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Inhibition Against Mosquito Cytochrome P450 Enzymes by Rhinacanthin-A, -B, and -C Elicits Synergism on Cypermethrin Cytotoxicity in *Spodoptera frugiperda* Cells

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ABSTRACT *Rhinacanthus nasutus* (Acanthaceae) is a shrub reported to contain insecticidal activities. The current study was conducted to determine whether *R. nasutus* constituents could inhibit benzyloxyresorufin *O*-debenzylation (BROD) mediated by CYP6AA3 and CYP6P7. Both enzymes have shown pyrethroid degradation activity and been implicated to play role in pyrethroid resistance in *Anopheles minumus* (Theobald) mosquito, a malaria vector. Three compounds, rhinacanthin-A, -B, and -C that exhibited potent inhibitory activity were isolated and purified from aerial part of *R. nasutus*. Their kinetic parameters and modes of inhibition were determined. Rhinacanthin-B was the most potent inhibitor in in vitro inhibition assay and exhibited mechanism-based inhibition against both CYP6AA3 and CYP6P7. Rhinacanthin-C which is a major compound of *R. nasutus* reversibly inhibited both enzymes in vitro with 2–4 folds less inhibitory potency than rhinacanthin-B. In contrast, rhinacanthin-A reversibly inhibited CYP6AA3, but inhibition against CYP6P7 was a mechanism-based inhibition type. Where mechanism-based inhibition was found, the inhibition showed characteristic of time-, concentration-dependence, and requirement of NADPH. Using 3-(4, 5-dimethylthiazol-2-y-l)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay in intact *Spodoptera frugiperda* (Sf9) cells, the three compounds increased susceptibility of CYP6AA3- and CYP6P7-expressing cells to cypermethrin cytotoxicity because of inhibition effect on mosquito enzymes. The combined inhibition effect on mosquito cytochrome P450 enzyme and synergistic effect on cypermethrin cytotoxicity of the three *R. nasutus* compounds could be beneficial for resistance management strategies in mosquito vector control.

KEY WORDS *Anopheles minimus*, *Rhinacanthus nasutus*, cytochrome P450, inhibition

Long-term use of synthetic insecticides to control mosquitoes could promote resistance and hinder control of associated diseases. In particular pyrethroid resistance is widespread in several vector species including *Anopheles gambiae* (Giles), *Culex quinquefasciatus* (say), and *Aedes aegypti* (Linnaeus) (Sathantriphop et al. 2006, Coleman and Hemingway 2007, Yadouleton et al. 2011). A common resistance mechanism to synthetic insecticides is increased activity of detoxification enzymes including the heme-containing cytochrome P450 monooxygenases (Feyereisen 1999). Inhibition of the P450 monooxygenases enzyme defense mechanism may thus overcome or at least restrict insecticide resistance (Bingham et al. 2007). Mechanism-based inhibition by covalent modification of the heme group of P450s and/or the active site amino acid residue of enzymes can result in irreversible loss of enzyme activity (Correia and Ortiz de Montellano 2005) and effectively prevent insecticide detoxification. Piperonyl butoxide (PBO) that is a P450 inhibitor has been used as a synergist of pyrethroids against mosquito vectors (N′Guessan et al. 2010, Tungu et al. 2010). Combinations of PBO and another insecticide with different mode of action completely restore the efficacy of pyrethroid on resistant *An. gambiae* mosquitoes (Darriet and Chandre 2011). Piperine found in *Piper nigrum* and *P. tuberculatum* that possesses insecticidal activities against *Cx. pipiens pallens* (Coquillett), *Ae. aegypti*, and *Ae. togoi* (Theobald) larvae (Park et al. 2002) is another example reported containing inhibition activity against insect and human cytochrome P450 enzymes, but toxicity test has shown that it is acutely toxic to rodents (Piyachaturawat et al. 1983, Scott 1996, Daware et al. 2000, Bhardwaj et al. 2002).

Rhinacanthus nasutus (Linn.) Kurz, a shrub of the Acanthaceae family, is widely distributed in Southeast

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Asia including Thailand and has long been used in the treatment of several diseases. These therapeutic potentials of *R. nasutus* have been further verified through experimental studies, for example, antiviral and antiproliferative activity (Sendle et al. 1996; Siripong et al. 2006a, 2006b). Recently, extract of this plant has been reported to possess larvicidal activity against various mosquito species including *Ae. aegypti*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus* (Giles), and *An. dirus* (Peyton & Harrison) (Komalamisra et al. 2005; Kamaraj et al. 2008a, 2008b; Bagavan et al. 2009), but chemical compounds that possess larvicidal activity have not been identified. Chemical constituents from different parts of this plant suggest the main bioactive compounds to be a group of naphthoquinone esters (rhinacanthins A–D, G–Q). Flavonoid compounds, oroxylin A and wogonin, found in *R. nasutus* were reported to inhibit diclofenac 4′-hydroxylase activity of human CYP2C9 (Kim et al. 2002, Si et al. 2009). Whether these compounds and/or other *R. nasutus* constituents possess inhibitory effect toward mosquito P450 activity has not been reported.

We have previously isolated CYP6AA3 and CYP6P7 genes from laboratory-selected deltamethrin-resistant *An. minimus* mosquito and the heterologously expressed enzymes contain pyrethroid metabolizing activities in vitro, implicating their roles in pyrethroid resistance in this mosquito species (Duangkaew et al. 2011b). Examination in *Spodoptera frugiperda* (Sf9) insect cells using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assays revealed that CYP6AA3 and CYP6P7 expressed in Sf9 cells could cytoprotect cells against pyrethroid toxicity because of ability of both enzymes in pyrethroid degradation (Boonseupsakul et al. 2008, Duangkaew et al. 2011a). Addition of PBO inhibited enzyme cytoprotection against pyrethroid toxicity (Duangkaew et al. 2011a). Moreover, preliminary studies of enzyme inhibition revealed that flavonoids, furanocoumarins, and methylenedioxyphenyl compounds also have the potential to inhibit both enzymes based on benzyloxyresorufin-*O*-debenzylation (BROD) assays. Plant compounds in the flavonoid and furanocoumarin groups also have inhibition potentials against the housefly, *Musca domestica* CYP6D1, *An. gambiae* CYP6Z2 and *Papilio polyxenes* CYP6B3 enzymes that could metabolize pyrethroids suggesting that phytochemicals could be a source of insect P450 inhibitors (Scott et al. 2000, Wen et al. 2006, Mclaughlin et al. 2008).

The current study investigated inhibition by *R. nasutus* against *An. minimus* BROD activity of CYP6AA3 and CYP6P7 enzymes using in vitro reconstitution assays. Chemical constituents that showed inhibitory activity against both enzymes were isolated, purified, and identified. Mode of inhibition and kinetic parameters of purified compounds were determined and synergistic effect on susceptibility to cypermethrin cytotoxicity of intact Sf9 cells expressing mosquito P450s was investigated.

Materials and Methods

Chemicals. Benzyloxyresorufin, cypermethrin, leupeptin, nicotinamide adenosine diphosphate reduced form (NADPH), phenylmethylsulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), α-naphthoflavone, and piperonyl butoxide (PBO) were purchased from Sigma–Aldrich (St. Louis, MO), and 3-(4, 5-dimethylthiazol-2-y-l)-2, 5-diphenyltetrazolium bromide (MTT) from USB (Cleveland, OH). Analytical grade hexane, ethyl acetate (EtOAc), ethanol, methanol, diethyl ether, and high performance liquid chromatography (HPLC) grade acetonitrile were supplied by RCI Labscan (Bangkok, Thailand). The Sf9 insect cell line and SF-900 II SFM culture media were provided by Invitrogen (Carlsbad, CA).

Extraction and Isolation of Bioactive Compounds. Aerial parts of *R. nasutus* were purchased from traditional medicine market (Bangkok, Thailand). Extractions were done as previously described (Komalamisra et al. 2005) with some modifications. Air-dried tissue (0.5 kg) was homogenized and macerated in 95% ethanol (1 liter × 5 d) at room temperature, filtered (Whatman no. 1) and the residue re-extracted twice using the same procedure. Filtrates were combined and solvent removed by vacuum rotary evaporator yielding 18.5 g of EtOH extract. The concentrated EtOH extract was resuspended in 30% ethanol followed by successive extractions with *n*-hexane (0.4 liters × 2) and ethyl acetate (EtOAc, 0.4 liters × 2) yielding *n*-hexane (5.66 g), EtOAc (1.05 g), and aqueous fractions (11.32 g). Fractions were tested for inhibitory effects against CYP6AA3 and CYP6P7 enzymes.

Bioassay-Guided Fractionation of *n*-Hexane- and EtOAc-Soluble Fractions. Stepwise gradient elution of each *n*-hexane extract (0.4 g) was conducted in a chromatography column containing silica gel (silica 60; 0.063–0.200 mm, Merck, Germany), using a *n*-hexane/EtOAc/methanol solvent system. This provided six fractions; fr.1: *n*-hexane, 26 mg; fr.2: *n*-hexane-EtOAc (16:3), 2 mg; fr.3: *n*-hexane-EtOAc (7:3), 92 mg; fr.4: *n*-hexane-EtOAc (1:1), 139 mg; fr.5: EtOAc, 65.2 mg; fr.6: EtOAc-methanol (1:1), 27.6 mg. Among these, fr.3 and fr.4 that showed potent inhibition activities were subjected to analysis by HPLC (Symmetry RP-18 column; 3.9 × 150 mm, Waters, Ireland). HPLC gradient was initiated from 50% acetonitrile (ACN) in water (vol:vol) to 100% over 20 min, 100% ACN was held for 25 min, followed by a linear gradient returning to 50% ACN over 30 min and a final equilibration with 50% ACN for 35 min with flow rate of one ml per min. Four major absorption peaks at 254 nm were obtained. The two peaks at retention time, 18.3 min (rhinacanthin-B) and 19.3 min (rhinacanthin-C), that were associated with inhibition effect were further purified by thin-layer chromatography (TLC) on precoated Kiesel gel 60 F_{254} sheets (Merck, Germany) using *n*-hexane-diethyl ether (6:4, vol:vol) as mobile phase and traveled at retardation factor (R_f) values of 0.59 and 0.7, respectively.

The EtOAc fraction (400 mg) was separated by TLC yielding four subfractions with only one fraction containing inhibitory activities against both mosquito P450s. This fraction was further analyzed by HPLC. Gradient of HPLC was initiated from 10% ACN in water (vol:vol) to 100% over 18 min, 100% ACN was held for 22 min, followed by a linear gradient returning to 10% ACN over 26 min and a final equilibration with 10% ACN for 30 min with flow rate of 1 ml/min from which four major absorption peaks were obtained. A peak at retention time 13.8 min (rhinacanthin-A) that showed inhibitory effect was further purified by HPLC (Symmetry RP-18 column; 7.8 × 100 mm, Waters, Ireland) using a gradient initiated from 25% ACN in water (vol:vol) to 100% over 17 min, 100% ACN was held for 20 min, followed by a linear gradient returning to 10% ACN over 23 min and a final equilibration with 10% ACN for 27 min with flow rate of 1.8 ml/min. Rhinacanthin-A was collected at retention time 10.01 min. The three purified compounds were subjected to NMR analyses and their chemical structures elucidated by comparing ^{1}H- and ^{13}C-NMR spectral data with those previously reported (Sendl et al. 1996, Wu et al. 1998).

Fluorescence-Based Inhibition Assays of CYP6AA3 and CYP6P7. Expression of CYP6AA3 and CYP6P7 were carried out using baculovirus-mediated insect cell expression system in Sf9 cells as previously described (Kaewpa et al. 2007, Duangkaew et al. 2011b). Microsomal fractions containing either CYP6AA3 or CYP6P7 were prepared and used with purified *An. minimus* NADPH-dependent cytochrome P450 reductase (CPR) enzyme as P450 redox partner for inhibition assays against BROD of CYP6AA3 and CYP6P7 using benzyloxyresorufin substrate at the concentration approximately K_m value (2 μM for CYP6AA3 and 0.5 μM for CYP6P7; Duangkaew et al. 2011b). Different concentrations of test compounds dissolved in DMSO (1% final concentration) were used and the co-incubation inhibition assay was performed in 50 mM Tris-HCl buffer, pH 7.5 in a total volume of 500 μl. The reaction was initiated by addition of NADPH to a final concentration of 0.1 mM. Resorufin production was measured for 5 min using RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan) at λ_{ex} = 530 and λ_{em} = 590 nm. The amount of resorufin and rate of resorufin formation was calculated from standard curve and expressed as picomole resorufin/min/pmol P450. The IC_{50} values of *R. nasutus* fractions were determined graphically by nonlinear regression analysis of logarithm of extract concentrations against the relative residual enzyme activity using GraphPad Prism 5 (GraphPad Co. Ltd., San Diego, CA, USA). Enzyme activities in the presence of test compounds were compared with reaction with DMSO as vehicle control. When tested with silica and HPLC eluted fractions, inhibition of CYP6AA3 and CYP6P7 was performed with a final concentration of 10 μg/ml of each *R. nasutus* fraction.

Time-dependent inhibition of P450 activity was investigated to preliminarily determine mechanism-based inhibition by plant extracts. Assays were performed by preincubation of various concentrations of each extract with P450 enzyme for 30 min in the presence of NADPH or absence of NADPH before addition of benzyloxyresorufin substrate, and IC_{50} values of extracts were calculated. The indication of mechanism-based inhibition is the IC_{50} shift to lower range in preincubation with inhibitor in the presence of NADPH compared with co-incubation assay (Fowler and Zhang 2008).

Determination of Inhibition Kinetics. Apparent K_i values and modes of inhibition were determined from enzymatic assays with various concentrations of benzyloxyresorufin substrate (0.75, 1.5, 3, or 4.5 μM for CYP6AA3 and 0.25, 0.5, 1, or 2 μM for CYP6P7) conducted with different concentrations of rhinacanthin-A (4–16 μM) and -C (5–20 μM). Mode of inhibition on each enzyme activity of these three compounds was estimated graphically from a double reciprocal plot of velocity against substrate concentrations (Lineweaver-Burk plot), K_i values were calculated via second plots of the slopes from Lineweaver–Burk plots versus inhibitor concentrations.

The kinetic constant for mechanism-based inhibition of rhinacanthin-A and rhinacanthin-B was determined from time-dependent assay by varying rhinacanthin-A and rhinacanthin-B concentrations (2–16 μM of rhinacanthin-A and 0.3–2.4 μM of rhinacanthin-B) and preincubation times (0, 10, 20, and 30 min). A logarithm of the percentage of the remaining activity was plotted against preincubation time and the slope of the lines was obtained from linear regression analysis. The apparent inactivation rate constants (k_{obs}) were taken from the slope of the line ($-k_{obs}$). 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.

Inhibition of P450 Activities in P450-Expressing Sf9 Cells. In this study, extracts of *R. nasutus* and the isolated compounds, rhinacanthin-A, -B, and -C, that could inhibit CYP6AA3- and CYP6P7-mediated BROD in vitro, were evaluated their effects on susceptibility to cypermethrin insecticide in Sf9 parent and P450-expressing cells. Before cell-based inhibition assays, cytotoxicity of cypermethrin, *R. nasutus* extract, rhinacanthin-A, rhinacanthin-B, rhinacanthin-C, and two P450 inhibitors (α-naphthoquinone and PBO that were previously shown high inhibitory potency against CYP6AA3 and CYP6P7) toward Sf9 parental cells were evaluated by MTT assays as previously described (Duangkaew et al. 2011a). The Sf9 cells were seeded at 2 × 10^5 cells per well in 24-well culture plates. Cells were allowed to attach for 3 h followed by addition of test compounds to each well and DMSO was used as vehicle control. After 24-h exposure, cells in each well were washed twice with Luckhorff's buffer pH 7.2 (132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES), followed by addition of 200 μl MTT solution (5 mg/ml in Luckhorff's buffer). After incubation (4 h, 28°C), MTT was removed and DMSO was added to dissolve

Table 1. IC_{50} values of purified compounds, fractions, and ethanolic extract of *R. nasutus* and test compounds in inhibition against BROD of CYP6AA3 and CYP6P7

Sample	IC_{50}[a] (μg/ml)			
	CYP6AA3		CYP6P7	
	Coincubation	Preincubation	Coincubation	Preincubation
Ethanolic extract	18.78 ± 2.44	21.44 ± 3.30[b]	14.63 ± 0.29	13.82 ± 0.79[b]
n-hexane fraction	12.25 ± 0.98[c]	6.33 ± 0.54[c]	15.44 ± 2.43[c]	6.74 ± 1.52[c]
EtOAc fraction	6.20 ± 0.14[b]	5.89 ± 0.46[b]	14.70 ± 1.07[b,c]	7.75 ± 0.49[b,c]
Aqueous fraction	>100	ND[e]	>100	ND[e]
Rhinacanthin-A (μM)	9.15 ± 0.23[b]	8.59 ± 0.21[b]	35.14 ± 0.91[b,c]	6.41 ± 0.91[b,c]
Rhinacanthin-B (μM)	2.39 ± 0.28[b,c]	0.29 ± 0.05[c]	3.69 ± 0.07[b,c]	0.67 ± 0.01[c]
Rhinacanthin-C (μM)	10.64 ± 0.84	10.10 ± 0.76	9.56 ± 0.27	8.92 ± 0.10
α-Naphthoflavone (μM)	0.37 ± 0.06[d]	0.38 ± 0.06[d]	2.90 ± 0.27[d]	3.03 ± 0.45[d]
PBO (μM)	9.91 ± 0.81[c,d]	4.04 ± 0.31[c,d]	31.77 ± 3.21[c,d]	16.22 ± 1.81[c,d]

[a] Each value represents mean ± SD of triplicate tests.
[b] Significant differences between enzymes, $P < 0.05$.
[c] Significant differences between co-incubation and pre-incubation, $P < 0.05$.
[d] Values obtained from Duangkaew et al. (2011b).
[e] ND, not determined.

formazan product. The absorbance of formazan was measured at 540 nm using Multiskan EX microtiter plate reader (Thermo Labsystems, Finland). Cell viability was expressed as percentage of viable cells relative to cells treated with DMSO alone that was assigned as 100% viability.

In the cell-based inhibition study, *R. nasutus* extract (1.25 μg/ml) and inhibitors (24 μM of rhinacanthin-A, 7.5 μM of rhinacanthin-B, 0.8 μM of rhinacanthin-C, 6.25 μM of α-naphthoflavone, and 3.125 μM of PBO) that caused ≈20% mortality in Sf9 parent cells were used. CYP6AA3- and CYP6P7-expressing cells and Sf9 control cells were treated with selected doses of each inhibitor followed by addition of various concentrations of cypermethrin (3–500 μM). Inhibition of P450s was measured as cytotoxicity of cypermethrin to cells in the presence of inhibitor (after normalization with that of cells treated with each inhibitor alone and that of Sf9 cells treated with both cypermethrin and test compound) compared with cells treated with cypermethrin and vehicle (DMSO).

Statistical Analysis. Data were statistically analyzed by GraphPad Prism 5 using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. Results with $P \leq 0.05$ were considered to be significantly different.

Results

Inhibition of CYP6AA3 and CYP6P7 by *R. nasutus* Extracts. Inhibition studies using time-dependent inhibition assay, by means of preincubation of enzyme mixture with each test extract/compound for 0 and 30 min in the presence of NADPH before addition of benzyloxyresorufin substrate, was performed with CYP6AA3 and CYP6P7 expressed in baculovirus-insect cell expression system. This was to primarily determine whether inhibition of *R. nasutus* extracts/constituents followed a mechanism-based inhibition pattern. Inhibition of crude ethanolic extract of *R. nasutus* did not differ dramatically against BROD mediated by mosquito CYP6AA3 and CYP6P7 enzymes (Table 1). However, significant increase in inhibition by the *n*-hexane fraction against both enzymes occurred when preincubation time was increased to 30 min, a characteristic of mechanism-based inactivation. The EtOAc fraction inhibited CYP6AA3 with higher potency than CYP6P7 with the IC_{50} for the latter shifting to a lower range. It should be noted that there was no inhibitory activity against enzymatic activity of CPR-mediated cytochrome *c* reduction to 200 μg/ml of crude ethanolic extract (data not shown), suggesting inhibition effect was not attributable to CPR redox partner enzyme.

Modes and Kinetics of CYP6AA3 and CYP6P7 Inhibition by Rhinacanthin-A, -B, and -C. Strong inhibitory potential against both CYP6AA3 and CYP6P7 was associated with each of two compounds in the *n*-hexane fraction and one in the EtOAc fraction. The ^{1}H and ^{13}C NMR spectra of these compounds sug-

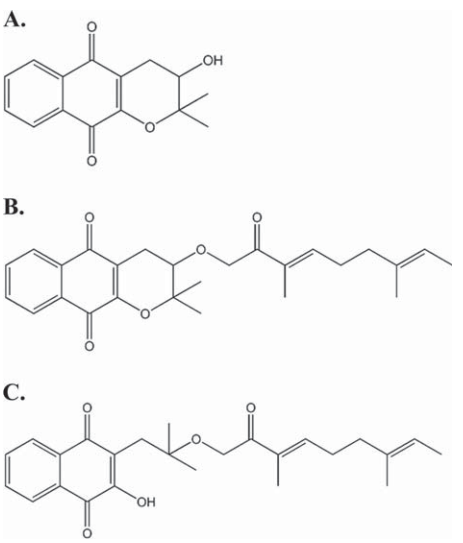

Fig. 1. Chemical structures of rhinacanthin-A (A), rhinacanthin-B (B), and rhinacanthin-C (C).

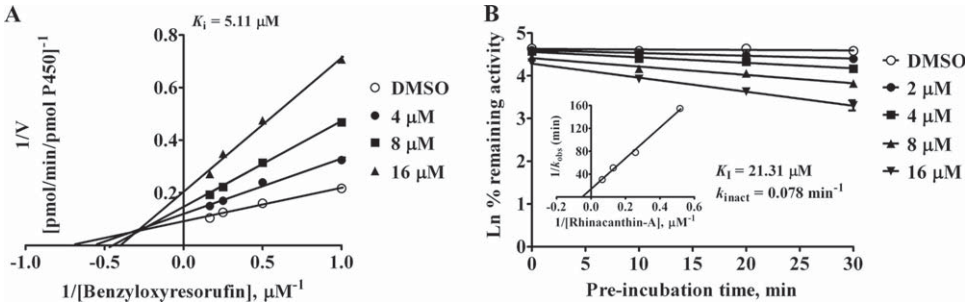

Fig. 2. Inhibition of CYP6AA3 and CYP6P7 by rhinacanthin-A. (A) Lineweaver–Burk plot of inhibition on CYP6AA3. (B) Plot of natural log percentage of remaining CYP6P7 activity in the presence of rhinacanthin-A versus preincubation times. Inlet in (B) is a plot of reciprocal of the k_{obs} values against the reciprocal of rhinacanthin-A concentrations. Each fitted line was produced by linear regression analysis of each point. Each point represents the mean ± SD of triplicate experiments.

gested structures consistent with those reported for rhinacanthin-A, -B, and -C (Sendl et al. 1996, Wu et al. 1998; see structural formulas in Fig. 1). Modes and kinetics of inhibition by these three compounds against CYP6AA3 and CYP6P7 were determined. BROD enzymatic assays indicated CYP6AA3 activity was inhibited by rhinacanthin-A in the mixed-type manner with K_i values of 5.11 μM, while that on CYP6P7 was in a preincubation time-dependent manner (Fig. 2). Preincubation in the absence of NADPH abolished the time-dependent inhibition pattern. Rhinacanthin-B also inhibited both CYP6AA3 and CYP6P7 activities in a time- and NADPH-dependent manner. The k_{inact} and K_I values of rhinacanthin-A against CYP6P7 and rhinacanthin-B against both enzymes determined from double-reciprocal plots of k_{obs} and inhibitor concentrations are shown in Figs. 2 and 3. Efficiency of enzyme inactivation from the ratio of k_{inact} to K_I by rhinacanthin-A was 3.71 min/nM for inhibition of CYP6P7, and efficiency by rhinacanthin-B on CYP6AA3 was 96.07 min/nM and on CYP6P7 was 32.94 min/nM, indicating that rhinacanthin-B has higher inhibition potency than rhinacanthin-A. However, rhinacanthin-C noncompetitively inhibited CYP6AA3 with a K_i value of 7.95 μM, and competitively inhibited CYP6P7 with a K_i value of 7.32 μM (Fig. 4).

Effect of Rhinacanthin-A, -B, and -C on Susceptibility to Cypermethrin in P450-Expressing Cells. Crude ethanolic extract, rhinacanthin-A,-B, and -C significantly decreased cypermethrin LC_{50} values in cells expressing mosquito P450s (Table 2), as a result of synergistic action of *R. nasutus* compounds with cypermethrin cytotoxicity. It should be noted that rhinacanthin-C has a synergistic effect on cypermethrin cytotoxicity in the control uninfected Sf9 cells, but this was not prominent for rhinacanthin-A and rhinacanthin-B. Thus, we normalized percent cell viability of P450-expressing cells upon treatment with cypermethrin and each of rhinacanthin compounds with that of Sf9 parent cells. Synergistic ratios of rhinacanthin-B and rhinacanthin-C with cypermethrin cytotoxicity on P450-expressing cells was ≈6–10 folds higher than Sf9 cells and 2–4 folds higher than those obtained from α-naphthoflavone and PBO inhibitors. Synergism of rhinacanthin-A with cypermethrin cytotoxicity was much less than that with rhinacanthin-B and -C and was comparable to those of α-naphthoflavone and PBO inhibitors.

Discussion

Mosquito enzymes, CYP6AA3 and CYP6P7, were inhibited by rhinacanthin-A, -B, and -C isolated from *R. nasutus* but differed in their modes of action. Rhinacanthin-C reversibly inhibited both CYP6AA3 and CYP6P7. In contrast, rhinacanthin-B inhibited both enzymes in a mechanism-based manner, while rhinacanthin-A reversibly inhibited CYP6AA3 and irreversibly inhibited CYP6P7. In particular the mechanism-based inhibition of rhinacanthin-A and rhinacanthin-B is time-, concentration-, and NADPH-

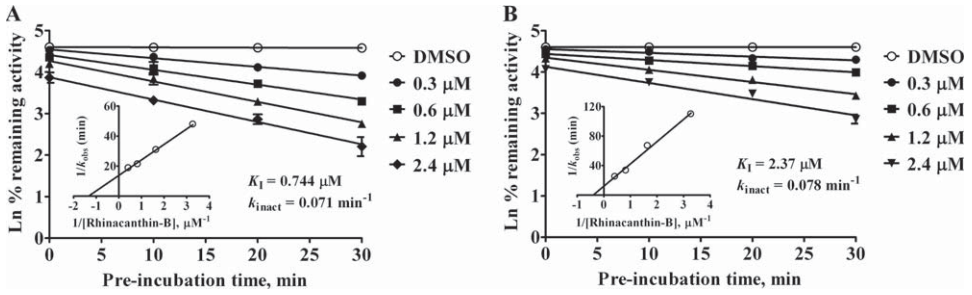

Fig. 3. Kinetic analysis of CYP6AA3 and CYP6P7 inhibition by rhinacanthin-B. Plots of inhibition on CYP6AA3 (A) and CYP6P7 (B) were performed as described in Fig. 2B. Each point represents the mean ± SD of triplicate experiments.

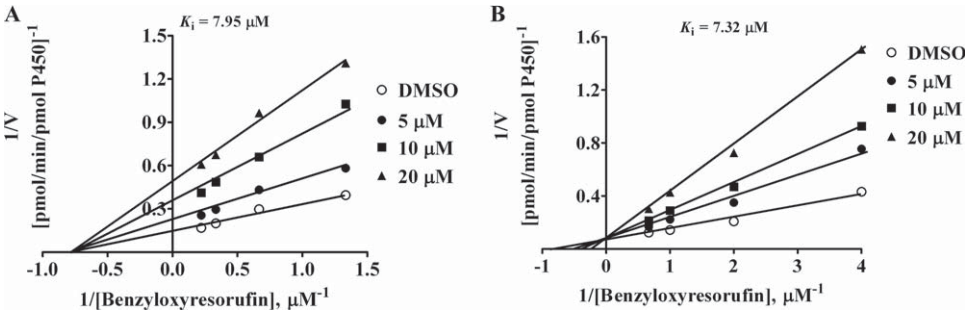

Fig. 4. Lineweaver–Burk plots of CYP6AA3- or CYP6P7-mediated BROD in the presence of rhinacanthin-C. Inhibitions of CYP6AA3 (A) and CYP6P7 (B) are shown. Each point represents the mean ± SD of triplicate experiments.

dependent. *R. nasutus* extracts do not possess inhibition activity on CPR-mediated cytochrome c reduction, suggesting the inhibition effect was on mosquito P450 enzymes. By using MTT cytotoxicity assays on Sf9 cells (Duangkaew et al. 2011a), rhinacanthin-A, -B, and -C inhibited both enzymes and showed a synergistic effect on susceptibility of P450-expressing cells toward cypermethrin cytotoxicity. The synergism of rhinacanthin-C using cell-based assays was approximately twice as efficient as PBO and α-naphthoflavone, while rhinacanthin-B possessed >2–4 folds higher synergism than PBO and α-naphthoflavone. This is consistent with results of in vitro inhibition assays in that rhinacanthin-B possessed higher inhibitory effect than rhinacanthin-C. Synergism of these compounds with pyrethroid toxicity can be further investigated in vivo with pyrethroid resistant mosquitoes. With PBO as a synergist, a 4–20-fold decrease in LC_{50} value of deltamethrin was observed in *Ae. aegypti*, *An. stephensi*, *An. culicifacies*, *An. vagus*, *Cx. tritaeniorhynchus*, and *Cx. quinquefasciatus* mosquitoes (Fakoorziba et al. 2009).

Naphthoquinones are the major chemically bioactive constituents in *R. nasutus.* Rhinacanthin-A and -B possess anticancer activity against several human cell lines (Wu et al. 1988, 1998), while rhinacanthin-C, which is a major constituent, contains various activities including antiviral and antiproliferative activities against human cancer cells (Sendl et al. 1996; Siripong et al. 2006a, 2006b). It has been suggested that antiproliferation activity of rhinacanthin-C was because of apoptosis induction causing cell death (Siripong et al. 2006a). The *R. nasutus* extracts have been found containing insecticidal activities with LC_{50} values of 20–170 mg/liter against *An. stephensi* and 15–90 mg/liter against *Ae. aegypti* (Pushpalatha and Muthukrishnan 1999; Kamaraj et al. 2008a, 2008b). In this study crude ethanolic extract of *R. nasutus* was cytotoxic to parent

Table 2. Effect of *R. nasutus* extract and compounds on cypermethrin susceptibility of Sf9, CYP6AA3-, and CYP6P7-expressing cells

Cells	Treatment	LC_{50} [a] (μM)	SR[b]	Ratio[c]
Sf9 cells	Cypermethrin	130.7 ± 8.13[d,e]	—	—
	+ Ethanolic extract	173.4 ± 13.7[d,e]	0.79	NA
	+ Rhinacanthin-A	123.0 ± 5.52[e]	1.06	NA
	+ Rhinacanthin-B	107.6 ± 3.89[e]	1.21	NA
	+ Rhinacanthin-C	20.3 ± 0.01[d,e]	6.44	NA
	+ α-Naphthoflavone	162.4 ± 9.42[d]	0.80	NA
	+ PBO	144.9 ± 11.3[e]	0.90	NA
CYP6AA3-expressing cells	Cypermethrin	397.9 ± 16.7[d,e]	—	—
	+ Ethanolic extract	61.8 ± 2.30[d,e]	6.44	8.15
	+ Rhinacanthin-A	125.2 ± 3.08[d]	3.18	2.99
	+ Rhinacanthin-B	46.1 ± 0.19[d,e]	8.63	7.13
	+ Rhinacanthin-C	9.3 ± 0.95[d,e]	42.78	6.64
	+ α-Naphthoflavone	162.7 ± 1.08[d]	2.45	3.06
	+ PBO	182.7 ± 13.2[d,e]	2.17	2.4
CYP6P7-expressing cells	Cypermethrin	402.4 ± 42.5[d,e]	—	—
	+ Ethanolic extract	126.1 ± 6.10[d,e]	3.19	4.04
	+ Rhinacanthin-A	137.9 ± 4.45[d,e]	2.92	2.75
	+ Rhinacanthin-B	33.3 ± 2.37[d,e]	12.08	9.98
	+ Rhinacanthin-C	11.0 ± 0.63[d,e]	36.58	5.68
	+ α-Naphthoflavone	178.5 ± 14.5[d]	2.25	2.80
	+ PBO	209.6 ± 14.3[d,e]	1.92	2.13

[a] Each value is mean ± SD of triplicate experiments.
[b] Synergism ratio: LC_{50} cypermethrin alone/LC_{50} cypermethrin in the presence of inhibitors.
[c] Synergism ratio for P450-expressing cells after normalization with Sf9 cells.
[d] Significant difference between cypermethrin alone and with inhibitors, $P < 0.05$.
[e] Significant difference of each treatment between Sf9 and P450-expressing cells, $P < 0.05$.
NA, not applicable.

Sf9 cells with LC_{50} value of 4.5 mg/liter, but it is not known whether cytotoxicity of crude *R. nasutus* extract observed in this study is inherent to apoptosis induction and to insecticidal activity observed in mosquitoes.

The chemical nature of these three rhinacanthins is naphthoquinone ester derivatives that share a common 1, 4-naphthoquinone core structure (Fig. 1). Rhinacanthin-A and -B possess a pyranonaphthoquinone ring, while rhinacanthin-B and -C share an octadienoic acid attached to the naphthoquinone core structure. It is conceivable that the naphthoquinone core might partly be responsible for insecticidal activity. The derivatives of 1, 4-naphthoquinone such as isoshinanolone and plumbagin, isolated from rhizome of *Plumbago capensis*, have shown insecticidal activity toward *Ae. aegypti* with LC_{50} values of 1.26 and 5.43 μg/ml, respectively (Sreelatha et al. 2010). Other naphthoquinone derivatives such as lapachol, 6-hydroxyplumbagin, 3-*O*-methyldroserone, and maritinone exert toxicity in the LC_{50} range of 15–40 μg/ml against *Ae. aegypti* (Ribeiro et al. 2009). These compounds also show antifeedant activity against cabbage looper, *Trichoplusia ni* (Akhtar et al. 2012). Under our experimental conditions, we observed cytotoxicity of rhinacanthin-A, -B and -C against Sf9 parental cells with LC_{50} values $\approx$35 μg/ml, 14.57 and 0.8 μg/ml, respectively, suggesting that naphthoquinone core structure might play a role in cytotoxicity toward Sf9 insect cells.

Toxicity of naphthoquinones has been suggested to be because of formation of reactive oxygen species (ROS), and electrophilic attack to critical cellular nucleophiles leading to oxidative stress and cell death (Thor et al. 1982). Other mechanisms attributed to toxicity of quinone moiety have been redox cycling (Tokunaga et al. 2004) and inhibition of mitochondrial respiration (Khambay et al. 2003). Whether rhinacanthins might involve in the described mechanisms in insect cells thereby causing insect mortality remains to be investigated. Taken together rhinacanthins A–C in combination could exert high potency in inhibition against mosquito P450s and contain synergistic effects on cypermethrin cytotoxicity in cell-based assays. Results of this study could have an implication for use of *R. nasutus* extract to combat pyrethroid resistance in mosquitoes or other insecticide resistance that is mainly mediated by P450 detoxification. Moreover, preliminary investigation of *R. nasutus* on nontarget organisms such as *Poecilia reticulate* showed toxicity at least 10-fold less than against *Ae. aegypti* (Rongsriyam et al. 2006). However, further studies on insecticidal mode of action and effects on other nontarget organisms and environment of *R. nasutus* is needed for practical use as mosquito control agent and for resistance management strategies in mosquito vector control.

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