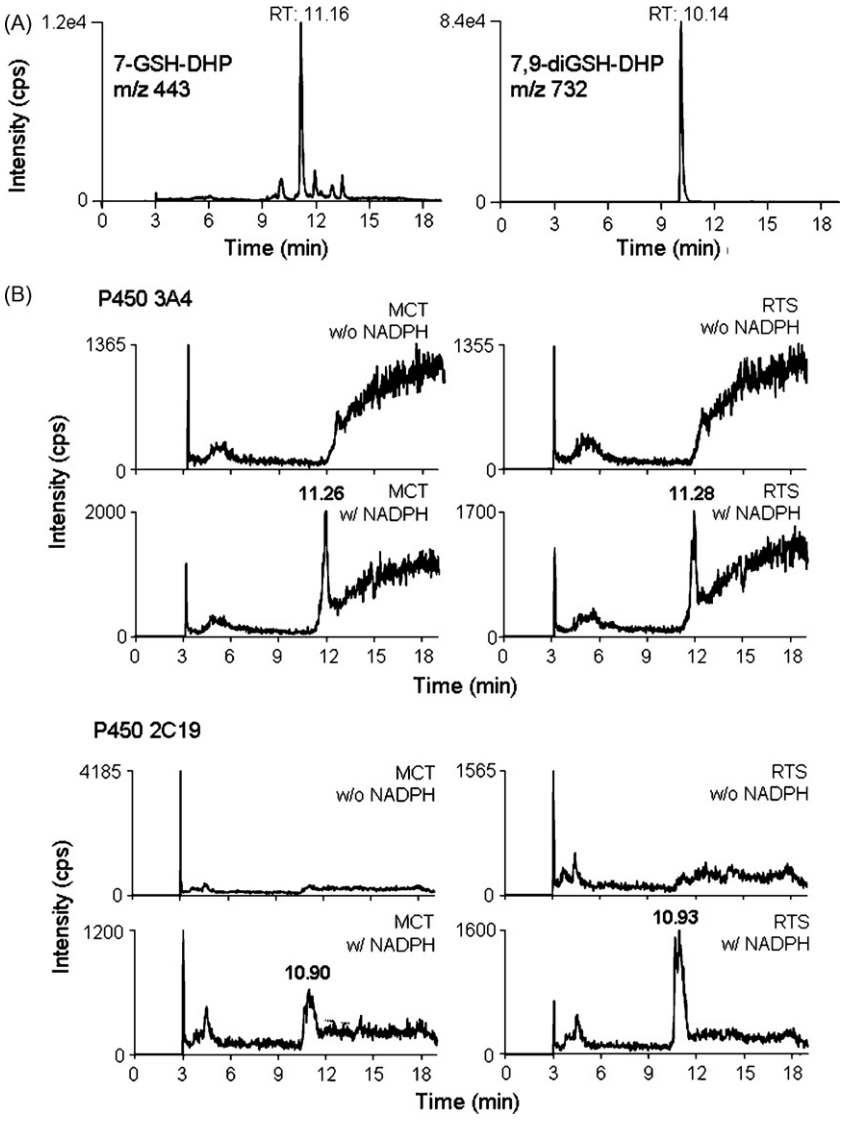


Fig. 4. LC/MS chromatograms of (A) GSH conjugate standards and (B) 7-GSH-DHP (m/z 443) formed in supersome incubation mixtures in the presence or absence of NADPH.

3.6. GSH conjugate trapping

RTS or MCT was incubated with individual P450s, including 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4, in the presence or absence of NADPH for 60 min and the resulting reactive metabolites were trapped with GSH *in situ*, followed by LC/MS analysis (Fig. 4). The retention times of 7-GSH-DHP and 7,9-GSH-DHP (refer to Fig. 1 for their structures) were 11 and 10 min, respectively. The peak responsible for 7-GSH-DHP (*m/z* 443) as the major GSH conjugate was observed in the incubation of either RTS or MCT with P450s 3A4 or 2C19 in the presence of NADPH, but such peak was neither found in incubations without NADPH nor incubations of any other P450 enzymes tested. In P450 3A4 incubation mixtures, the formation of 7-GSH-DHP from RTS was 7.86 pmol/min/pmol P450, and that from MCT was 9.52 pmol/min/pmol P450. In P450 2C19 incubations, the GSH conjugate formed from RTS and MCT were 15.6 pmol/min/pmol P450 and 6.34 pmol/min/pmol P450, respectively. Nonetheless, bis-GSH conjugate 7,9-diGSH-DHP was not found in the incubations of all P450s carried out.

4. Discussion

Although P450-mediated metabolic activation has been suggested to play a critical role in the toxicities of retronecine-type PAs, little attention has so far been paid to the interaction of PAs with individual P450 isoenzymes. The present study started with screening tests which allowed us to determine rapidly whether RTS and MCT showed time-dependent inhibition of selective cytochromes P450, including P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4. RTS was found to inactivate P450 3A4 but not the other isoenzymes tested. The subsequent kinetic studies further demonstrated that RTS produced a time- and concentration-dependent inactivation of P450 3A4 with a K_I of 7.7 μM and a k_{inact} of 0.025 min^{-1}. The enzyme inactivation was also found to be NADPH-dependent. This implies the enzyme inhibition by RTS is mediated by biotransformation. The enzyme inactivation reached a plateau at the concentration of approximately 100 μM. The inactivation of P450 3A4 could not be prevented by the addition of GSH in the preincubation system, indicating that the inactivation by electrophilic metabolites of RTS took place before escaping from the active site of the enzyme. In addition, catalase and SOD, scavengers of reactive oxygen species, failed to protect P450 3A4 against the inactivation by RTS, implying P450 3A4 inactivation was not caused by the formation of reactive oxygen species during metabolism. However, an excess of dextromethorphan, an alternate substrate of P450 3A4, protected P450 3A4 against the inactivation, suggesting that dextromethorphan competes with RTS binding to the active site of P450 3A4 and therefore reduces the formation of the reactive metabolite responsible for the modification of the enzyme *in situ*. As expected, the dialysis did not reverse the enzyme inhibition by RTS. The observed irreversibility of the enzyme inhibition suggests that the reactive metabolite generated from metabolic activation may covalently modify the enzyme responsible for the bioactivation of RTS. Taken together, these results support the conclusion that RTS inhibited P450 3A4 as a mechanism-based inactivator.

Interestingly, MCT, unlike RTS, failed to inhibit the activity of P450 3A4, although it has the same core structure (necine base and diester) as RTS. The GSH trapping experiments showed that both MCT and RTS were metabolized by P450 3A4 to the corresponding dehydropyrrolizidines. We believe that the differences in the reactivity of the electrophonic metabolites of RTS and MCT is a factor for the modification of nucleophilic amino acid residues in the active site of P450 3A4, leading to the loss of enzyme activity. Kinetic studies showed that dehydroretrorsine was more reactive than dehydromonocrotaline when they were incubated with 4-(*p*-nitrobenzyl)pyridine, a model nucleophile as a trapping reagent [23]. This supports the scenario that dehydromonocrotaline is not reactive enough to modify nucleophilic amino acid residue(s) before it is released from the active site of the enzyme. However, we cannot exclude other factors, for example, (1) the distance of the electrophilic centers of the corresponding dehydropyrrolizidine to the nucleophile(s) in the active site of the enzyme; and (2) an appropriate conformational orientation required for the formation of covalent bond between reactive metabolites of pyrrolizidine alkaloids and the target amino acid residues.

Besides P450 3A4, P450s 1A2, 2A6, 2C9, 2C19, 2D6, and 2E1 are the most abundant P450 isoenzymes in human liver and most important in the metabolism of clinically relevant drugs as well [24]. Additionally, P450 2B family was reported to be involved in the metabolic activation of senecionine, another retrornecine-type PA [25]. Therefore, we examined whether RTS and MCT showed mechanism-based inactivation of P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, and 2E1. Our results showed neither RTS nor MCT exhibited time-dependent inhibition on P450 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, and 2E1 in the presence of NADPH. In order to clarify whether these enzymes participate in the metabolic activation of RTS and MCT, we incubated MCT or RTS with individual P450s using GSH as a trapping agent. Among the enzymes, only P450s 3A4 and 2C19 were found to catalyze the formation of the corresponding dehydrogenated products. However, it appears that neither MCT nor RTS showed time-dependent inhibition on P450 2C19.

In summary, pyrrolizidine alkaloids RTS and MCT are metabolically activated by P450 3A4 and 2C19 to form chemically reactive dehydrogenated intermediates. RTS is a mechanism-based inactivator of P450 3A4 but not 2C19. Unlike RTS, MCT failed to show the mechanism-based inactivation of P450 3A4.

Conflict of interest

These authors declare that there are no conflicts of interest.

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