Identification of mosquito sterol carrier protein-2 inhibitors

Minsik Kim, Vilena Wessely, and Que Lan
Department of Entomology, University of Wisconsin–Madison, Madison, Wisconsin

Abstract A mosquito sterol carrier protein-2, AeSCP-2, has been shown to aid in the uptake of cholesterol in mosquito cells. The discovery of chemical inhibitors of AeSCP-2 is reported here. AeSCP-2 inhibitors (SCPIs) belong to several chemotypes of hydrophobic compounds. Those inhibitors competed with cholesterol for AeSCP-2, binding with relatively high binding affinities. In cultured insect cells, SCPIs reduced cholesterol uptake by as much as 30% at 1–5 μM concentrations. SCPIs were potent larvicides to the yellow fever mosquito, Aedes aegypti, and to the tobacco hornworm, Manduca sexta, with 50% lethal doses (LD₅₀) of 5–21 μM and 0.013–15 ng/mg diet, respectively. The results indicate that sterol carrier protein-2 has functional similarity in two different insect species.—Kim, M-s., V. Wessely, and Q. Lan. Identification of mosquito sterol carrier protein-2 inhibitors. J. Lipid Res. 2005. 46: 650–657.

Supplementary key words cholesterol • insecticide • high throughput screening

Sterol carrier protein-2 (SCP-2) belongs to a family of proteins containing a sterol binding domain (SCP-2 domain). The SCP-2 domain is found in the following vertebrate proteins: SCP-2, SCP-x, 17β-hydroxysteroid dehydrogenase type IV (HSD17B4), and stomatin (1, 2, 3). The vertebrate SCP-2, SCP-x, and HSD17B4 have a peroxisome localization sequence in the C terminus, targeting these proteins to the peroxisome (1, 2, 4). The mosquito SCP-2 (AeSCP-2) appears to represent a unique nonperoxisomal and low-molecular-weight protein in the SCP-2 gene family (5, 6).

The vertebrate SCP-2 is characterized as a nonspecific lipid carrier protein, which has affinity for different ligands in the order cholesterol >> straight-chain fatty acid > kinked-chain fatty acid (7). Like the vertebrate SCP-2, AeSCP-2 binds cholesterol (5) and fatty acid (8). Similarly, both the vertebrate and AeSCP-2 increase cholesterol uptake in SCP-2-overexpressing cells (5, 9). However, AeSCP-2 differs from the vertebrate SCP-2 in several aspects. In both cultured Aedes aegypti cells and in the larval midgut, AeSCP-2 localizes mostly in the cytosol, which is consistent with the fact that AeSCP-2 lacks the C-terminal peroxisome targeting sequence (6). The coordination site for a ligand in AeSCP-2 is different from the vertebrate SCP-2, in which the hydrophobic moieties of these ligands are oriented at opposite ends of the protein (8). AeSCP-2 seems to be a vital gene for the survival and development of mosquitoes, whereas the vertebrate SCP-2 is not critical for survival and fertility (10, 11). Knockdown of AeSCP-2 expression in larvae led to a high mortality rate in developing adults, and silencing of AeSCP-2 in adults lowered fertility (Q. Lan, unpublished observations). AeSCP-2 expression is high in the midgut during feeding stages in larvae (5), when cholesterol and sterols are absorbed (12). Targeting cholesterol metabolism for the development of new insect growth regulators for insect population control is one of the goals of this study. Inhibitors are useful tools for elucidating the mode of action and molecular mechanism of a functional protein. To search for inhibitors of AeSCP-2, we have developed a 384-well microplate format for screening small molecular chemical libraries using high-throughput technology. Several AeSCP-2 inhibitors (SCPIs) were identified. SCPIs belong to several chemotypes of hydrophobic compounds. Based on the inhibitory effect of SCPIs on AeSCP-2 cholesterol binding in vitro and on cholesterol uptake in cultured insect cells, it is assumed that SCPIs might reduce cholesterol absorption in vivo. SCPIs showed high larvicidal activities in the yellow fever mosquito, Aedes aegypti, and in the tobacco hornworm, Manduca sexta, even though SCPIs had very low cytotoxicity in cultured mouse cells. We report here the first successful attempt at targeting the cholesterol transport pathway in insects for the development of specific inhibitors.

MATERIALS AND METHODS

Chemicals

Chemicals and reagents were purchased from Sigma (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), and ICN (Costa Mesa, CA). Methanol and chloroform were used for HPLC analysis. [³H]cholesterol and [²H]fatty acids were obtained from New England Nuclear (Boston, MA). 

Abbreviations: GST, glutathione S-transferase; HDLp, high density lipophorin; RBA, relative binding affinity; SCP, sterol carrier protein; SCP-2, sterol carrier protein-2; SCP-x, sterol carrier protein-x; SCPI, sterol carrier protein-2 inhibitor.

To whom correspondence should be addressed.
e-mail: qlan@entomology.wisc.edu

[6] The online version of this article (available at http://www.jlr.org) contains an additional two figures.

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CA) if their origins are not mentioned in the text. NBD cholesterol was purchased from Molecular Probes (Eugene, OR). ChemBridge Corporation (San Diego, CA) provided the Chembridge DIVERSet small molecular chemical library, and individual compounds with at least 90% purity were purchased from ChemBridge Corporation.

**Mosquitoes**

The yellow fever mosquito, *Aedes aegypti*, is an inbred laboratory strain (Rockefeller). The mosquitoes were maintained as described (5).

**The tobacco hornworm, Manduca sexta**

*Manduca sexta* eggs were a gift from Dr. Walter G. Goodman, University of Wisconsin–Madison. Larvae were fed commercial gypsy moth wheat germ diet (ICN Biomedicals, Irvine, CA) and reared at 25°C and 60% relative humidity under a 16:8 light/dark cycle. Fresh food was provided every other day.

**Insect cell lines**

The *A. aegypti* cell line Aag-2 was maintained in Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) at 28°C under a 5% CO₂ atmosphere as described (13). Cells were passed every 7 days using a 1:4 dilution of cells.

The *M. sexta* cell line GV1 was maintained in modified Grace’s medium (Invitrogen) supplemented with 10% FBS at 28°C as described (14). Cells were passed every 7 days with a 1:4 dilution of cells.

**Chemical library screening**

NBD cholesterol (Molecular Probes, Eugene, OR) is a fluorescent cholesterol analog that is essentially nonfluorescent in water. However, upon binding to cholesterol or lipid binding protein, NBD cholesterol emits fluorescence at a distinct wavelength (excitation/emission = 470/530). AeSCP-2 was purified as described (8).

Stock solution of NBD cholesterol was made in 100% ethanol at 200 μM concentration, and the working solution of NBD cholesterol was made by diluting the stock solution in double-distilled water to desired concentrations. Compounds in the chemical library were dissolved in 100% DMSO at 0.1 mM concentration.

Laboratory robotics for high-throughput screening were performed using a Biomek FX (Beckman-Coulter, Inc., Fullerton, CA) liquid handler, a uFill reagent dispenser (Bio-Tek Instruments, Inc., Winooski, VT), and an EnVision plate reader (Perkin Elmer, Inc., Wellesley, MA). The positive control was AeSCP-2 with NBD cholesterol and 3.3% DMSO, and negative controls from the NDB cholesterol-AeSCP-2 complex in the absence or presence of an inhibitor. The data were plotted with the NBD cholesterol intensity (bound NBD cholesterol) as the y-axis and molarity of inhibitor as the x-axis. Bacterial GST protein was used as a negative control for the assays. It showed that the presence of a nonbinding protein did not affect the NBD cholesterol fluorescence (Fig. 1). The 50% inhibition concentration (IC₅₀) for NBD cholesterol binding to AeSCP-2 was calculated for each SCPI. A separate test was set up to use cholesterol competing with NBD cholesterol for AeSCP-2 binding, assessing the relative binding affinity (RBA) of cholesterol to AeSCP-2 under the same experimental conditions. The RBA of each SCPI to AeSCP-2 was estimated by dividing the IC₅₀ of cholesterol by the IC₅₀ of the individual SCPI and was expressed as a percent (RBA of cholesterol = 100%).

Parallel recombinant SCP-2 domain from the AeSCP-x (16) was also used for SCPI binding assays to verify whether SCPIs interact with other sterol carrier proteins (SCPs). The assays were conducted as described above.

**Cell-based biological assays**

AeSCP-2-overexpressing Aag-2 cells (6) were used to assess the biological activity of a potential SCPI. All experiments were performed in triplicate and repeated at least two times. Aag-2 cells were seeded at 2 ml of 5 × 10⁵ cells/ml in 35 × 10 mm culture dishes. Transfection of AeSCP-2 expression vector was conducted as described (6). AeSCP-2-overexpressing cells were dislodged, diluted 2-fold (5 × 10⁴ cells/ml), and dispersed at 0.5 ml/well into 24-well culture dishes. The cells were allowed to grow in 24-
well dishes for an additional 24 h, and the medium was changed to sterol-free medium (13). After overnight culture in sterol-free medium, the medium was replaced with 250 μl of fresh sterol-free medium containing 0.33 μCi [3H]cholesterol/ml (40 Ci/mM) and a potential SCPI at various concentrations (diluted from a 10 mM DMSO stock solution). Control cells were incubated in 250 μl of fresh sterol-free medium containing [3H]cholesterol and a correspondingly diluted DMSO solution. After 16 h labeling with [3H]cholesterol, cells were washed twice with 2 ml cold PBS and the total cellular lipids were extracted and determined as described (6). The relative [3H]cholesterol incorporation was calculated by normalizing compound-treated to [3H]cholesterol of DMSO-treated cells, which was set as 100%. The IC50 (the concentration of compound that inhibits [3H]cholesterol incorporation by 50%) was determined using standard methods.

Manduca sexta GV1 cells were seeded at 0.2 ml of 5 × 10⁵ cells/ml in each well of a 96-well culture plate and incubated overnight, and the medium was changed to sterol-free medium. After overnight culture in sterol-free medium, the medium was replaced with 50 μl of fresh sterol-free medium containing 0.33 μCi [3H]cholesterol/ml (40 Ci/mM) and a potential SCPI at various concentrations (diluted from a 10 mM DMSO stock solution). Control cells were incubated in 50 μl of fresh sterol-free medium containing [3H]cholesterol and a correspondingly diluted DMSO solution. After 24 h labeling with [3H]cholesterol, cells were washed and collected, and cellular [3H]cholesterol was extracted as described above for Aag-2 cells.

Cytotoxicity assays in mammalian cells
Normal mouse breast epithelial NMuMG cells were used to assess cellular toxicity of SCPI to vertebrate cells using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). NMuMG cells were maintained in DMEM medium from CellGro (Herndon, VA) supplemented with 10% FBS from ICN and 10 μg/ml insulin (Sigma). Cells were harvested by trypsinization using 0.25% trypsin and 0.1% EDTA (CellGro) and counted in a hemacytometer. Cells were plated at a density of 8,000 cells/well of each 96-well microtiter plate. Cells were grown for 4 h at 37°C with 5% CO₂ in a humidified incubator to allow cell attachment to occur before compound addition.

SCPIs were diluted in series with 100% DMSO to obtain 100× stock solutions of the dosages tested. Compound stocks were diluted 10-fold with DMEM medium with 10% FBS and penicillin-streptomycin to ensure full mixing of DMSO stocks with culture media using the FX liquid handler (Beckman). Ten microliters of each compound stock was added to duplicate wells in microtiter plates using the FX liquid handler. The final concentration of DMSO in all wells was 1%. Cells were incubated with the test compounds for 72 h before the assay was conducted. Test plates were removed from the incubator and allowed to equilibrate to room temperature for 30 min. Seventy microliters of culture medium was removed from each well, leaving 30 μl remaining in the well. An equal volume of 30 μl of room temperature CellTiter-Glo assay reagent was added to each well and incubated for 10 min at room temperature. The plates were read on the EnVision plate reader for luminescence counts.

For each compound tested, the percentage of inhibition of cell proliferation was calculated at each concentration of compound tested using the formula % inhibition = 100 - (100 × (relative light units or absorbency of sample)/(relative light units or absorbency of negative control)). The average percent inhibition at each concentration was calculated by taking the average of the percent inhibition calculated for each replicate. The IC50 (the concentration of compound that inhibits the growth of cells by 50%) was determined.

RESULTS
Identification of SCPIs
Cholesterol competes with NBC cholesterol for AeSCP-2 binding in a dose-dependent fashion (Fig. 1), which indicates that NBD cholesterol interacts with AeSCP-2 much like cholesterol itself. Using NBC cholesterol as a ligand and purified recombinant AeSCP-2, a 384-well microplate format was developed for screening chemical inhibitors using high-throughput technology. Of the 16,000 compounds of the Chembridge DIVERSet small molecular chemical library (ChemBridge Corporation, San Diego, CA), 396 interfered with NBC cholesterol-AeSCP-2 binding. To narrow down the number of candidate compounds, NBD cholesterol fluorescence intensity <20% of the positive control (NBD cholesterol-AeSCP-2) was used as the cutoff, and 57 potential SCPIs were identified through the high-throughput screening. Those SCPIs belong to several chemotypes, five of which are shown in Table 1. A common feature of SCPIs is that they are relatively hydrophobic and the molecular weight is close to that of cholesterol.

The binding affinity of each SCPI for AeSCP-2 was measured using NBD cholesterol competitive binding assays. The IC50 of each SCPI to block NBD cholesterol-AeSCP-2 binding was determined. The IC50 of tested SCPIs were between 0.042 and 0.35 μM, which is comparable to the IC50 of 0.11 μM for cholesterol (Table 1), suggesting that SCPIs were able to compete with NBD cholesterol for AeSCP-2 binding at a level comparable to cholesterol (Fig. 1). The RBA of each SCPI to AeSCP-2 was estimated by dividing the IC50 of cholesterol by the IC50 of individual SCPIs, which was expressed as a percent (RBA of cholesterol = 100%). Based on the RBA values, the estimated affinity of SCPIs to AeSCP-2 was in the order SCPI-5 > SCPI-2 > SCPI-4 > SCPI-3 > SCPI-1 (Table 1).
cholesterol uptake in AeSCP-2-overexpressing Aag-2 cells (Table 1); however, SCPI-5 was less effective in inhibiting cholesterol uptake much more effectively in GV1 cells than in Aag-2 cells (Fig. 2A, B; Table 2). The results indicate that SCPI-2, -4, and -5 had higher specificity in inhibiting cholesterol binding in AeSCP-2.

**Effects of SCPIs on cholesterol incorporation in insect cells**

Fifty-seven potential SCPIs were first tested for their inhibitory effect on cholesterol incorporation in AeSCP-2-overexpressing Aag-2 cells, and 12 of the SCPIs showed varied inhibitory effects on cholesterol uptake. Five SCPIs that had >20% inhibition of cholesterol incorporation in Aag-2 cells were chosen as biologically active SCPIs. SCPI-5 had the highest RBA to AeSCP-2 in the in vitro assays (Table 1); however, SCPI-5 was less effective in inhibiting cholesterol uptake in AeSCP-2-overexpressing Aag-2 cells (Fig. 2A and Table 2). Based on the IC₅₀ values, the effectiveness in inhibiting cholesterol uptake in Aag-2 cells was in the order SCPI-3 > SCPI-2 > SCPI-4 > SCPI-1 > SCPI-5. The inhibitory effect of SCPIs on cholesterol uptake was dose dependent (Fig. 2A). At 1 μM, SCPI-1, -2, and -3 inhibited >25% of cholesterol uptake, compared with controls (Fig. 2A), consistent with the level of decreased cholesterol uptake in AeSCP-2-silenced Aag-2 cells (6).

To test whether the effect of SCPIs on cellular cholesterol uptake was specific to mosquito cells or whether they had broader specificity to other insect species, the *M. sexta* GV1 cells were treated with various concentrations of individual SCPIs. *M. sexta* GV1 cells express low levels of SCP-2 (immuno-reactive to affinity-purified anti-AeSCP-2 antibody; data not shown). SCPIs showed similar inhibitory effects on cholesterol uptake in GV1 cells (Fig. 2B and Table 2), and the order of the effectiveness of SCPI in GV1 cells was SCPI-5 > SCPI-3 > SCPI-4 > SCPI-1 > SCPI-2. SCPI-5 inhibited cholesterol uptake more effectively in GV1 cells than in Aag-2 cells (Fig. 2A, B; Table 2).

**Cytotoxicity of SCPIs in cultured mouse cells**

The vertebrate SCP-2 has 46% identity and 69% similarity to the mosquito AeSCP-2 in the sterol binding domain (5). It is speculated that SCPIs may inhibit the function of the vertebrate SCPs, although the vertebrate SCP-2/RCP-x gene is not vital for the survival (10, 11). Because SCPIs selected for inhibiting AaSCP-2 function were lethal to cultured insect cells at 10 μM concentration (data not shown), cytotoxicity of SCPIs to mouse normal breast epithelium NmuMG cells was tested. NmuMG cells were treated with various concentrations of SCPIs for 72 h, and cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay. Only SCPI-1 and -2 showed moderate cytotoxicity in NmuMG cells, with IC₅₀ for cell proliferation at 57.3 μM and 55.3 μM, respectively (Table 2), implying that SCPIs for AeSCP-2 had little or no toxicity in normal mouse cells. The result is consistent with the observation that silencing of the vertebrate SCP-2/SCP-x does not affect the survival of mice (10, 11).

### Table 1. RBA of SCPIs to AeSCPs

| Chemotype | SCPI-1 | SCPI-2 | SCPI-3 | SCPI-4 | SCPI-5 | Cholesterol |
|-----------|--------|--------|--------|--------|--------|-------------|
| AeSCP-2   | IC₅₀ (μM) | 0.347 ± 0.026 | 0.059 ± 0.021 | 0.159 ± 0.173 | 0.065 ± 0.001 | 0.042 ± 0.008 | 0.113 ± 0.067 |
| RBA (%)   | 55     | 192    | 71     | 226    | 245    | 100         |
| Log RBA   | 1.51   | 2.28   | 1.85   | 2.24   | 2.43   | 2.00        |
| AeSCP-x   | IC₅₀ (μM) | 0.527 ± 0.210 | 0.268 ± 0.203 | 0.285 ± 0.202 | 0.329 ± 0.094 | 0.816 ± 1.046 | 2.470 ± 1.314 |
| RBA (%)   | 468.7  | 921.6  | 866.7  | 753.0  | 303.1  | 100         |
| Log RBA   | 2.67   | 2.96   | 2.94   | 2.88   | 2.48   | 2.00        |

IC₅₀, 50% inhibition concentration; RBA, relative binding affinity.

*IC₅₀* derived from assays described in Fig. 1 = mean ± SD (N = 8–12).

*RBA*: dividing the IC₅₀ of cholesterol by the IC₅₀ of each chemical, expressed as percent (cholesterol = 100%).
To assess the biological activity of SCPIs, A. aegypti larvae were treated with different SCPIs. Results from toxicity assays in A. aegypti larvae showed that SCPIs had high lethality in mosquitoes (Table 2). The LD50 of SCPIs were between 6 and 16 μM, <10 ppm concentrations. The lethality of SCPIs is consistent with the observation that knockdown of AeSCP-2 resulted in a high mortality rate (Q. Lan, unpublished observations). Interestingly, SCPI-5 was slightly less toxic to mosquito larvae, which is consistent with the lesser effectiveness of SCPI-5 in inhibiting cholesterol uptake in cultured mosquito cells (Fig. 2A and Table 2).

The toxic effects of SCPIs can be classified into two types, depending on the timing of the acute toxicity in mosquitoes. The “fast-killing” effect (SCPIs-1 and -3), represented by SCPI-1, and the “slow-killing” effect (SCPI-2, -4 and -5), typified by SCPI-2. The same cohort of newly hatched first-instar larvae were treated with SCPI-1 and -2 separately. SCPI-1 killed the larvae within 3 days of treatment, while most of the larvae were still in the second through third larval stages (Fig. 3B); whereas SCPI-2 caused most death at the larva-to-pupa transition period (Fig. 3A). The slow-killing SCPIs had higher RBA to AeSCP-2 than did the fast killing SCPIs (Table 1). The results indicate that these two types of SCPIs may have slightly different modes of action in A. aegypti. However, there was no significant difference between SCPIs-1 and -2 in the speed of toxic action in M. sexta larvae (data not shown). Comparison of the speed of SCPI toxic activity in A. aegypti and M. sexta suggests that the activity of each SCPI may vary in different insect species.

**DISCUSSION**

**SCPIs and cholesterol uptake**

Insects do not synthesize cholesterol de novo, because they lack key enzymes in the cholesterol biosynthesis pathway (17). The critical difference between vertebrates and insects is that the vertebrates internalize cholesterol-loaded low density lipoprotein (LDL) via endocytosis, whereas insects do not internalize high density lipoprotein (HDLp), which transports lipids to insect cells (18, 19). The exception is in the ovary, where a fraction of HDLp is internalized (20, 21). On the other hand, the insect midgut does not synthesize lipophorin to carry dietary sterol into the hemolymph; instead, lipids bind to the HDLp at the interface of the hemolymph and the cellular membrane (19, 22). Influx/efflux cholesterol in insect cells has to be shuttled intracellularly by a sterol carrier to and from subcellular locations. Insects appear to have multiple genes encoding the small intracellular SCPs, including AeSCP-2 (16), in contrast to vertebrates, which have only a single-copy SCP gene, the vertebrate SCP-2 (1, 23).

**Table 2. Biological activities of SCPIs**

|                  | SCPI-1 | SCPI-2 | SCPI-3 | SCPI-4 | SCPI-5 |
|------------------|--------|--------|--------|--------|--------|
| Aedes aegypti LD<sub>50</sub> (μM)<sup>a</sup> | 4.8 ± 1.3 | 10.6 ± 5.8 | 12.3 ± 9.8 | 21.8 ± 28.9 | 19.4 ± 15.6 |
| IC<sub>50</sub> (μM) for cholesterol uptake in Aag-2 cells<sup>b</sup> | 5.53 ± 5.85 | 3.97 ± 3.22 | 3.09 ± 2.06 | 4.36 ± 0.91 | 6.92 ± 3.18 |
| Manduca sexta LD<sub>50</sub> (ng/mg diet)<sup>c</sup> | 0.22 | 0.013 ± 0.001 | 15 | ND | 15 |
| IC<sub>50</sub> (μM) for cholesterol uptake in GV1 cells | 3.3 | 3.4 | 2.3 | 2.7 | 2.0 |
| IC<sub>50</sub> (μM) for cell viability in NmuMG cells<sup>d</sup> | 57.3 ± 5.2 | 55.3 ± 22 | 214 ± 2.4 | 256.9 ± 129.5 | 452.5 ± 190.2 |

LD<sub>50</sub>, 50% lethal dose.

<sup>a</sup>LD<sub>50</sub> = mean ± SD (N = 4–6).

<sup>b</sup>IC<sub>50</sub> = mean ± SD (N = 2).

<sup>c</sup>IC<sub>50</sub> = mean ± SD (N = 2).

<sup>d</sup>IC<sub>50</sub> = mean ± SD (N = 2).
The relatively high affinity of SCPIs to AeSCP-2 measured in vitro indicates that SCPIs may compete with cholesterol or sterol for AeSCP-2 binding and may inhibit AeSCP-2 function in vivo. Moreover, SCPIs reduced cholesterol uptake in AeSCP-2-overexpressing Aag-2 cells by as much as 30%, similar to the effect of AeSCP-2 RNA interference (6). These results suggest that SCPIs inhibit AeSCP-2-mediated cholesterol transport in mosquito cells, consistent with the observed correlation between increased AeSCP-2 expression and elevated levels of cholesterol uptake (6). It should be noted that the IC50 for cholesterol binding in in vitro assays (Table 1) cannot be directly compared with the IC50 for cholesterol uptake in cultured cells (Table 2). This is because each SCPI might have different efficiency in entering the cells and might be broken down in the cell at a different rate. Thus, the inhibitory effect of each SCPI in vivo (i.e., in cultured cells) may not be equal to its efficiency in interfering with cholesterol binding to SCP-2 in vitro.

AeSCP-2 is not solely responsible for cholesterol uptake in Aag-2 cells, because the cell continues to incorporate cholesterol at a slightly lower rate without 95% of its AeSCP-2 (6). Silencing of AeSCP-2 in Aag-2 cells does not decrease cell growth, as measured by total cellular protein concentration (6). However, SCPI treatments at concentrations >10 μM significantly decreased cell growth (S2). Although SCPIs and AeSCP-2 silencing have similar effects on cellular cholesterol incorporation, the biological activities of SCPIs should be interpreted with much caution, as discussed in the following section.

**SCPI interaction with other member of the SCP-2 gene family**

The SCP-2 domains in the SCP-2 gene family have high sequence and structural similarity, underscoring the constraining of ligand binding of this functional domain (8, 24, 25). Because of the high similarity in the SCP-2 domains of AeSCP-2 and AeSCP-x (16), it was speculated that SCPIs might bind to AeSCP-x. Results of in vitro competitive binding assays revealed that SCPIs (except for SCPI-1) bind to AeSCP-x at a much lower affinity, based on the IC50 values (Table 1). AeSCP-2 and AeSCP-x may have little overlapping function, because of the segregated cellular localization of those two proteins. AeSCP-2 is mostly cytosolic, whereas AeSCP-x associates heavily with peroxisomes (6). AeSCP-x may have little to do with cholesterol metabolism in insects, because insects do not process cholesterol via bile acid formation (22), which requires the cleavage of 24-keto-THCA-CoA into choloyl-CoA by SCP-x in the peroxisome (10). AeSCP-x may be involved in fatty acid metabolism, similar to the vertebrate SCP-x (1). Its cytosolic localization makes AeSCP-2 a likely intracellular sterol carrier, aiding in the absorption and redistribution of cholesterol within the cell. At low concentrations, the inhibitory effects of SCPIs on cellular cholesterol uptake may be due to the dysfunction of AeSCP-2 alone. However, at higher concentrations, SCPIs may result in a loss of function in both AeSCP-2 and AeSCP-x.

**Toxicity of SCPIs to insect larvae**

Affinity-purified anti-AeSCP-2 antibodies did detect a 13 kDa protein in the protein extracts from GV1 cells and the midgut of *M. sexta* (data not shown), indicating that a protein structurally similar to AeSCP-2 may exist in *M. sexta*. SCPIs suppress cholesterol uptake in both *A. aegypti* and *M. sexta* cells (Table 2). The LD50 of SCPIs are in the μM and ng/mg diet ranges in *A. aegypti* and *M. sexta*, respectively, indicating that SCPIs have potent insecticidal activity (Table 2). The slow-killing SCPIs had much higher RBA to AeSCP-2 than did the fast-killing SCPIs (Table 1 and Fig. 3), indicating that the speed of larvicidal action of the SCPIs in mosquitoes was not correlated with the affinity of each SCPI to AeSCP-2. However, there was no significant difference between SCPI-1 and -2 in the speed of toxic action in *M. sexta* larvae (data not shown). It is possible that the absorption rate or metabolic rate of each SCPI in mosquito larvae is different, which may influence the required length of time for the acute toxic action of the SCPI. On the other hand, it also possible that SCPI-1...
and -2 have different interactive spectrums with other SCP-2-like proteins in the SCP-2 gene family (6), which may render varied physiological effects on treated larvae.

Toxicity of SCPIs to mammalian cells

SCPI-3, -4, and -5 are essentially nontoxic to normal mouse cells, based on cell viability assays after 72 h of treatment (Table 2), indicating that those SCPIs are specific to insects. However, SCPI-1 and -2 had moderate cytotoxicities to normal mouse cells. The dosages of SCPI-1 and -2 required for inhibiting cell proliferation in mouse cells are much higher (Table 2) than those needed to cause cellular stress in insect cells (data not shown). The cytotoxicity of SCPI-1 and -2 in mammalian cells could be the result of SCPI-1 and -2 bound to the vertebrate SCP-2 or SCP-x and interference with the vertebrate SCP-2/x function, which may lead to the inhibition of cellular growth. There was no anti-AeSCP-2 immunoreactive protein in NmuMG cellular protein extract (data not shown). On the other hand, there was a 60 kDa anti-AeSCP-x immunoreactive protein in NmuMG cells (S3), indicating that NmuMG cells might express the mouse SCP-x. Or the cytotoxicity could result from the inhibitory effect of SCPI-1 and -2 on normal mammalian cell proliferation via interaction with other members of the vertebrate SCP-2 gene family. However, the inhibition of the vertebrate SCP-2/SCP-x may not be the cause of SCPI-1 and -2-induced cytotoxicity in mouse cells, as in a human SCP-2 and SCP-x do not appear to be essential for survival (10, 11). It is possible, because of the high degree of similarity in the sterol binding domain, that SCPI-1 and -2 may bind to other members of the vertebrate SCP-2 gene family. The vertebrate SCP-2 gene family includes SCP-2, SCP-x, 17β-hydroxysteroid dehydrogenase type IV, and stomatin (23, 26), which has a conserved sterol binding domain (SCP-2-like proteins in the SCP-2 gene family (6), which and -2 have different interactive spectrums with other SCP-2-like proteins in the SCP-2 gene family (6), which may render varied physiological effects on treated larvae.

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