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Mechanism-based Inactivation of Cytochrome P450 2A6 and 2A13 by *Rhinacanthus nasutus* Constituents

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Summary: Human cytochrome P450 CYP2A6 and CYP2A13 catalyze nicotine metabolisms and mediate activation of tobacco-specific carcinogens including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). In this study, we found rhinacanthins A, B, and C isolated from *Rhinacanthus nasutus* potentially inhibited coumarin 7-hydroxylation mediated by reconstituted purified recombinant CYP2A6 and CYP2A13. Rhinacanthins A–C are mechanism-based inactivators of CYP2A6 and CYP2A13 as they cause concentration, time and NADPH-dependent inhibition. Among the three rhinacanthins, rhinacanthin-B possessed highest inhibitory potency against CYP2A13 with apparent K_I and k_{inact} of 0.16 μM and 0.1 min^{-1}, respectively, while values of 0.44 μM and 0.12 min^{-1} were found against CYP2A6. Rhinacanthin-C had least inhibition potency, with apparent K_I and k_{inact} of 0.97 μM and 0.07 min^{-1} for CYP2A6, respectively, and values of 1.68 μM and 0.05 min^{-1} for CYP2A13. Rhinacanthin-A inhibited CYP2A6 and CYP2A13 with apparent K_I values of 0.69 and 0.42 μM, respectively and apparent k_{inact} of 0.18 and 0.06 min^{-1}, respectively. The inhibition of both enzymes by rhinacanthins A–C could not be prevented by addition of trapping agents or reversed by dialysis or potassium ferricyanide. These findings demonstrated that rhinacanthins A–C, which are 1,4-naphthoquinone derivatives, irreversibly inhibited CYP2A6 and CYP2A13 in a mechanism-based inhibition mode.

Keywords: CYP2A; human CYP enzymes; mechanism-based inhibition; NADPH cytochrome P450 reductase

Introduction

Tobacco smoking is widely prevalent due to the highly addictive properties of nicotine, a major constituent of tobacco. Tobacco smoke contains a vast array of chemical contaminants including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), naphthalene, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) that are reported in association with increased risk of lung cancer and respiratory diseases.[1)] In humans, nicotine is primarily metabolized by the cytochrome P450 2A6 (CYP2A6) and CYP2A13 enzymes.[2–5)] CYP2A6, primarily expressed in the liver, is also responsible for metabolisms of compounds such as coumarin (a CYP2A6 probe substrate) and quinolone, and mediates activation of tobacco-specific carcinogenic compounds such as NNK.[6,7)] NNK has been reported to have highly mutagenic, genotoxic and carcinogenic properties in animals and humans, through its α-hydroxylation and formation of reactive metabolites that form DNA adducts.[1)]

CYP2A13 is 93.5% identical to CYP2A6 in its deduced amino acid sequence, has high expression in the respiratory tract and lungs, and shares several substrates with CYP2A6.[8)] Genetic polymorphisms of CYP2A6 and CYP2A13 have been identified.[3,9,10)] Polymorphic variations of CYP2A6 gene are associated with inter-individual differences in nicotine metabolism, affecting nicotine dependence and smoking habits; for example, individuals with CYP2A6 gene deletion alleles have impaired nicotine metabolism

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and reduced smoking.[3] In addition both CYP2A6 and CYP2A13 play a significant role in NNK metabolic activation and their genetic polymorphisms are associated with a decrease in cancer risk, suggesting that both enzymes might have a significant impact on human susceptibility to lung cancer in smokers.[7,9,10] Thus inhibition of CYP2A6 and CYP2A13 by natural products, mimicking gene variations, may help lower the risk of becoming tobacco-dependent and of exposure to metabolically activated NNK in target tissues.[11] In search of CYP2A modulating agents in natural products, including *Acanthaceae*, *Asteraceae*, *Apiaceae*, *Fabaceae*, *Poaceae*, and *Rutaceae* families, we obtained promising results with *Rhinacanthus nasutus* (L.) Kurz (Acantaceae), among a few plants that effectively inhibited both enzymes (unpublished results). *R. nasutus*, a medicinal shrub widely distributed in Southeast Asia, has been reported to possess several biological activities including anti-viral, hepatoprotective, and anti-proliferative activities.[12–16] Major bioactive compounds found in *R. nasutus* are rhinacanthins which are 1,4-naphthoquinone derivative esters.[17] 1,4-naphthoquinone derivatives are found in various parts of plants with bioactive pharmacological and physiological activities.[18] These compounds have been reported to possess inhibitory potency against CYPs; for example, 1,4-naphthoquinone derivatives showed inhibition against CYP1A and CYP2B1,[19,20] and naphthalene competitively inhibits human CYP2A6.[21] In the present study, we investigated the role of *R. nasutus* constituents in the inhibition of coumarin 7-hydroxylation mediated by the human CYP2A6 and CYP2A13 enzymes using a reconstitution system consisting of purified recombinant human CYP and rat NADPH-dependent cytochrome P450 reductase (CYPOR). We obtained rhinacanthins from *R. nasutus* that exert inhibition against recombinant CYP2A6 and CYP2A13 enzymes and their modes of inhibition were determined.

Materials and Methods

Chemicals: Coumarin, *δ*-aminolevulinic acid (*δ*-ALA), 1,2-dilauroyl-rac-glycero-3-phosphocholine (DLPC), dimethyl sulfoxide (DMSO),7-hydroxycoumarin, isopropyl-*β*-D-thiogalactopyranoside (IPTG), nicotinamide adenosine diphosphate reduced form (NADPH), phenylmethylsulfonyl fluoride (PMSF), 8-methoxypsoralen (8-MOP), pilocarpine, (S)-nicotine, glutathione (GSH), catalase, superoxide dismutase (SOD) and potassium ferricyanide were purchased from Sigma-Aldrich (St. Louis, MO). Analytical grade ethyl acetate, *n*-hexane, ethanol, methanol, diethyl ether and high performance liquid chromatography (HPLC) grade acetonitrile (ACN) were supplied by RCI Labscan (Bangkok, Thailand).

Isolation of rhinacanthins from *Rhinacanthus nasutus*: Stems and leaves of *R. nasutus* were obtained from the Chao Krom Poe traditional medicine market, Bangkok, Thailand and identified by Dr. Thaya Jenjittikul, Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand. A voucher sample (7801) was deposited at Suanluang Rama IX herbarium, Bangkok. The dry *R. nasutus* (100 g) material was homogenized, macerated in 95% ethanol to obtain 5.2 g ethanol extract, and further partitioned with *n*-hexane and ethyl acetate (EtOAc), yielding *n*-hexane (1.1 g), EtOAc (0.7 g) and aqueous (3.1 g) extracts. The *n*-hexane extract was subjected to silica gel column chromatography (silica 60; 0.063–0.200 mm Merck) with a solvent system containing *n*-hexane/EtOAc/methanol to yield 6 fractions (fr. 1: *n*-hexane-EtOAc (100:0) eluate, 170 mg; fr. 2: *n*-hexane-EtOAc (85:15) eluate, 60 mg; fr. 3: *n*-hexane-EtOAc (70:30) eluate, 410 mg; fr. 4: *n*-hexane-EtOAc (50:50) eluate, 170 mg; fr. 5: *n*-hexane-EtOAc (0:100) eluate, 65 mg; fr. 6 EtOAc-methanol (0:100) eluate, 145 mg). Fractions (10 µg/ml each) that contained at least 50% inhibition effect against CYP2A6 and CYP2A13-mediated coumarin 7-hydroxylation were processed for compound purification. Fractions 3 and 4 were subjected to HPLC (Symmetry RP-18 column: 47.8 mm × 100 mm, Waters Corporation, Dublin, Ireland) with detection at 254 nm. A mobile phase (1.8 ml/min) consisting of ACN and water was set as follows: 0 min, 50% v/v ACN/water; 0–20 min, a linear gradient from 50% ACN/water to 100% ACN; 20–25 min, 100% ACN; 25–30 min, a linear gradient from 100% ACN to 50% ACN; and 30–35 min, a final equilibration at 50% ACN. The purity of each compound was checked by thin layer chromatography (TLC using precoated Kiesel gel 60 F_{254} sheets from Merck, Billerica, MA) with *n*-hexane-diethyl ether (4:6) and nuclear magnetic resonance (NMR, AVANCE 400 MHz NMR Spectrometer; Bruker, Berlin, Germany). Chemical structures of compounds in fr. 3 were identified as rhinacanthin-B and frs. 3 and 4 as rhinacanthin-C, based on NMR spectra previously reported.[13,22] Under the HPLC conditions described, rhinacanthin-B and rhinacanthin C were eluted at 18 and 19 min, respectively.

Fraction 5 was chromatographed by HPLC analysis with detection at 254 nm. The mobile phase (1.8 ml/min) was ACN/water and set as follows: 0 min, 35% ACN/water (v/v); 0–15 min, a linear gradient from 35% to 80%; 15–18 min, a linear gradient from 80% to 100% ACN; 18–25 min, 100% ACN; 25–30 min, a linear gradient from 100% to 35% ACN; and 30–35 min, a final equilibration at 35% ACN. The compound eluted at a retention time of 6 min was found to be an inseparable mixture of rhinacanthins H and I isomers (further identified by an electrospray ionization source equipped with a tandem mass spectrometer (ESI-MS/MS; Bruker Daltonics GmbH, Bremen, Germany). ESI-MS/MS ions were recorded in both positive and negative modes on the system. The ESI-MS and MS^n *m/z* (rel. int.) were MS^+: 426 (12), 409 (100), 243 (4); MS^2 [426]: 409 (100); MS^2 [409]: 243 (86) and MS^-: 407 (100); MS^2 [407]: 165 (100); MS^3 [407→165]: 120 (18), 109 (100), 91 (46), indicating a mixed structure of previously reported rhinacanthins H/I.[22]

The EtOAc fraction was subjected to separation by TLC resulting in four fractions and only one fraction contained inhibition activities. The fraction was subjected to HPLC using the following conditions: the mobile phase (1.8 ml/min) was ACN/water and set as 0 min, 25% v/v ACN/water; 0–17 min, a linear gradient of 25% to 100%ACN; 17–22 min, 100%ACN; 22–25 min a linear gradient from 100% to 25% ACN; and 25–30 min, a final equilibration at 25% ACN. The purified compound eluted at 11.7 min on HPLC was identified as rhinacanthin-A based on its NMR spectra.[22]

Inhibition assay: *R. nasutus* extracts and constituents were tested for inhibitory activity on coumarin 7-hydroxylation mediated by CYP2A6 and CYP2A13 using enzymatic reconstitution assay. The CYP2A6 and CYP2A13 enzymes were obtained from Dr. Emily Scott, and each was heterologously expressed in *Escherichia coli*.[23,24] The enzymes were expressed and purified using a nickel nitrilotriacetic acid (Ni-NTA) affinity column according to previously published methods.[23,25] Each CYP2A (10 pmol each) was reconstituted with rat NADPH-dependent cytochrome P450 oxidoreductase (CYPOR, a gift from Dr. Jung-Ja

Kim[26]) in a ratio of 1:2 in 500 µl 50 mM Tris-HCl (pH 7.4) and the presence of DLPC, and incubated at room temperature for 10 min prior to initiation of each reaction with 50 µM NADPH as described.[27] Formation of 7-hydroxycoumarin fluorescent product was measured by spectrofluorometer (Shimadzu, Kyoto, Japan) at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm, and the rate of formation was calculated based on the 7-hydroxycoumarin standard curve. The K_m values of coumarin substrate and the rate of coumarin-7-hydroxylation for CYP2A6 (1 µM) and CYP2A13 (2 µM) were comparable to those of prior reports.[27–29]

Inhibitory effects of *R. nasutus* ethanol extract, fractions (aqueous, *n*-hexane fraction and sub-fractions, ethyl acetate fraction and sub-fractions) and rhinacanthins A, B, C, and H/I were examined with coumarin (1 µM for CYP2A6 and 2 µM for CYP2A13) in the reaction mixture and enzyme activities determined as previously described.[30] A 10 min pre-incubation with each test compound (10 µg each of fractions and sub-fractions, and 0–20 µM each of rhinacanthins A–C, H/I dissolved in absolute ethanol) was performed to primarily determine if inhibition conformed to a mechanism-based inactivation. The pre-incubation process was allowed to occur for 10 min before addition of coumarin substrate and NADPH. 8-MOP was used as a control mechanism-based inhibitor of both CYP2A6 and CYP2A13.[31,32] Inhibitory effect was calculated as percent relative inhibition compared with vehicle control reaction and quantified to obtain IC_{50} values (GraphPad Prism 5). For NADPH-dependent inactivation, 0 and 5 µM each of rhinacanthins A–C was added to the reaction mixture in the presence or absence of NADPH. After pre-incubation, aliquots of the incubated mixture were withdrawn and diluted 5-fold with fresh reaction mixture followed by determination of enzymatic activities.

Effects of trapping agents were investigated by pre-incubation with each rhinacanthin (2.5 µM for rhinacanthins A and B, 5 µM for rhinacanthin-C) for 10 min in the presence and absence of 2 mM reduced glutathione (GSH), 2,000 U catalase, or 500U superoxide dismutase (SOD). For the dialysis experiment, dialysis tubing (MW cut off of 6,000–8,000) was bought from Spectral Medical Industries, Los Angeles, CA. The pre-incubation reactions were dialyzed at 4°C for 12 h against 2 liters of 50 mM Tris-HCl (pH 7.4) with the buffer changed twice during dialysis. Protein content and enzymatic activities were determined after dialysis. Dialysis was carried out with 2.5 µM each of 8-MOP or pilocarpine as known irreversible and reversible inhibitors, respectively, of both CYP2A6 and CYP2A13.[25,31,32] To determine an effect of ferricyanide, a 100 µl aliquot of each pre-incubation reaction was transferred to a secondary reaction containing 70 µM potassium ferricyanide and incubated at room temperature for 10 min, followed by 5-fold dilution in the reaction mixture containing coumarin substrate and NADPH.

Determination of inactivation rate constants: Kinetic studies were performed with rhinacanthins A–C at concentrations of 0, 0.31, 0.63, 1.25, 2.5, 5 and 10 µM (or as defined in **Fig. 2**) and at pre-incubation times of 0, 10, 20, and 30 min. The apparent inhibition constants K_I and k_{inact}, were determined following the inactivation assay as described.[29] The maximal inactivation rate constant (k_{inact}) and the inhibitor concentration required for half-maximal rate of inactivation (K_I) were calculated from double reciprocal plots of k_{obs} versus inhibitor concentrations by linear regression analysis using GraphPad Prism 5.

NADPH oxidation: A test of reduction of rhinacanthins at concentrations 0.31, 0.63, 1.25, 2.5, 5, 10 µM by rat CYPOR was performed in the presence of CYP2A6 or CYP2A13 or rat CYPOR alone and was measured by the decrease in absorbance at 340 nm, based on oxidation of NADPH, using an extinction coefficient of 6.26 $mM^{-1}\,cm^{-1}$ as described.[20,33]

Statistical analysis: Data were statistically analyzed by Statistix 8.0 using Student's *t* test for analyses of data in **Tables 1** and **2**, and Kruskal-Wallis One-Way Analysis of Variance (ANOVA) with all-pairwise comparisons of mean ranks for analyses of data in **Figure 4**. Results with $p \leq 0.05$ were considered to be significantly different.

Results

The inhibitory effect of *R. nasutus* crude ethanol extract on CYP2A6 and CYP2A13 was investigated using reconstituted recombinant enzyme and coumarin as the probe substrate. Pre-incubation of CYP-reconstitution reactions with the ethanol extract, *n*-hexane, and EtOAC fractions for 10 min displayed a significant increase in inhibition compared to co-incubation, indicating a time-dependent inactivation of CYP2A6 and CYP2A13 (**Table 1**). Similar significant enhancement of inhibition was observed with 8-MOP, a known mechanism-based inhibitor of CYP2A6 and CYP2A13. Inhibition effects of sub-fractions from *n*-hexane and EtOAC fractions were essentially similar for CYP2A6 and CYP2A13. Strong inhibitory potential against both CYP2A6 and CYP2A13 was found associated with rhinacanthin-A in the

Table 1. IC_{50} values of crude ethanol extract, fractions and purified rhinacanthins (A–C, H/I) from *Rhinacanthus nasutus* against coumarin 7-hydroxylation mediated by the purified cDNA-expressed CYP2As

Test samples	IC_{50} (µg/ml)			
	CYP2A6[a]		CYP2A13[a]	
	Co-incubation (µg/ml)	Pre-incubation (µg/ml)	Co-incubation (µg/ml)	Pre-incubation (µg/ml)
Crude ethanol	5.02 ± 0.62	1.36 ± 0.53[b]	3.93 ± 0.22	1.87 ± 0.23[b]
Hexane fraction	3.68 ± 0.02	1.02 ± 0.17[b]	4.06 ± 0.24	1.72 ± 0.14[b]
Ethyl acetate fraction	4.89 ± 0.44	1.46 ± 0.03[b]	2.47 ± 0.48	0.67 ± 0.11[b]
Aqueous fraction	>500	—[c]	>250	—[c]
8-methoxysoralen (µM)	1.53 ± 0.01	0.39 ± 0.11[b]	0.79 ± 0.01	0.27 ± 0.01[b]
Rhinacanthin-A (µM)	1.88 ± 0.13	0.63 ± 0.12[b]	1.42 ± 0.05	0.41 ± 0.07[b]
Rhinacanthin-B (µM)	2.00 ± 0.03	0.54 ± 0.02[b]	1.58 ± 0.17	0.29 ± 0.08[b]
Rhinacanthin-C (µM)	5.60 ± 0.94	1.19 ± 0.17[b]	7.10 ± 0.81	4.28 ± 0.40[b]
Rhinacanthin-H/I (µM)	5.33 ± 0.76	1.18 ± 0.05[b]	6.50 ± 1.40	2.22 ± 0.18[b]

[a]All values represent the mean ± SD of triplicate experiments.
[b]Significantly different between co-incubation and pre-incubation at $p < 0.05$.
[c]Not determined.

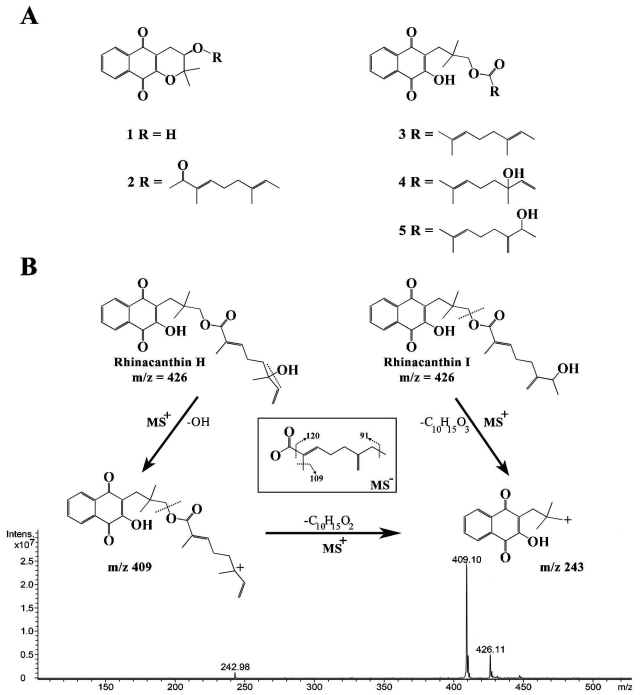

Fig. 1. Chemical structures of anthraquinones and 3-hydroxy-1,4-naphthoquinones (A) and determination of rhinacanthins H/I according to ESI-MS spectrum (B)

(A) Chemical structures of anthraquinone derivatives, rhinacanthin-A (1) and rhinacanthin-B (2); and 3-hydroxy-1,4-napthoquinone derivatives, rhinacanthin-C (3), rhinacanthin-H, (4) and rhinacanthin-I (5). (B) The ESI-MS spectrum of the rhinacanthin-H/I mixture is shown, with rhinacanthin-H (MW 426) promptly fragmented resulting in *m*/*z* 409, shown in the spectrum profile (bottom). Representations of the postulated fragmentations of rhinacanthin-H and rhinacanthin-I are shown above the spectrum. The postulated fragmentations of the side chain from ESI-MS/MS (negative mode, MS^-) are shown in the box.

EtOAc fraction and rhinacanthins B and C in the *n*-hexane fraction. The structures of these compounds shown in **Figure 1A** were identified based on their ^1H and ^{13}C NMR spectra, which were consistent with those of previous reports.[13,17,22] In the *n*-hexane fraction, a strong inhibition effect was also found associated with a mixture of two inseparable compounds with the same molecular mass. The mixture had the patterns of ionized mass fragmentation on ESI-MS (**Fig. 1B**) identified as structures of rhinacanthins H and I.[17]

A 10 min pre-incubation with rhinacanthins A, B, C, and H/I resulted in a significant increase in inhibition against CYP2A6- and CYP2A13-mediated coumarin 7-hydroxylation compared to co-incubation, indicating a time-dependent inhibition. Their IC_{50} values are shown in **Table 1**. Inhibition by the rhinacanthins H/I mixture was attributed to either rhinacanthin H or I, or to a synergistic action of both compounds, and was not investigated further. Further examination of the effects of time and inhibitor concentrations on CYP2A6 and CYP2A13 inhibition showed that inhibition by rhinacanthins A–C was time- and concentration-dependent (**Fig. 2**). Pre-incubations by rhinacanthins in the absence of NADPH abolished the time-dependent inhibition pattern (unreported data), suggesting a mechanism-based inhibition mode. Kinetics of inhibition by rhinacanthins A, B and C against CYP2A6 and CYP2A13 was determined and the apparent k_{inact} and K_I values are shown in **Figure 2**. Efficiency of CYP2A6 inactivation determined from the ratio of k_{inact} to K_I by rhinacanthin-A ($0.261\ min^{-1}\cdot\mu M^{-1}$) was approximately equal to rhinacanthin-B ($0.272\ min^{-1}\cdot\mu M^{-1}$), and was 3.6-fold greater than rhinacanthin-C ($0.072\ min^{-1}\cdot\mu M^{-1}$). Unlike CYP2A6, CYP2A13 was inactivated best by rhinacanthin-B ($0.625\ min^{-1}\cdot\mu M^{-1}$), followed by rhinacanthin-A ($0.143\ min^{-1}\cdot\mu M^{-1}$). Both rhinacanthins A and B had inhibitory efficiency against CYP2A13 of 4.7- and 20-fold greater than rhinacanthin-C ($0.03\ min^{-1}\cdot\mu M^{-1}$), respectively.

Several 1,4-naphthoquinone derivatives are known to stimulate oxidation of NADPH as 1,4-naphthoquinones undergo reduction by CYPOR, possibly forming a superoxide radical,[18] and could modulate CYP2A6 and CYP2A13. To see this effect, the reaction mixture containing rat CYPOR in the presence of NADPH and each of the rhinacanthins was tested and the molar absorption change of NADPH oxidation was measured.[33] The results revealed that rhinacanthins B and C did not accelerate NADPH oxidation by CYPOR (unreported data), suggesting no interference of electron transfer from CYPOR to CYP2A6 or CYP2A13 by either compound. Moreover rhinacanthins B and C did not inhibit cytochrome *c* reduction mediated by CYPOR (unreported data). Hence inhibition by rhinacanthins B and C was due to the inhibitory effect of CYP2A6 and CYP2A13 enzymes. In contrast rhinacanthin-A, at concentrations of 2.5, 5, and 10 μM, in the presence of rat CYPOR alone evidently showed accelerated NADPH oxidation (**Supplemental Fig. S1**). In the presence of CYP2A6 or CYP2A13, rhinacanthin-A could not significantly further accelerate NADPH oxidation compared to that in the presence of rat CYPOR alone, indicating CYP2As were not involved in the accelerated NADPH oxidation (**Supplemental Fig. S1**). The mixed-type inhibition by rhinacanthin-A shown in **Figure 3** suggested that rhinacanthin-A bound to both CYP2A enzymes, and a catalytic process was involved in the inhibition.

To determine whether inhibition of CYP2A6 and CYP2A13 by rhinacanthins A–C was affected by trapping agents including nucleophilic trapping agent (GSH) and oxygen scavenging agents (catalase and SOD), a reaction mixture pre-incubation with each rhinacanthin for 10 min in the presence of NADPH was performed with or without each of the trapping agents. The GSH, catalase and SOD failed to protect CYP2A6 and CYP2A13 from inactivation mediated by rhinacanthins A, B and C (**Fig. 4**). To determine whether inhibition by rhinacanthins A–C was due to covalent modification of enzymes by metabolically activated rhinacanthins, dialysis and addition of potassium ferricyanide in the reaction mixture were performed. Following overnight dialysis at 4°C, inactivation of CYP2A6 and CYP2A13 by rhinacanthins A–C was observed in the pre-incubation reactions in the presence of NADPH, while in the absence of NADPH, coumarin 7-hydroxylation activities were restored (**Table 2**). For control dialysis reactions, recovery of enzyme activities was observed with pilocarpine reversible inhibitor against CYP2A6 and CYP2A13 after dialysis, but activities were not recovered with 8-MOP mechanism-based inactivator. Addition of potassium ferricyanide in the reaction mixture did not significantly protect inactivation of CYP2A6 or CYP2A13 by rhinacanthins A–C. Taken together, these results are consistent with the irreversible mechanism-based inactivation by rhinacanthins A–C of CYP2A6- and CYP2A13-mediated coumarin 7-hydroxylation.

Discussion

In the present study we identified rhinacanthins A, B, and C, and H/I mixed compounds that possessed inhibitory potentials on

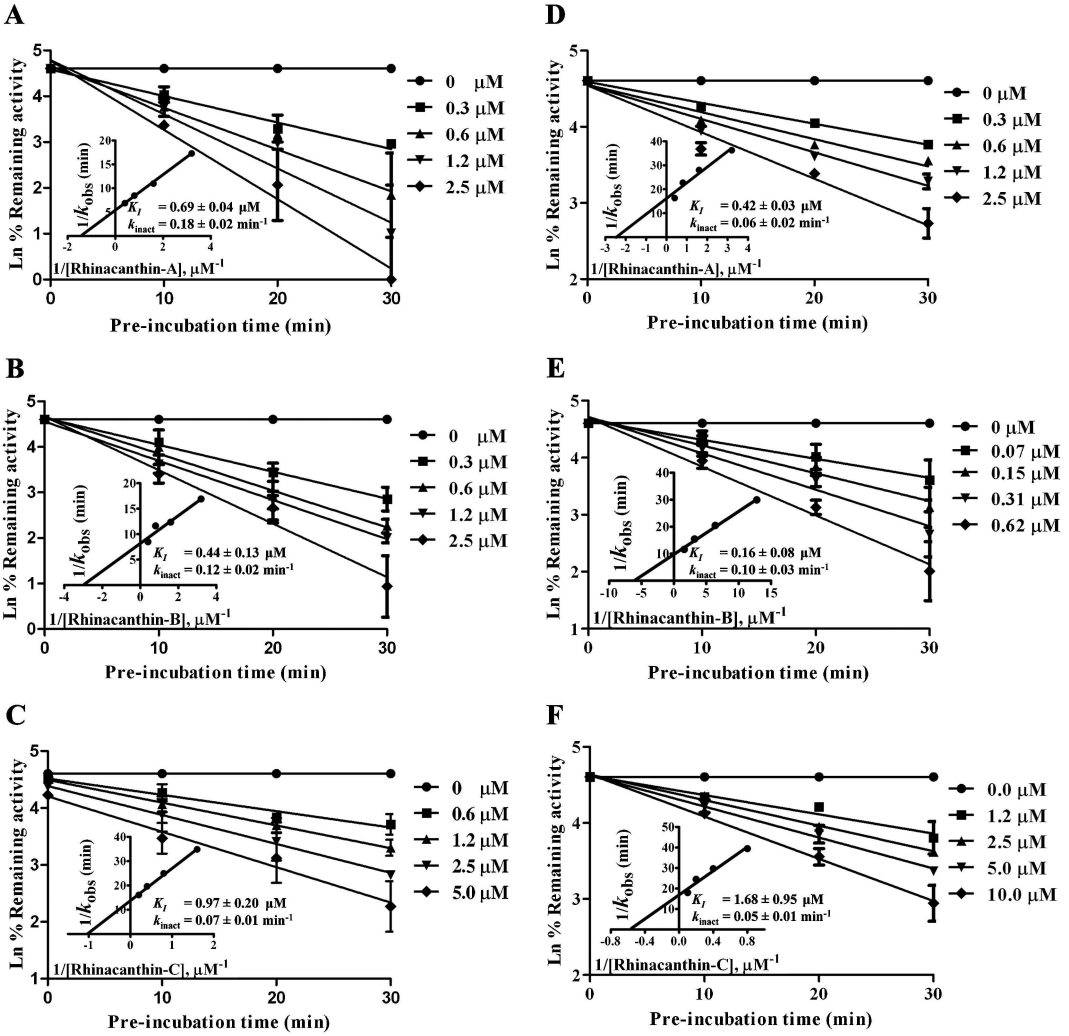

Fig. 2. Time- and concentration-dependent inactivation and kinetics of inhibition of CYP2A6 (A–C) and CYP2A13 (D–F)-mediated coumarin 7-hydroxylation by rhinacanthin-A (A, D), rhinacanthin-B (B, E) and rhinacanthin-C (C, F)

Various concentrations of each rhinacanthin were used as indicated. Inlets represent double-reciprocal plots of k_{obs} values against the reciprocal of rhinacanthins. Each data point represents the mean ± SD of three independent experiments.

CYP2A6- and CYP2A13-mediated coumarin 7-hydroxylation activities. We show the evidence indicating that rhinacanthins A, B, and C are potent mechanism-based inactivators of CYP2A6 and CYP2A13, as the criteria of time, concentration and NADPH dependence that define mechanism-based inactivation were met. The rhinacanthins H/I mixture inhibited both enzymes with an indication of time-dependent manner (**Table 1**). Inhibition of nicotine metabolism mediated by CYP2A6 and CYP2A13 was also detected for rhinacanthin-C (**Supplemental Fig. S2**); rhinacanthins A and B were not tested due to the insufficient amount of compounds obtained. The chemical constituents and biological activity of *R. nasutus* have been widely investigated; compounds found include 15 naphthoquinone esters (rhinacanthins A–D, G–Q), rhinacanthone, triterpenoids, and flavonoids.[17] Among rhinacanthins, all are 3-hydroxy-1,4-naphthoquinone esters, except rhinacanthins A, B, O and P, which are anthraquinones. Under our isolation conditions, rhinacanthin-C was most abundant in *R. nasutus*, comprising about 6% of crude ethanol extract, and the amount obtained for rhinacanthins A, B, and H/I was about 0.44%, 0.9%, and 0.4%, respectively. This is consistent with results previously reported and other rhinacanthins existed at levels lower than or comparable to that of rhinacanthin-I.[17] In this study, we could identify only rhinacanthins A–C and H/I as exhibiting potential inhibitory effects against CYP2A6 and CYP2A13. It is possible that other inhibitory compounds were also present, but not in sufficient amount for detection of inhibition; further investigation of inhibitory effects of other rhinacanthins against CYP2A6 and CYP2A13 could be useful to extrapolate their structure-function relationship.

The apparent K_I values of rhinacanthins A and B are within the 1 μM range and similar against both CYP2A6 and CYP2A13 (**Fig. 2**), while rhinacanthin C comprises 1.3–10.5-fold higher K_I. Previous studies reported that menthofuran, 8-MOP, and decursinol angelate furanocoumarins, all of which are mechanism-based inactivator of CYP2A6, had K_I values of 2.2, 0.8 and 2.42 μM, respectively.[30,32,34] Judging from these values we could infer that rhinacanthins A, B, and C bind CYP2A6 comparably to 8-MOP, menthofuran, and decursinol angelate. For CYP2A13, mechanism-

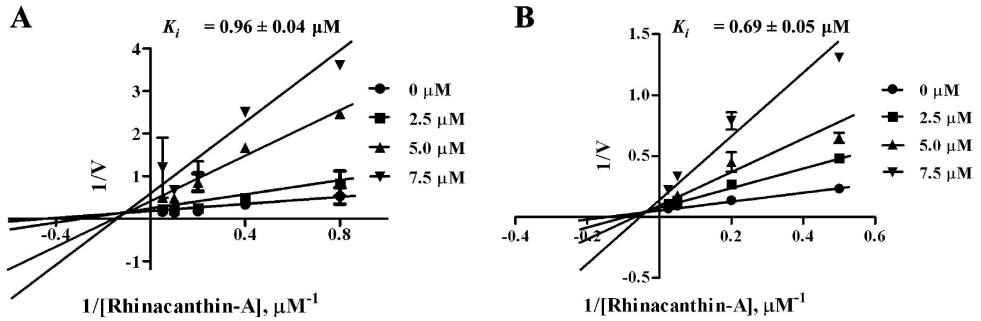

Fig. 3. Lineweaver-Burk plots of inhibition by rhinacanthin-A on CYP2A6 (A) and CYP2A13 (B)
Mode and kinetics of reversible inhibition of rhinacanthin-A were determined by incubation of the reconstituted enzyme mixture with various concentrations of rhinacanthin-A (2.5, 5, and 7.5 μM) and coumarin (2.5, 5, 10, 20, 40 μM). Rate (V) was measured as pmol/min/pmol P450. The data were fitted into the Lineweaver-Burk equation and the apparent K_i values were determined (GraphPad Prism 5). Each fitted line was produced by linear regression analysis of each point. Each point represents the mean ± SD of duplicate experiments.

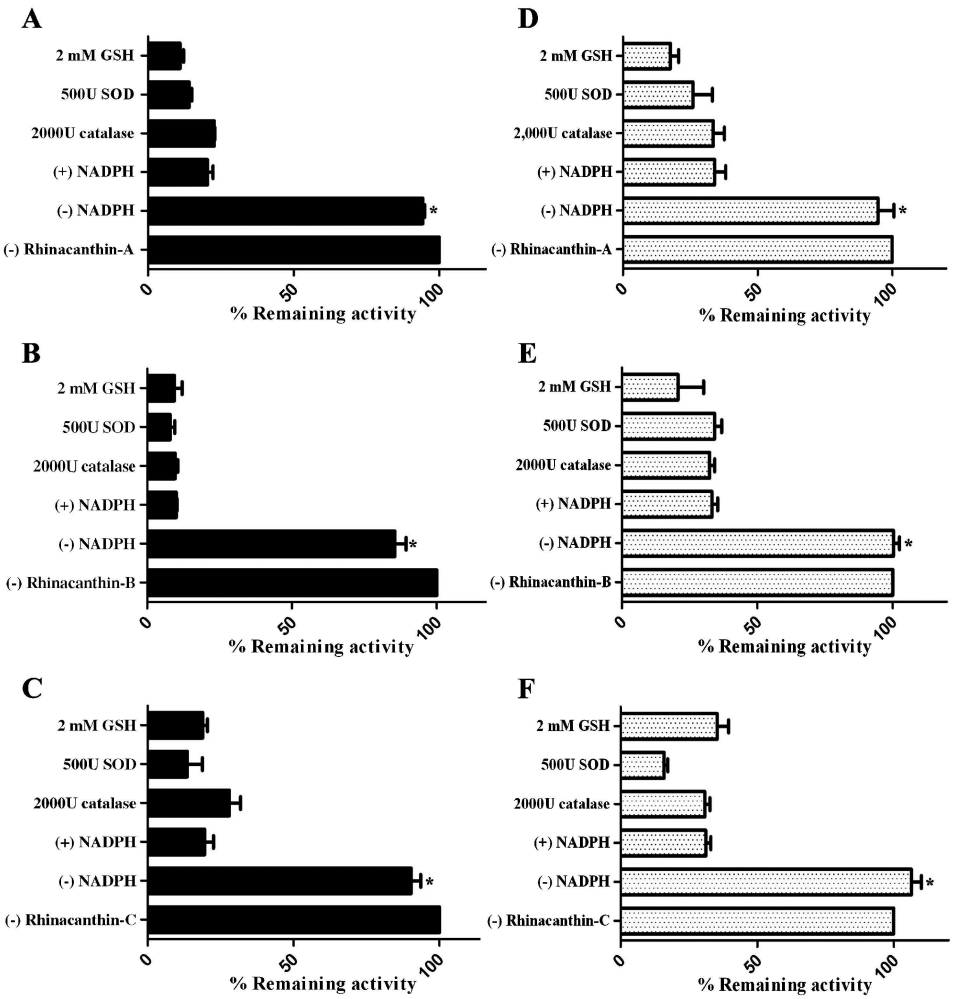

Fig. 4. Effects of various trapping or protecting agents on inactivation of recombinant CYP2A6 (A–C) and CYP2A13 (D–F) by rhinacanthins A (A, D), B (B, E) and C (C, F)
CYP2As were inactivated by rhinacanthins A and B (2.5 μM), rhinacanthin C (5 μM) and inactivation time (10 min) in the presence and absence of various trapping or protecting agents. The percent remaining activities of both CYP2As were measured. Each column represents the mean of triplicate determinations. Reactions in the absence of each rhinacanthin were set to 100% remaining activity. * Represents significant difference compared to reactions in the presence of NADPH (+NADPH) and of each trapping agent at $p < 0.05$.

Table 2. Effects of potassium ferricyanide and dialysis on rhinacanthin-mediated inactivation of CYP2As activity

Conditions	Percent remaining activity[a]	
	CYP2A6	CYP2A13
Before dialysis		
(−) Rhinacanthin	100	100
After dialysis		
(−) Rhinacanthins	91.4 ± 4.7	95.6 ± 0.8
(+) Rhinacanthins		
2.5 μM Rhinacanthin-A, − NADPH	85.6 ± 8.0	96.6 ± 8.5
2.5 μM Rhinacanthin-A, + NADPH	10.6 ± 5.3[b]	10.0 ± 0.8[b]
2.5 μM Rhinacanthin-B, − NADPH	83.5 ± 5.6	91.0 ± 0.9
2.5 μM Rhinacanthin-B, + NADPH	14.3 ± 2.5[b]	7.3 ± 4.2[b]
5.0 μM Rhinacanthin-C, − NADPH	86.2 ± 4.3	91.5 ± 4.5
5.0 μM Rhinacanthin-C, + NADPH	21.1 ± 1.6[b]	29.3 ± 5.4[b]
(+) 8-MOP		
2.5 μM 8-MOP, − NADPH	90.7 ± 0.1	89.0 ± 5.8
2.5 μM 8-MOP, + NADPH	12.0 ± 1.7[b]	22.0 ± 1.5[b]
(+) 2.5 μM Pilocarpine	91.1 ± 2.3	92.2 ± 2.3
70 μM $K_3Fe(CN)_6$		
+2.5 μM Rhinacanthin-A, +NADPH	16.7 ± 3.1	11.5 ± 3.9
+2.5 μM Rhinacanthin-B, +NADPH	8.9 ± 2.3	5.8 ± 2.4
+5.0 μM Rhinacanthin-C, +NADPH	9.01 ± 1.8	18.5 ± 0.6

[a]Percent remaining activity was compared to reactions without rhinacanthin (before dialysis). All values represent the mean ± SD of triplicate experiments. [b]Significantly different between −NADPH and +NADPH at $p < 0.05$.

based inhibition was reported for isothiocyanate derivatives including phenylpropyl isothiocyanate, phenylhexyl isothiocyanate, *tert*-butyl isothiocyanate, and 8-MOP, with K_I values of 0.14, 1.1, 4.3, and 0.11 μM, respectively.[31,35] These values may imply that rhinacanthins A and B bind to CYP2A13 with binding competency comparable to that of phenylpropyl isothiocyanate, phenylhexyl isothiocyanate and 8-MOP, while rhinacanthin-C is less efficient. Comparison of apparent K_I values among rhinacanthins A–C suggests that the octadienoic acid side chain presented in rhinacanthins B and C might not contribute significantly to inhibition potency. Alternatively it might be the overall configuration of rhinacanthins A and B that define inhibitory potency. This is different from decursinol derivatives of which the alkyl side chain of decursinol angelate plays a role in the formation of an intermediate metabolite that is responsible for CYP2A6 mechanism-based inhibition.[30] It should be noted that rhinacanthins H/I inhibited both enzymes with IC_{50} values closer to that of rhinacanthin-C in parallel to their correlated structures. In the present investigation the naphthoquinone ring of rhinacanthins might be important for catalytic processing leading to generation of true inhibitory intermediate compounds, since rhinacanthins A–C share a 1,4-naphthoquinone ring. Determination of reactive metabolites derived from catalytic transformation of rhinacanthins might elucidate the mechanism of CYP2As inactivation.

Inactivation of CYP2A6 and CYP2A13 by rhinacanthins A–C was irreversible and unaffected by various trapping agents and dialysis, suggesting that any reactive intermediate formed might not involve peroxidative reactions and could not be scavenged prior to the enzyme inactivation event. Previous studies revealed that 1,4-naphthoquinones could stimulate NADPH oxidation pertinent to its structural derivatives.[20] In a series of naphthoquinone derivatives, 5-hydroxy and 2-methyl derivatives of 1,4-naphthoquinones existing in juglone (5-hydroxy-1,4-naphthoquinone), menadione (2-methyl-1,4-naphthoquinone) and plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) displayed potential effect in promoting NADPH oxidation to a different extent, whereas the 2-hydroxy group of lawsone (2-hydroxy-1,4-naphthoquinone) does not exert acceleration of NADPH oxidation.[20] Our findings with rhinacanthin-C could add to the notion that 3-hydroxy-1,4-naphthoquinone ester with the octadienoic acid side chain does not stimulate reduction by CYPOR. In contrast rhinacanthin-A could be subject to accelerated reduction by CYPOR, but rhinacanthin-B at concentration up to 40 μM was incapable of stimulating NADPH oxidation (unreported data), despite its sharing an anthraquinone structure with rhinacanthin-A but with a side chain attachment. In a study with several antitumor anthraquinone derivatives, for example ametantrone, benzoperimidine, anthrapyridone, they were found to stimulate NADPH oxidation catalyzed by CYPOR.[33] That rhinacanthin-A anthraquinone might be reduced by CYPOR to a radical has yet to be explored. However acceleration of NADPH oxidation was negligible at K_I concentrations of rhincanthin-A (within a 1 μM range) and might not affect CYP2As inhibition as the inactivation could not be rescued by trapping agents. Moreover the mechanism-based inhibition of rhinacanthin-A was supported by the pattern of mixed-type inhibition (**Fig. 3**) suggesting that rhinacanthin-A could competitively bind to the CYP2As pockets and generate a reactive intermediate inhibitor through a catalytic process mediated by CYP2As.

In conclusion, rhinacanthins A–C are mechanism-based inactivators of CYP2A6 and CYP2A13, as they showed time, concentration, and NADPH-dependent inhibition. Overnight dialysis, addition of potassium ferricyanide, and trapping agents did not recover coumarin 7-hydroxylation activity. Inhibition of both enzymes by rhinacanthins, A–C and H/I, which are 1,4-naphthoquinone derivative esters, might give useful insights into structural characteristics of CYP2A6 and CYP2A13 inactivators. Whether these rhinacanthins are selective inhibitors of CYP2A6 and CYP2A13 remains to be elucidated by inhibition studies with other human CYP isoforms. Finally the potential of these compounds as chemopreventive agents for smokers bears further exploration.

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