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Mosquitocidal Properties of Natural Product Compounds Isolated From Chinese Herbs and Synthetic Analogs of Curcumin

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Abstract

Because of resistance to current insecticides and to environmental, health, and regulatory concerns, naturally occurring compounds and their derivatives are of increasing interest for the development of new insecticidal compounds against vectors of disease-causing pathogens. Fifty-eight compounds, either extracted and purified from plants native to China or synthetic analogs of curcumin, were evaluated for both their larvicidal activity against *Aedes aegypti* (L.) and their ability to inhibit binding of cholesterol to *Ae. aegypti* sterol carrier protein-2 in vitro. Of the compounds tested, curcumin analogs seem especially promising in that of 24 compounds tested five were inhibitors of *Ae. aegypti* sterol carrier protein-2 with EC₅₀ values ranging from 0.65 to 62.87 μ M, and three curcumin analogs exhibited larvicidal activity against fourth instar *Ae. aegypti* larvae with LC₅₀ values ranging from 17.29 to 27.90 μ M. Adding to the attractiveness of synthetic curcumin analogs is the relative ease of synthesizing a large diversity of compounds; only a small fraction of such diversity has been sampled in this study.

Keywords

sterol carrier protein-2; mosquitocide; natural product; curcumin analog

The mosquito *Aedes aegypti* (L.) is a known vector in the transmission of yellow fever, dengue hemorrhagic fever, and the reemerging disease chikungunya (Simon et al. 2008). Over a third of the world population lives in areas at risk for epidemic transmission of these mosquito-transmitted viral diseases (CDC 2011). Controlling mosquito populations with insecticides has been effective in preventing transmission of disease (Gurtler et al. 2009) and as there is no dengue vaccine is the only effective means of combating this disease (Tan and Alonso 2009). However, the efficacy of insecticide-based control methodologies has been compromised by the emergence of insecticide resistance (Rivero et al. 2010), leading to the need to discover new insecticidal compounds.

All insects lack the enzymatic pathway to synthesize their own cholesterol (Zdobnov et al. 2002), requiring them to obtain this vital nutrient from dietary sources. Being a highly

hydrophobic molecule, absorption of cholesterol from the gut and subsequent aqueous transport requires a carrier protein. Sterol carrier protein two (SCP-2) is at least partially responsible for this role (Blitzer et al. 2005). As the function of SCP-2 is not vital for mammalian survival (Seedorf et al. 1998), SCP-2 is an especially attractive target for the development of biorational insecticides. Sterol carrier protein-2 inhibitors (SCPIs) have been identified through high-throughput screening (Kim et al. 2005) and have demonstrated larvicidal activity against four species of mosquitoes (Larson et al. 2008). Similarly, a botanical SCPI, α -mangostin, has also been found to possess larvicidal activity against different mosquito species (Larson et al. 2010).

Botanical insecticides such as pyrethrum and neem have garnered significant attention in part because of their reputed lower environmental and health risks compared with synthetic insecticides (Isman 2006). A further attraction is that many essential oils, especially those used for culinary purposes, and their constituents have fewer regulatory restrictions (USEPA 2010). Some research on botanical or natural product compounds has focused on the creation of synthetic analogs and investigation for biological activity. This is exemplified by curcumin, a yellow pigment present in the spice turmeric. Both curcumin and its analogues have demonstrated many different biological properties including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities (Anand et al. 2008).

In this study, 58 compounds, either extracted and purified from plants native to China or synthetic analogs of curcumin, were evaluated for both their larvicidal activity against *Ae. aegypti* and their ability to inhibit binding of cholesterol to *Ae. aegypti* SCP-2 (AeSCP-2) in vitro. New insights into the nature of the SCP-2:ligand interaction were gained and it is suggested that curcumin analogs in particular are a rich ground for development of SCPIs and larvicidal compounds.

Materials and Methods

Chemicals

Chemicals and reagents were purchased from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), and ICN (Costa Mesa, CA) if their origins are not mentioned in the text. Selected on the basis of Chinese ethnomedical records, plants were the source of 34 compounds that were isolated and purified as described (Song et al. 2006, Cao 2007, Cao et al. 2007, He 2007, He et al. 2007, Y. M. Li 2007, Liu 2007, Song 2007, Chen et al. 2008, Li 2008, Zhang 2008). Twenty-four compounds are analogs of curcumin synthetically prepared as described (S. B. Li 2007, Li et al. 2008, Song et al. 2009). A summary of the source of the tested compounds is listed in Table 1; for a complete list of compounds see Supp. Table 1 (online only). SCPI-1 (*N*-(4-{[4-(3,4-dichlorophenyl)-1,3-thiazol-2-yl]amino}phenyl)acetamide) was purchased from Chembridge (San Diego, CA) and NBD-cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesterol-3 β -ol) was purchased from Molecular Probes (Eugene, OR).

AeSCP-2 Binding Assays

Recombinant AeSCP-2 was purified as described (Dyer et al. 2003). Assays were conducted as previously described (Kim et al. 2005), except that all fluorescence measurements were performed using a Synergy HT Multi-Mode Microplate Reader (Biotek Instruments, Winooski, VT) using 485 nm (25 nm bandpass) excitation and 528 nm (20 nm bandpass) emission filters. For initial ligand competition studies, all 58 compounds were assayed at 100 μ M in a 100 μ l volume in a 96-well plate (Corning Costar 3356), with 5 μ M purified recombinant AeSCP-2 and 2 μ M NBD-cholesterol. Compounds were also assayed for their ability to quench NBD-cholesterol fluorescence by repeating the experiment without

AeSCP-2; any compounds that quenched residual NBD-cholesterol fluorescence were omitted from further consideration. Compounds were also tested for intrinsic fluorescence by omitting AeSCP-2 and NBD-cholesterol from the above binding assay.

Compounds that reduced NBD-cholesterol:AeSCP-2 fluorescence by at least 30% and neither quenched NBD-cholesterol fluorescence in the absence of AeSCP-2 nor were intrinsically fluorescent themselves were then subjected to ligand competition assays. In a 96-well plate, a 100 μ l volume containing 5 μ M AeSCP-2 and 1.25 μ M NBD-cholesterol was incubated with varying concentrations of the test compounds. A blank composed of the compound at each individual concentration, 1.25 μ M NBD-cholesterol, and 5 μ M purified recombinant bacterial glutathione-S-transferase (GST) was subtracted to produce the net change in fluorescence because of competition of the compound with the NBD-cholesterol:AeSCP-2 interaction. Data were collected in quadruplicate for each compound concentration tested. The EC₅₀ (concentration causing 50% reduction of NBD-cholesterol:AeSCP-2 binding) was calculated in GraphPad Prism version 4.0 (GraphPad Software Inc., La Jolla, CA) using a single-site competition, nonlinear regression model.

Insecticidal Assays in Mosquito Larvae

For initial screening of all 58 compounds, three *Ae. aegypti* (Rockefeller strain) second instars were placed into each well of a 96-well plate with 150 μ l double-distilled water and either 33 μ M of tested compound, 33 μ M SCPI-1 (positive control), or no compound (negative control). The numbers of dead larvae were counted every 24 h. Compounds that were active at this concentration (killing at least two out of three larvae) were further tested at four concentrations ranging from 4.2 to 33.3 μ M. Compounds showing a clear time and dose-response then had LC₅₀ values determined using fourth instars. Twenty early fourth instars were placed in 20 ml double-distilled water along with 25 mg Tetramin fish food and four different concentrations of test compound, in triplicate, and reared in an incubator maintained at 26°C, 70% RH, and a photo-period of 16H:8H (L:D). A negative control was set up identically save for absence of the test compound. Mortality was assessed after 72 h in all treatments. Abbott's correction (Abbott 1925) was applied to all treatments to adjust for natural mortality. Assays were repeated if untreated control mortality exceeded 20%. Bioassay data were analyzed with Log₁₀ probit analysis (Finney 1971) to obtain LC₅₀ values and 95% confidence intervals (PROC PROBIT; SAS Institute 2006). All models presented were tested for goodness-of-fit using Pearson χ^2 .

Results and Discussion

Biological Activity of Synthetic Analogs of Cur-cumin

Five out of 24 tested synthetic curcumin analogs were inhibitors of AeSCP-2 with 0.65–62.87 μ M EC₅₀ values (Table 2). Of these five compounds, all possessed a central carbonyl group within either a 2,6-dimethylene cyclohexanone or a 1,4-dien-3-one that links two aromatic rings. None of the nine tested compounds with a central oxime or oxime ether were found to inhibit AeSCP-2. Only one inhibitor contained a furan ring; all of the other compounds had benzene rings that usually bore additional hydroxy or methoxy functional groups. With the same central carbonyl group, EC₅₀ values of methoxybenzal compounds were lower than that of other derivatives. Trimethoxyphenyl compounds were more effective than hydroxyl-, chloro-, or trifluoromethyl substituted derivatives. The EC₅₀ values of trimethoxyphenyl compounds (0.65 μ M) were about one-third of that of hydroxybenzyl compounds (2.02–2.38 μ M). All five synthetic curcumin analog SCPIs were markedly hydrophobic and had limited ability to be hydrogen bond donors (Table 2). These characteristics are shared by most (Kim et al. 2005) but not all (Larson et al. 2010) previously described SCPIs. The natural product quercetin was also found to inhibit

AeSCP-2 (see below), but has four hydroxyl groups that can act as hydrogen donors (Table 2).

Three of the tested synthetic curcumin analogs showed larvicidal activities against fourth instar *Ae. aegypti*, 17.29–27.90 μM LC_{50} values (Table 3). Of the three, only one, (2E, 6E)-2,6-bis(furan-2-ylmethylene) cyclohexanone, was also an inhibitor of SCP-2 (Table 2). The EC_{50} value (62.87 μM) of this compound was the weakest of any compound that showed inhibition of AeSCP-2 in this study (Table 2). Given the low AeSCP-2 inhibitory activity the larvicidal activity of (2E,6E)-2,6-bis(furan-2-ylmethylene) cyclohexanone might not be a result of inhibition of AeSCP-2, but might be because of inhibition of other members of the SCP-2 family (Lan and Wessely 2004, Vyazunova et al. 2007), or through some other unknown mode of action. Similarly, the mechanism behind the other two larvicidal curcumin analogs is unknown. Why only one SCP-2 inhibitor was also larvicidal is unknown but similar to a previous study where only five out of 57 SCPIs were larvicidal (Kim et al. 2005).

That seven out of 24 tested curcumin analogs showed some biological activity is both encouraging and in keeping with other reports of bioactivity of curcumin and its analogs. Curcumin is the primary active ingredient of turmeric and comprises 2–5% of the spice which has long been used in traditional Indian and Chinese medicine for its anti-inflammatory properties (Shishodia et al. 2005). While turmeric has also been used to control various agricultural and animal pests this activity has been attributed to tumerone and *ar*-tumerone rather than curcumin (Jilani and Su 1983). Curcumin has demonstrated a diversity of biological activities and is being used to treat a variety of medical conditions such as cancer, Alzheimer's disease, psoriasis, diabetes, and other conditions (as reviewed in Shishodia et al. 2005 and Jurenka 2009). Recently curcumin has been found to inhibit growth of desert locust *Schistocera gregaria* Forskal and red cotton bug *Dysdercus koenigii* Walker nymphs (Chowdhury et al. 2000), and may play an important role to control insecticide-resistant pests by inhibiting P-glycoprotein ATPase as demonstrated in the bollworm *Helicoverpa armigera* (Hübner) (Aurade et al. 2010).

While curcumin has diverse pharmacological activities it also has several undesirable properties. Solubility is low as is bioavailability in mammals (reviewed by Jurenka 2009), and curcumin is unstable at neutral and basic pH (Wang et al. 1997). These issues have led to intense efforts to discover both natural and synthetic curcumin analogs (Anand et al. 2009). Dienone analogs are attractive not only in that a great diversity of compounds can be created from relatively simple reactants using Claisen-Schmidt reaction conditions, but they also remove the unstable diketone moiety of curcumin thereby increasing stability in neutral and basic conditions (Liang et al. 2009). This is of special interest in the development of mosquito larvicides in that any ingested compound faces a larval gut that ranges from pH 10 in the anterior midgut to pH seven in the hindgut (Dadd 1975).

Biological Activity of Natural Products Compounds

Of the 34 natural products compounds tested only quercetin, isolated from *Saxifraga stolonifera* (Liu 2007, Li 2008), demonstrated a dose-dependent inhibition of AeSCP-2 with an EC_{50} of 18.35 μM (Table 2). Quercetin is insecticidal to the pulse beetle *Callosobruchus chinensis* L. (Upasani et al. 2003) and has displayed antifeedant and insecticidal activity against the termite *Coptotermes formosanus* Shiraki (Adfa et al. 2010), as well as antifeedant activity against the South American ladybird beetle *Epilachna paenulata* Germar (Diaz Napal et al. 2009). The mechanisms of these activities are unknown. Although quercetin inhibited AeSCP-2s sterol-binding function and has demonstrated activities against other insects, it displayed no larvicidal activity against *Ae. aegypti* (Table S1, available online only).

Non inhibitory Natural Products and SCP-2 Protein: Ligand Interactions

Several natural product compounds investigated are interesting because they did not inhibit AeSCP-2. Stigmast-4-en-3-one was not found to inhibit AeSCP-2 at concentrations as high as 100 μ M despite having a structure quite similar to cholesterol. However, 7-dehydrocholesterol bears even greater similarity to cholesterol but also does not inhibit binding of NBD-cholesterol to AeSCP-2 (Radek et al. 2010).

More intriguing are methyl and ethyl palmitate, neither of which inhibited AeSCP-2 at up to 100 μ M concentration. Palmitic acid is known to bind SCP-2 in solution (Radek et al. 2010) and has been observed bound to SCP-2 both in solution (Singarapu et al. 2010) and crystal structures (Dyer et al. 2003). In the AeSCP-2:palmitic acid crystal structure, the carboxylic acid group of palmitic acid has extensive interactions with the protein, forming hydrogen bonds to the main chain amides of Gln 25 and Val 26, a salt bridge with Arg 24, and water-mediated hydrogen bonds to the main chain carbonyls of Asp 20 and Asn 23, and the side chain of Arg 15. Methyl and ethyl palmitate would not be able to form the salt bridge with Arg 24 because of loss of the negative charge by replacement of the carboxylic acid moiety with an ester. The extra bulk of the methyl or ethyl group may also complicate participation in the hydrogen bond network seen in the palmitic acid:AeSCP-2 structure. The inability of methyl and ethyl palmitate to inhibit the binding of NBD-cholesterol to AeSCP-2 suggests the importance of satisfying these polar interactions and that size and shape of the hydrophobic moiety of a fatty acid may be insufficient for tight binding to AeSCP-2.

The amino acids that interact with the carboxylic acid group of palmitic acid are part of the first α -helix and the loop connecting it to the first β -strand in AeSCP-2. In mammalian SCP-2 structures, however, this loop is instead an α -helix (Haapalainen et al. 2001, and references therein). Furthermore, the orientation of the lipid binding cavity in the mammalian apo and Triton X-100 bound forms is roughly orthogonal to the lipid binding cavity in AeSCP-2 and similar to the “horizontal” cavity found in the *Ae. aegypti* SCP-2-like2 structure (Dyer et al. 2008). It is unclear from our experiments whether or not methyl or ethyl palmitate might bind to those proteins.

Despite the small size of the set of 58 compounds that, to our knowledge none of which has demonstrated any prior SCPI or mosquitocidal behavior, we found three larvicidal compounds and six SCPIs against *Ae. aegypti*. Most of the biologically active compounds were synthetic analogs of curcumin. As these compounds are relatively easy to synthesize and have diverse chemical structure, synthetic curcumin analogs may be a rich source of future insecticidal compounds.

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Table 1

Sources of compounds tested

Plant species	Family	Collected part(s)	No. isolated compounds	Reference(s)
<i>Chimonanthus praecox</i> (L.)	Calycanthaceae	Roots, stems	4	Li 2008
<i>Jatropha curcas</i> (L.)	Euphorbiaceae	Stem bark, roots, leaves	17	Cao 2007, Song 2007, Zhang 2007, Song et al. 2006, Cao et al. 2007
<i>Marsdenia tenacissima</i> (Roxburgh) Wight et Arn.	Asclepiadaceae	Stems	3	Zhang 2007, Y. M. Li 2007
<i>Ribes henryi</i> Franch	Grossulariaceae	Roots	2	He 2007, He et al. 2007
<i>Saxifraga stolonifera</i> (L.) Meeb	Saxifragaceae	Whole plant	8	Liu 2007, Chen et al. 2008
Synthetic curcumin analogs	—	—	24	S. B. Li 2007, Li et al. 2008, Song et al. 2009

Table 2

Inhibitors of AeSCP-2

Structure	Name	EC ₅₀ (μM)	95% confidence interval (μM)
Quercetin	18.35	12.76–26.38
(2E,6E)-2-6-bis(3,4,5-trimethoxybenzylidene) cyclohexanone	12.11	9.67–15.16
(2E,6E)-2-6-bis(furan-2-ylmethylene) cyclohexanone	62.87	39.84–99.20
(1E,4E)-1,5-bis(4-hydroxyphenyl) penta-1,4-dien-3-one	2.38	1.80–3.16
(1E,4E)-1-(2-hydroxyphenyl)-5-phenylpenta-1,4-dien-3-one	2.02	1.38–2.94
1,5-Bis-(3,4,5-trimethoxy-phenyl)-penta-1,4-dien-3-one	0.65	0.47–0.92

Table 3

Larvicidal compounds against fourth instar *Ae. aegypti*

Structure	Name	N	Slope (SE)	LC ₅₀ (95% confidence interval, μ M)	χ^2 ^a
(1E,4E)-1,5-bis(2-fluorophenyl) penta-1,4-dien-3-one O-heptanoyl oxime	240	1.58 $\pm$ 0.56	27.90 (21.56–77.16)	9.64
(2E,6E)-2,6-bis(furan-2-ylmethylene) cyclohexanone	240	2.32 $\pm$ 0.74	20.07 (17.03–31.43)	2.60
(1E,4E)-1-(2-hydroxyphenyl)-5-(2-(trifluoromethyl)phenyl) penta-1,4-dien-3-one	240	4.13 $\pm$ 0.81	17.29 (13.91–19.44)	12.16

^aPearson χ^2 ; each compound had 10 degrees of freedom.