Rhinacanthin-C Mediated Herb-Drug Interactions with Drug Transporters and Phase I Drug-Metabolizing Enzymes

Wilasinee Dunkoksung, Nontima Vardhanabhuti, Pongpun Siripong, and Suree Jianmongkol

Departments of Pharmacology and Physiology (W.D., S.J.) and Pharmaceutics and Industrial Pharmacy (N.V.), Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; and National Cancer Institute, Bangkok, Thailand (P.S.)

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ABSTRACT

Rhinacanthin-C is a major active constituent in Rhinacanthus nasutus (L.) Kurz, a plant widely used in herbal remedies. Its potential for pharmacokinetic herb-drug interaction may exist with drug transporters and drug metabolizing enzymes. This study assessed the possibility for rhinacanthin-C-mediated drug interaction by determining its inhibitory effects against major human efflux and influx drug transporters as well as various human cytochrome P450(CYP) isofoms. Rhinacanthin-C demonstrated a moderate permeability through the Caco-2 monolayers [P\(_{\text{APP}}\) (AP→BL) = 1.26 × 10\(^{-6}\) cm/s]. It significantly inhibited transport mediated by both P-glycoprotein (P-gp) (IC\(_{50}\) = 5.20 μM) and breast cancer resistance protein (BCRP) (IC\(_{50}\) = 0.83 μM) across Caco-2 and BCRP-overexpressing Madin-Darby canine kidney II cells (MDCKII) cells. This compound also strongly inhibited uptake mediated by organic anion-transporting polypeptide 1B1 (OATP1B1) (IC\(_{50}\) = 0.70 μM) and OATP1B3 (IC\(_{50}\) = 3.95 μM) in OATP1B-overexpressing HEK cells. In addition to its inhibitory effect on these drug transporters, rhinacanthin-C significantly inhibited multiple human CYP isoforms including CYP2C8 (IC\(_{50}\) = 4.56 μM), 2C9 (IC\(_{50}\) = 1.52 μM), 2C19 (IC\(_{50}\) = 28.40 μM), and 3A4/5 (IC\(_{50}\) = 53 μM for midazolam and IC\(_{50}\) = 81.20 μM for testosterone), but not CYP1A2, 2A6, 2B6, 2D6, and 2E1. These results strongly support a high propensity for rhinacanthin-C as a perpetrator of clinical herb-drug interaction via inhibiting various influx and efflux drug transporters (i.e., P-gp, BCRP, OATP1B1, and OATP1B3) and CYP isoforms (i.e., CYP2C8, CYP2C9, and CYP2C19). Thus, the potential for significant pharmacokinetic herb-drug interaction should be addressed when herbal products containing rhinacanthin-C are to be used in conjunction with other prescription drugs.

Introduction

Herbal products have been used increasingly worldwide either as alternative medicines or dietary supplements. Coadministration of these products with therapeutic agents potentially leads to herb-drug interaction via pharmacokinetic interference on drug metabolism and/or transport (Oga et al., 2016; Sprouse and van Bremen, 2016; Wu et al., 2016). The common interference mechanisms involve inhibition and induction of drug metabolizing enzymes and drug transporters. Consequently, both therapeutic efficacy and safety can be affected (Zhou et al., 2007; Oga et al., 2016).

The superfamily of cytochrome P450 (CYP) enzymes—particularly CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5—represents the major drug-metabolizing enzymes in phase I oxidative metabolism. The metabolism of approximately 70% of drugs and exogenous substances in humans is attributed to these expressed CYP isoforms in the liver and extrahepatic tissues (i.e., intestines) (Wienkers and Heath, 2005). When taken orally, CYP substrates are metabolized by intestinal CYP enzymes as well as hepatic CYP enzymes. Both intestinal and hepatic metabolism will affect drug absorption and disposition, resulting in decreased bioavailability and altered pharmacokinetic profiles of those substrate drugs (Wienkers and Heath, 2005; Xie et al., 2016).

Moreover, drugs that are subjected to CYP enzyme metabolism are at high risk for drug-drug interactions (DDIs) when orally coadministered with CYP inhibitors. If CYP inhibitors are present in the gastrointestinal (GI) tract at high concentrations, they can effectively inhibit the intestinal CYP-mediated metabolism of concomitant substrate drugs. In addition, if these inhibitors reach the liver at high levels, they can also interfere with hepatic drug metabolism, leading to even higher plasma drug concentrations and alteration in therapeutic responses. Drugs with narrow therapeutic index such as phenytoin and warfarin are more vulnerable to such DDIs (Miners and Birckett, 1998).

Efflux transporters (e.g., breast cancer resistance protein [BCRP], multidrug resistance-associated protein [MRP], P-glycoprotein [P-gp]) and influx transporters (e.g., organic anion-transporting polypeptide [OATP]) also play important roles in drug absorption and disposition (Mizuno et al., 2003; König et al., 2013). These transporters are located in various organs, including the intestine, liver, and kidney. Several popularly used herbal products such as St. John’s wort, echinacea, goldenseal, grapefruit juice, ginseng, and milk thistle are potent inhibitors...
or modulators of various CYP enzymes and transporter proteins (Gurley et al., 2005; Brantley et al., 2014). Clinically, the incidence of CYP- or transporter-based drug interactions from herb-mediated pharmacokinetic alteration of prescription medicines has been reported (Oga et al., 2016; Sprouse and van Bremen, 2016). Hence, investigation of potential herb-drug interaction relating to CYP enzymes and transporters is necessary to support efficacy and safety of therapeutic agents in concurrent use with herbal products.

**Rhinacanthus nasutus** (L.) Kurz (Acanthaceae) has long been used in traditional medicines in the tropical region including India, Taiwan, Thailand, and the south of the People’s Republic of China. The plant has been used to treat various symptoms such as fever, fluid retention, hypertension, pneumonia, hepatitis, diabetes, and cancers (Siripong et al., 2006a,b; Horii et al., 2013). Rhinacanthin-C (Fig. 1) is a major bioactive naphthoquinone constituent found in this plant (Sendell et al., 1996; Siripong et al., 2006a,b; Panichayupakaranant et al., 2009). Recently, we demonstrated that rhinacanthin-C significantly enhanced doxorubicin-mediated cytotoxicity in vitro via inhibition of MRP2 and P-gp efflux transporters (Wongwanakul et al., 2013; Chaisit et al., 2017). In addition to its effect on efflux transporters, this naphthoquinone compound exerts inhibitory effect on a few CYP enzymes (i.e., CYP2A6 and 2A13) (Pouyfung et al., 2014). Thus, it can be anticipated that rhinacanthin-C may cause herb-drug pharmacokinetic interaction when concurrently used with CYP transporter drug substrates. Until now, there have been no reports on rhinacanthin-C inhibition against P-gp, BCRP, or OATP drug transporters or against human CYP enzymes, particularly those involved in drug metabolism.

This study assesses the potential for herb-drug interaction of rhinacanthin-C when taken orally. We examined the intrinsic properties of this compound as a perpetrator to interfere with drug absorption and disposition via drug transporters and phase I drug-metabolizing enzymes. The results of this mechanistic study on drug interaction were also used as a basis in predicting the potential for clinical pharmacokinetic interference between rhinacanthin-C and other coadministered drugs.

**Materials and Methods**

**Materials and Chemicals.** Rhinacanthin-C was isolated from the root of *Rhinacanthus nasutus* (L.) Kurz (R. nasutus), purified, and identified as previously described elsewhere (Siripong et al., 2006a,b). Other chemicals, including acetylcysteine, amiodarion dihydrochloride dihydrate, atenolol, dexamethasone hydrobromide, tert-butyl 3-((25S,8S)-14-methoxy-2-(2-methylpropyl)-4,7-dioxo-3,6,17-triazatetracyclo[8.7.0.03,8.011,16]heptadeca-1(10),11,13,15-tetraen-5-yl) hydrobromide, described elsewhere (Siripong et al., 2006a,b). Other chemicals, including *Rhinacanthus nasutus* Dulbecco MI). Midazolam was purchased from Cerilliant Corporation (Round Rock, TX). (Bremen, Germany), and (Bupropion hydrochloride, chlorzoxazone, coumarin, digoxin, digitoxin, doxazosin, labetalol, and 10 μM digitoxin for digoxin, 60 nM doxazosin for prazosin, and 1.25 μM mandelide sodium bisulfate for rhinacanthin-C. After centrifugation (12,000 g for 10 minutes at 4°C), the supernatants were analyzed by UHPLC-MS/MS.

**Cell Cultures.** The human colon adenocarcinoma (Caco-2, HB37) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in CYP enzymes containing 10% FBS, 1% non-essential amino acids, 1% L-glutamine, and 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere of 5% CO2. For the transport assays, cells (passage numbers 40–60) were seeded at a density of 6.0 × 10^4 cells/cm² onto Transwell inserts and cultured for 21 days. The integrity of cell monolayers was evaluated by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS (Millipore, Bedford, MA). Only Caco-2 monolayers having TEER values above 600 Ωcm² were used in our experiments.

The polarized Madin–Darby canine kidney II (MDCKII) parental cell line and subclone transduced with human BCRP (MDCKII-BCRP) were kind gifts from Dr. Alfred H. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands). The cells were maintained in CYP enzymes containing 10% FBS, 1% GlutaMax, and 0.5% penicillin/streptomycin solution in a humidified atmosphere of 5% CO2 at 37°C. For transport assays, cells were seeded at a density of 2.14 × 10^4 cells/cm² onto Transwell inserts and grown for 3 days. The cell monolayers with TEER values above 200 Ωcm² were used in our experiments.

The human embryonic kidney 293 (HEK293) cells stably overexpressing human OATP1B1 (HEK-OATP1B1), human OATP1B3 (HEK-OATP1B3), and the vector control cell lines HEKCoG418 and HEK-Co/Hygrozymogen were kind gifts from Dr. Jörg König (Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany). The cells were cultured in 10% FBS-MEM containing either geneticin or hygromycin at 37°C, as previously described elsewhere (König et al., 2011, 2012). For uptake assays, cells were seeded at densities of 12.5 × 10^4 cells/cm² (for HEK-OATP1B1 and HEKCoG418) and 8.0 × 10^4 cells/cm² (for HEK-OATP1B3 and HEK-Co/Hygrozymogen) onto poly-l-lysine coated plates and grown to their confluence for 3 days. The cells were further cultured in the presence of 10 nM sodium butyrate for 1 day and used for the uptake experiment (König et al., 2011).

**Permeability Assays.** Permeability assays were performed as previously described elsewhere (Hubatsch et al., 2007; Dunkoksung et al., 2019). The Caco-2 monolayers were treated with 10 μM HEPES-HBSS (pH 7.4) containing either rhinacanthin-C or a cocktail mixture of three permeability markers (10 μM acyclovir, 10 μM atenolol, and 10 μM propanolol) in the apical (AP) site at 37°C for 3 hours. Samples were collected from the basolateral (BL) side every 30 minutes with fresh buffer replacement. The collected samples were mixed with an equal volume of 100% acetonitrile (ACN) containing the internal standard (75 μM labeled for permeability markers; 1.25 μM mandelide sodium bisulfate for rhinacanthin-C). After centrifugation (12,000g for 10 minutes at 4°C), the supernatants were analyzed by ultra-high-pressure liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS).

**P-gp and BCRP Substrate Assays.** The bidirectional transports (AP to BL and BL to AP directions) of rhinacanthin-C were determined across cell monolayers (Caco-2 monolayers for P-gp, MDCKII-BCRP for BCRP) at 37°C, using a protocol described elsewhere (Hubatsch et al., 2007; Poller et al., 2011; Dunkoksung et al., 2019). Rhinacanthin-C was added to either the AP or the BL chamber, depending on the transport direction studied. A known substrate of each transporter (P-gp substrate digoxin, 5 μM; BCRP substrate prazosin, 5 μM) was used as a positive control group. Samples were taken from the relevant chamber every 30 minutes for 180 minutes and were mixed with an equal volume of 100% ACN containing internal standard of each compound (2 μM digitoxin for digoxin, 60 nM doxazosin for prazosin, and 1.25 μM mandelide sodium bisulfate for rhinacanthin-C). After centrifugation, the supernatants were analyzed by UHPLC-MS/MS.

**P-gp and BCRP Inhibition Assays.** The inhibitory action of rhinacanthin-C on either P-gp or BCRP activity was determined in the bidirectional transport assays, as described earlier. The cell monolayers were treated with probe P-gp or BCRP substrates (digoxin, 5 μM; prazosin, 5 μM) in the presence or absence of rhinacanthin-C. Samples were collected from the relevant chamber every
30 minutes for 180 minutes and were mixed with an equal volume of 100% ACN containing the internal standard. After centrifugation, the supernatants were used as positive control groups.

**OATP1B1 and OATP1B3 Inhibition Assays.** The inhibitory action of rhinacanthin-C on either OATP1B1 or OATP1B3 activities was determined in the uptake assay with a protocol described elsewhere (Seithel et al., 2007; Bednarczyk, 2010). The cells (HER-K293-OATP1B1, HER-K293-OATP1B3, and respective vector control cell lines) were treated with rhinacanthin-C for 30 minutes at 37°C, followed by the addition of the probe substrate 8-FCaA at a concentration of 2.5 μM for OATP1B1 or 1.25 μM for OATP1B3 for another 10 minutes. The treated cells were washed with ice-cold HBSS and lysed with methanol/10 mM LiCl solution (1:1) containing 1 mM EDTA. The fluorescent intensity of 8-FCaA was determined with a microplate reader (Wallac 1420 VICTOR 3; PerkinElmer, Waltham, MA) at 485/535 nm (excitation/emission wavelengths). Cyclosporin A-treated cells were washed with ice-cold HBSS and lysed with methanol/10 mM LiCl solution (1:1) containing 1 mM EDTA. The fluorescent intensity of rhinacanthin-C was used to determine the IC50 values of rhinacanthin-C on OATP1B1 and OATP1B3.

**CYP Cocktail Inhibition Assays.** The inhibitory action of rhinacanthin-C on CYP enzymes in HLMs was determined as described elsewhere (Li et al., 2015). HLMs (0.2 mg/ml, final concentration) were incubated with a cocktail of CYP substrates and rhinacanthin-C in 0.1 M phosphate buffer (pH 7.4) containing 3.3 mM MgCl2 at 37°C. The concentration of HLMs was kept at 0.2 mg/ml to minimize nonspecific binding to HLMs (Obach, 2008). The cocktail preparation of CYP substrates contained 100 μM phenacetin (CYP1A2), 1.5 μM coumarin (CYP2A6), 12 μM bupropion (CYP2B6), 1 μM amodiaquine (CYP2C8), 100 μM tolbutamide (CYP2C9), 50 μM (S)-mephenytoin (CYP2C19), 2.5 μM dextromethorphan (CYP2D6), 15 μM chloroxazone (CYP2E1), 2.5 μM midazolam, and 50 μM testosterone (CYP3A4/5). The reaction was initiated by the addition of NADPH (1.3 mM, final concentration). At the end of the 10-minute incubation period, the reaction was stopped with ice-cold 3% formic acid in 5% ACN solution containing 0.1 μM orphenadrine as internal standard. The reaction products were collected and centrifuged before UHPLC-MS/MS analysis.

**UHPLC-MS/MS Analysis.** UHPLC-MS/MS analysis was conducted on a Eksigent Ekspt ultra LC 100 with QTRAP 6500 system (AB Sciex, Framingham, MA). A rapid UHPLC gradient with an ACE C18 column (3 μm, 50 × 1.0 mm i.d.) was used to perform a quick reverse-phase separation (10%–95% ACN with 0.1% formic acid for all metabolites, rhinacanthin-C and prazosin; 20%–95% ACN with 2 mM ammonium formate for digoxin). The flow rate was set at 200 μl/minute, and the column oven temperature was set at 45°C. The injection volume was either 5 μl (prazosin) or 10 μl (all metabolites, rhinacanthin-C, and digoxin). Detection was performed using electrospray ionization with polarity switching, collision-induced dissociation, and selected reaction monitoring. The mass transitions of the metabolites, digoxin (ammonium adduct), prazosin, rhinacanthin-C, and internal standards are listed in Tables 1 and 2.

**Calculation.** For the transport assays, the apparent permeability coefficient, Papp (centimeter per second), for both AP to BL and BL to AP directed transport of each test compound was calculated from the following equation:

\[
P_{app} = (\frac{dQ}{dt}) \times (\frac{1}{C_{in}})
\]

where dQ/dt is the cumulative transport rate (nanomoles per minute), C<sub>in</sub> is the initial drug concentration on the drug-introducing side (micromolar), and A is the surface area of the inserts (1.12 cm<sup>2</sup> in 12-wells). The efflux ratio was the ratio of P<sub>app</sub>(BL-to-AP) to P<sub>app</sub>(AP-to-BL).

**Mechanism-Based CYP Inhibition Assays.** Mechanism-based inhibition of CYP enzymes was assessed by IC<sub>50</sub> shift method (de Ron and Rajaraman, 2012; Haque et al., 2017). HLMs (0.2 mg/ml, final concentration) were incubated for 30 minutes with rhinacanthin-C in 0.1 M phosphate buffer (pH 7.4) containing 3.3 mM MgCl2 at 37°C in the presence or absence of 1.3 mM NADPH. The reaction was initiated by addition of a cocktail of CYP2C8, 2C9, 2C19, and 3A4/5 substrates. At the end of the 10-minute incubation period, the reaction was terminated with ice-cold 3% formic acid in 5% ACN solution containing 0.1 μM orphenadrine as internal standard. The samples were collected and centrifuged before UHPLC-MS/MS analysis.

### Table 1

<table>
<thead>
<tr>
<th>Transporter/Permeability Marker</th>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Mass Transition</th>
<th>Mass Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;sub&gt;app&lt;/sub&gt; substrate</td>
<td>Digoxin</td>
<td>5</td>
<td>798.309 &gt; 651.2 (+)</td>
<td>782.312 &gt; 635.3 (+)</td>
</tr>
<tr>
<td></td>
<td>Prazosin</td>
<td>5</td>
<td>384.139 &gt; 246.8 (+)</td>
<td>452.161 &gt; 343.9 (+)</td>
</tr>
<tr>
<td>Permeability marker</td>
<td>Low</td>
<td>10</td>
<td>226.043 &gt; 151.8 (+)</td>
<td>329.060 &gt; 161.8 (+)</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>10</td>
<td>267.075 &gt; 144.7 (+)</td>
<td>329.060 &gt; 161.8 (+)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>10, 100</td>
<td>409.100 &gt; 167.0 (+)</td>
<td>252.840 &gt; 80.9 (−)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration (μM)</th>
<th>Metabolite</th>
<th>Mass Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin</td>
<td>112.7 ± 10.9</td>
<td>100</td>
<td>Acetaminophen</td>
<td>152.0 &gt; 109.7 (+)</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>1.5 ± 0.2</td>
<td>1.5</td>
<td>7-Hydroxycoumarin</td>
<td>161.1 &gt; 133.2 (+)</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion</td>
<td>125.2 ± 14.0</td>
<td>12</td>
<td>Hydroxycoproprion</td>
<td>256.1 &gt; 138.8 (+)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>1.0 ± 0.1</td>
<td>1</td>
<td>N-deethylamodiaquine</td>
<td>328.1 &gt; 282.9 (+)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>110.7 ± 11.6</td>
<td>100</td>
<td>Hydroxytolbutamide</td>
<td>285.1 &gt; 186.2 (−)</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-mephenytoin</td>
<td>52.5 ± 10.6</td>
<td>50</td>
<td>(±)-4-Hydroxymephenytoin</td>
<td>235.0 &gt; 132.6 (+)</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan</td>
<td>2.9 ± 0.5</td>
<td>2.5</td>
<td>Dextrophan</td>
<td>258.1 &gt; 157.2 (+)</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chloroxazone</td>
<td>149.8 ± 12.6</td>
<td>15</td>
<td>6-Hydroxychloroxazone</td>
<td>186.0 &gt; 119.7 (−)</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam</td>
<td>2.7 ± 0.1</td>
<td>2.5</td>
<td>1’-Hydroxymidazolam</td>
<td>342.0 &gt; 324.0 (+)</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>50.5 ± 5.6</td>
<td>50</td>
<td>6β-Hydroxytestosterone</td>
<td>304.8 &gt; 268.9 (+)</td>
</tr>
<tr>
<td></td>
<td>Orphenadrine (internal standard)</td>
<td></td>
<td></td>
<td></td>
<td>270.8 &gt; 180.9 (+)</td>
</tr>
</tbody>
</table>

*Li et al. (2015).*
was performed with either Student’s t test or ANOVA, followed by the post hoc Tamhane’s T2 test. *P < 0.05 vs. control.

Results

Permeability of Rhinacanthin-C Across Caco-2 Cell Monolayer.

Three permeability markers—namely acyclovir, atenolol, and propranolol (low, moderate, and high permeability, respectively)—were chosen as our references, based on the U.S. Food and Drug Administration (FDA) recommendation (CDER, 2017b). The Papp values across Caco-2 cell monolayers in the absorptive direction (AP-to-BL) of the three reference markers in ascending order were 0.06 ± 0.01 × 10⁻⁶ cm/s (acyclovir, 10 μM), 0.48 ± 0.06 × 10⁻⁶ cm/s (atenolol, 10 μM), and 19.25 ± 0.99 × 10⁻⁶ cm/s (propranolol, 10 μM). The Papp (AP-to-BL) of rhinacanthin-C (10 μM) was 1.26 ± 0.07 × 10⁻⁶ cm/s, suggesting that its permeability was likely in the same rank order with atenolol (moderately permeable compound).

P-gp and BCRP Substrate Assays. Expression of either P-gp in Caco-2 monolayers or BCRP in MDCKII-BCRP monolayers was clearly demonstrated by the bidirectional transport of specific probe substrates (digoxin, P-gp substrate; prazosin, BCRP substrate) and inhibitors (valsodar, ketoconazole, P-gp inhibitors; Ko143, BCRP inhibitor) (Figs. 2 and 3). The efflux ratio of digoxin (5 μM) across Caco-2 monolayers significantly decreased by approximately 10-fold in the presence of the positive control P-gp inhibitors (valsodar, 1 μM; ketoconazole, 25 μM) (Fig. 2). The known BCRP inhibitor Ko143 (1 μM) significantly hindered permeation of prazosin (5 μM) across MDCKII-BCRP monolayers by approximately 4-fold but not in the MDCKII-control cell monolayers (Fig. 3).

Permeability of rhinacanthin-C in the absorptive direction (Papp (AP-to-BL)) across Caco-2 monolayers increased without any significant change in Papp (BL-to-AP) upon increasing its concentration from 10 to 100 μM (Fig. 2). Consequently, the calculated efflux ratio of this compound somewhat decreased from 2.27 ± 0.28 (10 μM) to 0.88 ± 0.09 (100 μM). This result suggested that rhinacanthin-C could be a weak P-gp substrate. On the other hand, the permeability profiles of rhinacanthin-C (10, 100 μM) across MDCKII-BCRP and MDCKII-control monolayers were comparable, with the efflux ratio values of less than 2, suggesting that rhinacanthin-C is not a substrate for BCRP (Fig. 3).

P-gp and BCRP Inhibition by Rhinacanthin-C. The abilities of rhinacanthin-C to inhibit P-gp and BCRP activities were assessed by determining the net flux of digoxin and prazosin across cell monolayers. Rhinacanthin-C was able to inhibit both P-gp–mediated transport of digoxin and BCRP–mediated transport of prazosin in a concentration-dependent manner, with IC₅₀ values of 5.20 ± 0.44 and 0.83 ± 0.09 μM, respectively (Fig. 4; Table 3). Our results suggested that rhinacanthin-C was approximately 6-fold more selective for BCRP than for P-gp. At 10 μM, rhinacanthin-C inhibited transport of digoxin across Caco-2 monolayers by 64%, and transport of prazosin across MDCKII-BCRP monolayers by 75%.

OATP1B1 and OATP1B3 Inhibition by Rhinacanthin-C. Inhibition of OATP1B1 and OATP1B3 by rhinacanthin-C was investigated...
by monitoring the uptake of probe substrate 8-FcA in the HEK293 cell line heterologously expressing the human OATP1B1 or OATP1B3. Under our conditions, the positive control inhibitor cyclosporin-A inhibited OATP1B1 and OATP1B3 with IC\textsubscript{50} values of 0.71 \pm 0.19 and 0.31 \pm 0.11 \mu M, respectively. In addition, rifampicin inhibited OATP1B1 and OATP1B3, with IC\textsubscript{50} values of 1.44 \pm 0.49 and 1.47 \pm 0.52 \mu M, respectively. Rhinacanthin-C was able to inhibit both OATP1B1-mediated and OATP1B3-mediated uptake of 8-FcA in a concentration-dependent manner (Fig. 5). This compound inhibited OATP1B1 with IC\textsubscript{50} values of 0.70 \pm 0.12 \mu M and inhibited OATP1B3 with IC\textsubscript{50} values of 3.95 \pm 1.36 \mu M (Table 3). Apparently, rhinacanthin-C was approximately 8-fold more selective for OATP1B1 than for OATP1B3. At 10 \mu M, rhinacanthin-C suppressed activities of OATP1B1 by 87% and OATP1B3 by 65%.

**CYP Inhibition by Rhinacanthin-C.** Inhibition of human CYP enzymes by rhinacanthin-C was investigated by measuring the metabolite formation of each selective probe substrates in HLMs (Table 2). Rhinacanthin-C inhibited the activities of the CYP2C family—namely CYP2C8, CYP2C9, and CYP2C19—in a concentration-dependent manner, with IC\textsubscript{50} values of 4.45 \pm 0.44, 1.57 \pm 0.22, and 29.40 \pm 4.16 \mu M, respectively (Fig. 6B; Table 4). In contrast, rhinacanthin-C at all concentrations tested (0.1–50 \mu M) did not inhibit the activities of CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2E1, or CYP3A4/5 (IC\textsubscript{50} > 50 \mu M) (Fig. 6, A, C, and D; Table 4). Because CYP3A4/5 is highly expressed in enterocytes, where high concentrations of rhinacanthin-C would be anticipated, the inhibition study of CYP3A4/5 by rhinacanthin-C was performed at an expanded concentration range (1–100 \mu M) in HLMs. Rhinacanthin-C was able to inhibit CYP3A4/5-mediated transformation of midazolam and testosterone, with IC\textsubscript{50} values of 53.00 \pm 4.22 and 81.20 \pm 6.42 \mu M, respectively (Fig. 6D).

**The Mechanism-Based CYP Inhibition by Rhinacanthin-C.** The IC\textsubscript{50} ratio was obtained from the ratio of the IC\textsubscript{50} values of rhinacanthin-C in the absence of NADPH and in the presence of NADPH during the 30-minute preincubation. As shown in Fig. 7, the IC\textsubscript{50} values of rhinacanthin-C-mediated inhibition of CYP2C family were comparable in the absence and presence of NADPH in the preincubating reaction mixture, resulting in the IC\textsubscript{50} ratio values of 1 (0.61 \pm 0.07–1.12 \pm 0.34) (Fig. 7, A–C; Table 5). It is worth noting that rhinacanthin-C in the preincubating reaction mixture with NADPH was apparently more potent than that without NADPH in reducing CYP3A4/5-mediated metabolism of testosterone, resulting in the IC\textsubscript{50} ratio value of 1.97 \pm 0.44 (Fig. 7E; Table 5). However, the effect of NADPH was not observed when midazolam was used as CYP3A4 substrate in place of testosterone. The IC\textsubscript{50} ratio of rhinacanthin-C for CYP3A4/5 (midazolam as the substrate) was less than 1 (Fig. 7D; Table 5).

### Discussion

Rhinacanthin-C is a major active constituent in *R. nasutus*, which has been commonly used in complementary therapy for various symptoms such as fever, fluid retention, hypertension, pneumonia, hepatitis, diabetes, and cancers (Siripong et al., 2006a,b; Horii et al., 2013; Shah et al., 2018). This compound is very likely to be taken concomitantly with several other drug substances, leading to herb-drug interaction issues (Horii et al., 2013; Shah et al., 2018). In the present study, we assessed the potential for rhinacanthin-C as a perpetrator in herb-drug interactions.

### TABLE 3

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>Concentration (\mu M)</th>
<th>IC\textsubscript{50} (\mu M)</th>
<th>I\textsubscript{50}/IC\textsubscript{50}</th>
<th>Potential for DDI\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>Digoxin</td>
<td>5.00</td>
<td>5.20 \pm 0.44</td>
<td>8.00–32.06</td>
<td>Yes (intestinal)</td>
</tr>
<tr>
<td>BCRP</td>
<td>Prazosin</td>
<td>5.00</td>
<td>0.83 \pm 0.09</td>
<td>50.11–200.86</td>
<td>Yes (intestinal)</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>8-FcA</td>
<td>1.25</td>
<td>0.70 \pm 0.12</td>
<td>NA\textsuperscript{c}</td>
<td>NA\textsuperscript{c}</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>8-FcA</td>
<td>2.50</td>
<td>3.95 \pm 1.36</td>
<td>NA\textsuperscript{c}</td>
<td>NA\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}I\textsubscript{50} of rhinacanthin-C = 41.59–166.71 \mu M (Gotoh et al., 2004; Panichayupakaranant et al., 2009).

\textsuperscript{b}Possible DDI risk based on I\textsubscript{50}/IC\textsubscript{50} > 10, where I\textsubscript{50} is the intestinal luminal concentration of the interaction drug [calculated from dose (mol)/250 ml], as described in Zhang et al., 2008; Giacomini et al., 2010; CDHR, 2017a.

\textsuperscript{c}NA, not applicable: transporter not highly expressed in the gastrointestinal tract (Hilgendorf et al., 2007).
interaction via modulation of drug metabolizing enzymes and transporters.

To be a perpetrator in herb-drug interaction, a compound needs to be significantly absorbed through the GI epithelium (Zhou et al., 2007; Sprouse and van Breemen, 2016). Our study showed that rhinacanthin-C was not a substrate for P-gp or BCRP efflux transporters. In addition, it demonstrated moderate permeability through the model GI membrane when compared with the FDA-recommended bioavailability markers (CDER, 2017b). Thus, this compound could function as a perpetrator if it interfered with the metabolizing enzymes and transporters.

The intestinal efflux transporters play important roles in oral bioavailability and tissue distribution of their substrate drugs (Misaka et al.,

![Fig. 5.](image)

**Fig. 5.** Inhibitory effect of rhinacanthin-C (RN-C) on (A) OATP1B1- or (B) OATP1B3-mediated 8-fluorescein-cAMP (8-FcA) uptake. The OATP1B1- or OATP1B3-mediated uptake was obtained by subtracting the uptake into respective vector control cells from that into OATP-overexpressing cells. Values are expressed as percentage of vehicle control. Each value represents the mean ± S.E. of three independent experiments.

![Fig. 6.](image)

**Fig. 6.** Inhibitory effect of rhinacanthin-C (RN-C) on CYP450 in HLMs. The enzyme activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. All data represent the mean ± S.E. of three independent experiments. (A) CYP1A2 ( ), CYP2A6 ( ), and CYP2B6 ( ). (B) CYP2C8 ( ), CYP2C9 ( ), and CYP2C19 ( ). (C) CYP2D6 ( ) and CYP2E1 ( ). (D) CYP3A4 (midazolam substrate) ( ) and CYP3A4 (testosterone substrate) ( ).
AEROSOL OATP1B1 inhibitor cyclosporin A (IC50 0.71 μM) has been reported to be a major contributor to drug interaction issues (Han, 2011). In this study, we assessed the inhibitory potential of rhinacanthin-C on nine CYP isoforms (i.e., CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5) in HLMs. Our data clearly showed that rhinacanthin-C strongly inhibited several CYP2C isoforms (CYP2C8, IC50 4.45 μM; CYP2C9, IC50 1.57 μM; CYP2C19, IC50 29.40 μM), but not CYP1A2, CYP2A6, CYP2B6, CYP2D6, and CYP2E1. Although rhinacanthin-C was previously reported to inhibit recombinant CYP2A6 (Poufyung et al., 2014), we did not detect its CYP2A6 inhibition in our HLM-based assay system. This disparity may be attributed to the difference in CYP functionality in the enzyme sources used (i.e., recombinant CYP vs. HLMs). Critical differences in the sensitivity to detect time-dependent CYP inactivation arising from different enzyme sources were recently reported (Di et al., 2007; Kahma et al., 2019). The inhibitory action of rhinacanthin-C against CYP2C isoforms was NADPH-independent, suggesting a non-mechanism-based inhibition. Nevertheless, we could not rule out the possibility that rhinacanthin-C might be a substrate of these CYP isoforms.

In addition, this compound demonstrated weak inhibitory effect against CYP3A4/5 with the IC50 values of 53 μM (midazolam, substrate) and 81 μM (testosterone, substrate). It is interesting to note that rhinacanthin-C-mediated inhibition of testosterone metabolism was NADPH-dependent, suggesting mechanism-based inhibition (IC50 ratio = 1.97, Table 5) (Haque et al., 2017). However, this NADPH-dependent CYP3A4/5 inhibition was not observed when midazolam was used as CYP3A4/5 substrate (IC50 ratio <1, Table 5) (Haque et al., 2017). These findings indicate that rhinacanthin-C had low risk for herb-drug interaction via CYP3A4/5.

Our results suggest that a potential risk of herb-drug interaction arising from rhinacanthin-C-mediated CYP inhibition is likely to be associated with inhibition of CYP2C9. Inhibition of CYP2C9 could affect the metabolism of its drug substrates such as (S)-warfarin, tolbutamide, and phenytoin (van Booven et al., 2010). Inactivation of CYP2C9 by desethylamiodarone (IC50 5.5 μM) has been reported to be a major contributor to drug interaction between (S)-warfarin and amiodarone, leading to an increased plasma concentration of (S)-warfarin and risk of hemorrhage (Heimark et al., 1992; McDonald et al., 2012). It is very likely that rhinacanthin-C is capable of interfering with the CYP2C9-mediated metabolism of its substrate drugs when used concurrently.
CYP2C9 is primarily expressed in the liver and intestine (Paine et al., 2006). However, its catalytic activity and content in the intestine are known to be an order of magnitude lower than those in the liver. Though the intestinal CYP2C9 has a low contribution to first-pass metabolism of substrate drugs, this enzyme shows large interindividual variability in its activity and content (Paine et al., 2006; Xie et al., 2016). Thus, CYP enzyme inhibitors may cause drug interactions via intestinal CYP2C9 in some individuals, especially in cases of low oral bioavailability substrate drugs (Paine et al., 2006; Xie et al., 2016). For example, coadministration of fluvastatin with CYP2C9 inhibitors (e.g., ranitidine, cimetidine, and omeprazole) increased the bioavailability of fluvastatin (Scripture and Pieper, 2001). Based on the basic likelihood DDI model of CYP inhibition, the predicted ratio (R) can be calculated from $1 + \left( \frac{I_{\text{max,u}}}{K_i} \right)$, where $I_{\text{max,u}}$ is the maximum unbound plasma concentration of rhinacanthin-C and $K_i$ is the in vitro unbound inhibition constant (CDER, 2017a). The $K_i$ value of 0.79 μM was estimated from the Cheng-Prusoff equation for competitive inhibition, $K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$ (Hutzler et al., 2011). Given that the in vivo CYP inhibition is likely to occur if $R \geq 1.02$ (CDER, 2017a), the maximum plasma concentration of rhinacanthin-C should be less than 0.016 μM to prevent drug interaction issues with coadministered CYP2C9 substrate drugs. However, there is currently no information on plasma concentration of rhinacanthin-C available. Thus, the in vivo herb-drug interaction from this compound at the liver could not be predicted. On the other hand, in our study the expected concentration of rhinacanthin-C in the intestine was as high as 26-fold of its

![Fig. 7. The NADPH-dependent inhibition of rhinacanthin-C (RN-C) against (A) CYP2C8, (B) CYP2C9, (C) CYP2C19, (D) CYP3A4/5 for midazolam, and (E) CYP3A4/5 for testosterone with NADPH preincubation (•, –––) or without NADPH preincubation (○, – – –) in HLMs. The enzyme activity is expressed as a percentage of remaining activity compared with the control containing no inhibitor. All data represent the mean ± S.E. of three independent experiments.](image-url)


IC50 value for CYP2C9, and the herb-drug interaction at the intestinal site could be anticipated.

Rhinacanthin-C may contribute to the therapeutic efficacy of R. nasutus (Sendel et al., 1996; Siripong et al., 2006a,b; Panichayupakaranant et al., 2009). However, a potential safety risk stemming from herb-drug interaction may also exist when this natural substance is coadministered with drug substrates of the CYP2C family, OATP1B1/ OATP1B3 influx transporters, and P-gp/BCRP efflux transporters. The risk of adverse events may increase when those drug substrates are in the “narrow therapeutic” drug group such as digoxin (P-gp substrate), warfarin, and phenytoin (CYP2C9 substrate) (van Booven et al., 2010; Misaka et al., 2013). In addition, several drugs can be substrates of both CYP450 enzymes and drug transporters. For example, repaglinide is a substrate of CYP2C8 and OATP1B1 (Bidstrup et al., 2003). Pitavastatin and rosuvastatin are known substrates of OATP, BCRP, and CYP2C9 (Causevic-Ramosevac and Semiz, 2013; Hu and Tomlinson, 2014). Clinical pharmacokinetic drug interaction arising from CYP enzymes and OATP inhibition was reported in a case of combination use of gemfibrozil (CYP2C8 and OATP1B1 inhibitor) and cerivastatin (CYP2C9 and OATP1B1 substrate) (Backman et al., 2002; Shitara et al., 2004). Gemfibrozil and its metabolites increased the plasma concentration of cerivastatin via inhibiting both CYP2C8-mediated cerivastatin metabolism and OATP1B1-mediated cerivastatin hepatic uptake, leading to a high risk of rhabdomyolysis (Backman et al., 2002; Shitara et al., 2004). Because rhinacanthin-C can inhibit multiple CYP isoforms, the potential for significant pharmacokinetic herb-drug interactions must be kept in mind when it is used in conjunction with other drugs that are substrates for these enzymes. Special attention should be directed toward those that are substrates of CYP2C8, CYP2C9, and CYP2C19.

In conclusion, this in vitro study revealed that rhinacanthin-C is capable of inhibiting multiple efflux and influx drug transporters (i.e., P-gp, BCRP, OATP1B1, and OATP1B3) and CYP isoforms (i.e., CYP2C8, CYP2C9, CYP2C19, and CYP3A4/5). The safety profile associated with herb-drug interaction issues from rhinacanthin-C should not be ignored. Further studies on in vivo pharmacokinetic drug interaction should be pursued to support safe use of herbal products containing rhinacanthin-C in combination with other prescription drugs.

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Authorship Contributions
Participated in research design: Dunkoksung, Vardhanabhuti, Jianmongkol.
Conducted experiments: Dunkoksung.
Contributed new reagents or analytic tools: Dunkoksung, Siripong, Jianmongkol.
Performed data analysis: Dunkoksung.

Wrote or contributed to the writing of the manuscript: Dunkoksung, Vardhanabhuti, Jianmongkol.

References


IC50 values and ratio for inhibition of CYP2C8, 2C9, 2C19, and 3A4/5 with and without NADPH by rhinacanthin-C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Without NADPH Preincubation</th>
<th>With NADPH Preincubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C8</td>
<td>Amodiaquine</td>
<td>6.55 ± 1.22</td>
<td>10.68 ± 0.74</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide</td>
<td>2.71 ± 0.38</td>
<td>3.99 ± 0.58</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-mephentoin</td>
<td>25.03 ± 5.04</td>
<td>24.44 ± 3.79</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>Midazolam</td>
<td>32.70 ± 4.30</td>
<td>34.50 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>50.60 ± 4.42</td>
<td>27.20 ± 3.26</td>
</tr>
</tbody>
</table>

*IC50 ratio was calculated by the IC50 value of rhinacanthin-C without NADPH preincubation divided by the IC50 value of rhinacanthin-C with NADPH preincubation.
Interaction of Rhinacanthin-C with Transporters and P450


