Lepidopteran HMG-CoA reductase is a potential selective target for pest control

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As a consequence of the negative impacts on the environment of some insecticides, discovery of eco-friendly insecticides and target has received global attention in recent years. Sequence alignment and structural comparison of the rate-limiting enzyme HMG-CoA reductase (HMGR) revealed differences between lepidopteran pests and other organisms, which suggested insect HMGR could be a selective insecticide target candidate. Inhibition of JH biosynthesis *in vitro* confirmed that HMGR inhibitors showed a potent lethal effect on the lepidopteran pest *Manduca sexta*, whereas there was little effect on JH biosynthesis in *Apis mellifera* and *Diploptera punctata*. The pest control application of these inhibitors demonstrated that they can be insecticide candidates with potent ovicidal activity, larvicidal activity and insect growth regulatory effects. The present study has validated that Lepidopteran HMGR can be a potent selective insecticide target, and the HMGR inhibitors (especially type II statins) could be selective insecticide candidates and lead compounds. Furthermore, we demonstrated that sequence alignment, homology modeling and structural comparison may be useful for determining potential enzymes or receptors which can be eco-friendly pesticide targets.

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Abstract

22   As a consequence of the negative impacts on the environment of some insecticides,

23   discovery of eco-friendly insecticides and target has received global attention in recent years.

24   Sequence alignment and structural comparison of the rate-limiting enzyme HMG-CoA

25   reductase (HMGR) revealed differences between lepidopteran pests and other organisms,

26   which suggested insect HMGR could be a selective insecticide target candidate. Inhibition of

27   JH biosynthesis *in vitro* confirmed that HMGR inhibitors showed a potent lethal effect on the

28   lepidopteran pest *Manduca sexta*, whereas there was little effect on JH biosynthesis in *Apis*

29   *mellifera* and *Diploptera punctata*. The pest control application of these inhibitors

30   demonstrated that they can be insecticide candidates with potent ovicidal activity, larvicidal

31   activity and insect growth regulatory effects. The present study has validated that

32   Lepidopteran HMGR can be a potent selective insecticide target, and the HMGR inhibitors

33   (especially type II statins) could be selective insecticide candidates and lead compounds.

34   Furthermore, we demonstrated that sequence alignment, homology modeling and structural

35   comparison may be useful for determining potential enzymes or receptors which can be eco-

36   friendly pesticide targets.

1. Introduction

The traditional insecticides have made a major contribution to agriculture and health. But as a result of improper use and the inherent shortcomings of some insecticides, many showed negative impacts on the ecological environment. Therefore, eco-friendly insecticides have received global attention in recent years. But to predict and avoid potential ecological risk in the initial phase of insecticide discovery is a problem that has not been fully resolved to date.

The insect juvenile hormones (JHs) are methyl esters of farnesoic acid 10,11-epoxide (JH III) and related compounds, which function in the regulation of a number of insect physiological processes in insects including embryogenesis, larval and adult development, metamorphosis, reproduction, diapause, migration, polymorphism, and metabolism (*Nijhout, 1994*). JH biosynthesis proceeds in the corpora allata (CA) through the mevalonic acid (MVA) pathway, which insects share with most other organisms. By analogy with vertebrates, HMG-CoA reductase (HMGR) has been postulated to be a key enzyme in the regulation of the MVA pathway in insects (*Feyereisen, Pratt & Hamnett, 1981*). JH biosynthesis in insect CA is inhibited *in vitro* by compactin (*Monger et al., 1982*); mevinolin (*Feyereisen & Farnsworth, 1987*; *Couillaud, 1991*); or fluvastatin (*Debernard, Rossignol & Couillaud, 1994*). However, compactin shows poor inhibition of JH biosynthesis *in vivo*. Only repeated injections into *Manduca sexta* larvae induced the black pigmentation characteristic of JH deficiency; the black pigmentation is always followed by death within approximately 24 hours. In addition, compactin treatment by topical application has no effect on *M. sexta* larvae (*Monger et al.,*

59   *1982*). Fluvastatin injected into locusts inhibited JH biosynthesis *in vivo*, but by 12 hours, JH

60   biosynthesis had almost fully recovered, with no discernible effects on either JH-regulated

61   metamorphosis or oocyte maturation (*Debernard, Rossignol & Couillaud, 1994*). However,

62   the use of HMGR inhibitors for pest control has not been fully explored. In addition, because

63   HMGR is an enzyme which exists in most organisms, its status as an eco-friendly insecticide

64   target remains unclear.

65   In the present study, we predict the possibility of HMGR as an eco-friendly insecticide

66   target by using sequence alignment, homology modeling, and structural comparison. The

67   effects of three commercial HMGR inhibitors on JH biosynthesis was assayed by using *M.*

68   *sexta*, *Apis mellifera*, and *Diploptera punctata* as experimental animals *in vitro* to validate our

69   predictions. Finally, the possible applicability of these compounds for pest control was

70   demonstrated in this paper.

71   **2. Materials and methods**

72   **2.1. Insects**

73   Larvae of the tobacco hornworm, *M. sexta*, were raised from eggs provided by Carolina

74   Biological Supply Company (Burlington, NC, USA) and reared on an artificial diet (Bio-Serv,

75   NJ, USA) at 25°C under a long-day (16 h light/8 h dark) photoperiod (*Bell & Joachim, 1976*).

76   Pharate 5th instar larvae were set aside 4-7 h before lights off. The larvae molted within a few

77   hours and were designated day 0. At the start of wandering, indicated by the appearance of a

78   prominent dorsal vessel, the larvae were transferred to plastic vials containing vermiculite until

79 pupation. Freshly ecdysed pupae were transferred to a chamber containing a tobacco plant and

80 10% sucrose under a long-day photoperiod into which the adult moths emerged (*Lee,*

81 *Chamberlin & Horodyski, 2002*).

82   Newly emerged mated female *D. punctata* (day 0) were isolated from stock cultures.

83 Mating was confirmed by the presence of a spermatophore. Stocks and isolated females were fed

84 Lab Chow and water ad libitum, and were kept at 27±1°C and 50±5% relative humidity with a 12

85 h light/12 h dark cycle (*Kai et al., 2009*).

86   Worker larvae of *A. mellifera* were collected from apiaries in Shanghai, China, and placed

87 in an incubator at 34°C and 80% relative humidity, fed a diet that was prepared with 40% pollen

88 collected from combs and 60% honey. Fourth instar worker larvae were distinguished by the

89 differences in maximum width of their head capsules (*Rachinsky, Tobe & Feldlaufer, 2000*).

90 **2.2. Chemicals**

91      $L[^{14}C\text{-}S\text{-methyl}]$ methionine was purchased from Amersham Biosciences (Piscataway, NJ,

92 USA). HMGR inhibitors fluvastatin, pitavastatin and lovastatin and high-performance liquid

93 chromatography (HPLC)-grade isooctane was purchased from Sigma-Aldrich (St. Louis, MO,

94 USA).

95 **2.3. Bioassays**

96 ***2.3.1. Assays for JH biosynthesis assays* in vitro**

97   Rates of JH biosynthesis were determined *in vitro* by using the modified radiochemical

98   assay (*Tobe & Clarke, 1985*; *Tobe & Pratt, 1974*). The radiochemical assays for JH biosynthesis

99   were performed with CA from unfed day 1 fifth instars of *M. sexta*, day 7 adult female *D.*

100   *punctata* and fourth instar workers of *A. mellifera*, respectively. HMGR inhibitors were

101   dissolved in medium 199 (GIBCO) for assay as described previously (*Lee, Chamberlin &*

102   *Horodyski, 2002*; *Kai et al., 2009*) and used on the same day that the inhibitors were prepared.

103   Each pair of CA was incubated for 3 h at 30 °C in 100 μL of medium 199 with Hanks' salts, *L*-

104   glutamine, 25 mM HEPES buffer (pH 7.2), 1.3 mM Ca^{2+} and 2% Ficoll, containing $L[^{14}C\text{-}S\text{-}$

105   methyl] methionine (40 μM, specific radioactivity 1.48-2.03 GBq/mmol) in the dark with gentle

106   shaking. After incubation, both medium and CA were extracted with isooctane. The isooctane

107   phase was removed and its radioactivity determined by liquid scintillation spectrometry.

108   Inhibition of JH biosynthesis was calculated as percent activity compared with the control group

109   (i.e. no HMGR inhibitor added). The IC_{50} values for the test compounds were calculated by

110   using GraphPad Prism version 5.0.

111   **2.3.2. Assays for JH biosynthesis in vivo**

112   *2.3.2.1. Injection*

113   Injections of HMGR inhibitors (2 μL volume, and 1 μM concentration) in newly molted

114   fifth instar *M. sexta* (day 0) were carried out using a 10 μL Hamilton-syringe. The final

115   concentrations of the injected inhibitor in the hemolymph were approximately 4 nM. Control

116   larvae were similarly injected, but with 2 μL of double distilled water. Larvae were first

117   anesthetized by cooling on ice and then injected between the seventh and eighth spiracles near

118   the horn, close to the posterior heart chamber. These animals were assayed for JH biosynthesis at

119   day 1 using the method described in Section 2.3.1. Each group of inhibitor-injected animals was

120   compared with a group of water-injected animals treated concurrently.

121   *2.3.2.2. Topical Application*

122   Solutions of HMGR inhibitors (5 µL) were applied to the dorsal abdomen of *M. sexta* fifth

123   instars at day 0, and animals were assayed for JH biosynthesis at day 3 as described (see Section

124   2.3.1). The concentration of the inhibitors (in 20% DMSO and 80% acetone) used in the

125   bioassays was 100 µM. Each larva received 0.5 nmol inhibitor in the topical cuticular assays.

126   Controls were treated with the solvent.

127   *2.3.2.3. Oral administration*

128   Newly molted fifth instars of *M. sexta* were immediately fed with 5 µL inhibitor solution (1

129   µM concentration); subsequently, these animals were fed on the normal diet. Control larvae were

130   similarly fed, but with 5 µL of double distilled water. JH biosynthesis in these treated animals

131   was assayed one day 1ater by using the radiochemical assay.

132   **2.3.3. Assays for ovicidal activity on M. sexta**

133   *M. sexta* eggs that had been deposited on a paper filter were briefly immersed in solutions

134   of the HMGR inhibitor (H_2O containing 0.2% DMSO as co-solvent, concentrations ranged from

135   1 µM to 1000µM). After the test solution had dried, eggs were maintained in Petri dishes. Five

136   days later, the mortality (numbers of eggs that failed to hatch) was determined, relative to

137 untreated controls. (No eggs hatched after five days in either the treatment or control groups.

138 *2.3.4. Assays for impact of feeding on* **M. sexta** *larval growth and mortality*

139   Three groups of larvae were used for feeding assays. Newly hatched or newly molted *M.*

140 *sexta* larvae were fed with HMGR inhibitor solution (2 μL for first and second instars, 3 μL for

141 third instars, and 5 μL for fourth and fifth instars) at the beginning of the stadium, and then

142 reared on the normal artificial diet until the next ecdysis. Larval mortality and insect growth were

143 recorded after treatment.

144 *2.3.5. Statistics*

145   Data presented as percentages were log-transformed before statistical analyses. Data were

146 analysed by using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison

147 test as the post hoc determination of significance by using GraphPad Prism version 5.0. Dose-

148 response curves were prepared with GraphPad Prism. Values are expressed as mean ± standard

149 errors (S.E.M.) with *N* indicating the number of samples measured (*N* is 8-20).

150 **2.4. Sequence Alignment of HMGR**

151   A sequence database of all known HMGR was collected from the literature and GenBank by

152 using a combination of BLAST and keyword searches. Amino acid multiple sequence

153 alignments for HMGR were constructed with ClustalW (*Thompson, Higgins & Gibson, 1994*)

154 and adjusted by eye to ensure structural motifs were maintained. Poorly aligned regions and

155 major gaps were deleted.

156 **2.5. Homology modeling**

157   Because there was no crystal structure of insect HMGR, the homology models of HMGR of

158 *M. sexta*, *A. mellifera*, and *D. punctata* were prepared respectively, to explore the three-

159 dimensional structural differences of the HMGR from different organisms, especially the

160 differences at their active site. A crystal structure of human HMGR in complex with Fluvastatin

161 (PDB ID: 1HWI) was used as the 3D coordinate template for the homology modeling (*Istvan &*

162 *Deisenhofer, 2001*). The homology models for HMGRs were generated by using the FUGUE

163 and ORCHESTRAR modules in Sybyl. The initial model was optimized energetically by using

164 the minimize program with steepest descent algorithm, AMBER7 FF99 as the force field and

165 Gasteiger-Huckel as the atomic point charges. The minimization was terminated when the RMS

166 gradient convergence criterion of 0.05 kcal/(mol·Å) was reached. The qualities of these models

167 were analyzed by PROCHECK (*Laskowski et al., 1993*).

168 **2.6. Docking calculations**

169   A ligand lovastatin used for the docking studies with HMGRs from different organisms was

170 constructed by using the 2D sketcher module in Sybyl. Minimum energy conformations of all

171 structures were calculated with the Minimize module of Sybyl. The force field was MMFF94

172 with an 8 Å cutoff for nonbonded interactions, and the atomic point charges were also calculated

173 with MMFF94 (*Halgren, 1999*). Minimizations were achieved with the steepest descent method

174 for the first 100 steps, followed by the Broyden–Fletcher–Goldfarb–Shanno (BFGS) method

175 until the Root-Mean-Square (RMS) of the gradient became less than 0.005 kcal/(mol·Å) (*Head*

176 *& Zerner, 1985*; *Kai et al., 2006*).

177   The Surflex-Dock (*Spitzer & Jain, 2012*) module implemented in the Sybyl program was

178 used for the docking studies. The 3-D structures of *M. sexta*, *A. mellifera* and *D. punctata* were

179 performed with homology modeling. Each inhibitor was docked into the binding site of the

180 corresponding protein by an empirical scoring function and a patented search engine in Surflex-

181 Dock applied with the automatic docking. Other parameters were established by default in the

182 software.

183 **2.7. Molecular dynamics simulations**

184   Docking calculations as described above were performed for each inhibitor in complex

185 with HMGR in which energy was minimized and used the minimized program in Sybyl-X 2.0

186 with the optimization algorithm BFGS (*Head & Zerner, 1985*). The force field was AMBER7

187 FF99 and the atomic point charge was Gasteiger-Huckel for 500 steps to remove bad contacts

188 (*Kai et al., 2006*). The system was equilibrated at 400K for 0.1 ns followed by data collection,

189 at regular intervals, for 10 ns. Each structure collected was subjected to 0.1 ns of simulated

190 annealing to 300K. The final 100 structures were energy-minimized and clustered using cut-

191 off distance of <0.2 nm. AMBER7 FF99 was used for force field and Gasteiger-Huckel for

192 charges in molecular dynamics simulation using the dynamics program of Sybyl.

193 **3. Results**

194 **3.1. Sequence analysis**

195   As the aim of our study was to find an eco-friendly insecticide target, HMGR sequences

196   from different species were aligned relative to that of *M. sexta*, and the identity values were

197   recorded (Figure 1). The HMGR sequences of Blattaria, Isoptera, Coleoptera, Hymenoptera,

198   Homoptera and Diptera were significantly different from Lepidoptera, (Dunnett's multiple

199   comparison test of identity values) (Figure 1A). The identity values of more distantly related

200   organisms, i.e.,   Malacostraca, Actinopterygii, Amphibia, Aves, Mammalia, Monocotyledoneae,

201   and Dicotyledoneae were below 60% (Figure 1B), in comparison with the identity value of the

202   Lepidoptera (approximately 90%). This suggests that the HMGR sequences of Lepidopteran

203   insects are significantly different from other organisms.

204   **3.2. Homology modeling, docking and Molecular dynamics optimization**

205   With the goal of discovery of an eco-friendly insecticide target, the three-dimensional

206   structures (especially the active site structures) of different species were analyzed. The HMGR of

207   *M. sexta*, *A. mellifera* and *D. punctata* were selected for the structural comparison. The

208   homology models of *M. sexta*, *A. mellifera* and *D. punctata* were generated using the crystal

209   structure of *H. sapiens* (PDB ID: 1HWI) as the template. To select the best model, we checked

210   the structural validity by PROCHECK (http://services.mbi.ucla.edu/SAVES). The geometry of

211   the final refined models were evaluated with Ramachandran plot calculations computed using the

212   PROCHECK program. The torsion angles of φ and ψ (the two torsion angles of the

213   polypeptide chain, also called Ramachandran angles, describe the rotations of the polypeptide

214   backbone around the bonds between N-Cα called φ and Cα-C called ψ.) in the generated model

215   was represented in the Ramachandran plot as shown in Figure S1. The Ramachandran plot

216   showed 89.7% of the residues of *M. sexta*, 88.2% residues of *A. mellifera* and 90.5% residues of

217   *D. punctata* existed in the most favored regions. The percentages of residues in disallowed

218   regions of *M. sexta*, *A. mellifera* and *D. punctata* are 0.0%, 0.1% and 0.3%, respectively. This

219   indicated that the backbone dihedral angles, phi and psi, of the three homology models were

220   reasonably accurate.

221   One HMGR structure was assembled with the same four subunits. Each two adjacent

222   subunits constituted a ligand binding pocket, which means one HMGR contained the same four

223   binding pockets. Lovastatin, a commercial HMGR inhibitor, was used to identify the binding

224   pocket of the aforementioned HMGR structures with docking calculations. Molecular dynamics

225   (MD) simulation of the three homology models complexed with lovastatin as the ligand were

226   performed for 10 ns to obtain the stable and low energy conformations. By reporting the root

227   mean square deviation (RMSD) of the protein structure from the starting model, the receptor

228   changes in structure and reaches a relatively stable conformational minimum after approximately

229   3 ns. The conformations with the lowest energy of the final 100 structures from the MD

230   simulation were selected as the final structures.

231   **3.3. Structure comparison**

232   Both subunits that constituted the binding pocket of *A. mellifera* and *D. punctata* formed

233   hydrogen bonds with lovastatin, whereas only one subunit of *M. sexta* can form hydrogen bonds

234   with the ligand (Arg 579 and Lys 680 of chain A). This suggested that the binding pocket of *M.*

235 *sexta* is more flexible.

236   The surface properties of the binding pocket of the above three structures were defined

237 using MOLCAD calculations (an interactive visualization of molecular scenarios) in Sybyl to

238 analyse these binding pockets. Figure 2 sketches the molecular surfaces of the pockets of these

239 four HMGRs. The cavity of *M. sexta* was smooth and did not penetrate deep into the structure

240 compared with the cavities of *A. mellifera* and *D. punctata* (Figure 2). This result was in

241 accordance with the results of hydrogen bond interaction. The electrostatic potential of the

242 binding pockets of *M. sexta* was positively-charged, because the whole surface of the pocket was

243 colored in yellowish green, whereas that of *A. mellifera* was electroneutral (Figure 2). The front

244 of the binding pocket of *D. punctata* was colored with blue, which suggests that some part of this

245 pocket is electronegative. The lipophilic potential of the binding pocket of *M. sexta* was

246 lipophilic, colored with brown, whereas that pocket of *D. punctata* was more hydrophilic,

247 colored with blue. The green color suggested that the pocket of *A. mellifera* is neutral (Figure 2).

248 These results show that the active pockets of the three species are different, suggesting that it is

249 possible to design eco-friendly insecticides using differences in surface properties. Thus, the

250 insect HMGR may represent a potential eco-friendly insecticide target.

251   The surface properties of these three pockets suggests that increasing the molecular volume,

252 electronegativity and lipophilicity of the ligand can strengthen the binding affinity between

253 ligand and HMGR of *M. sexta*, whereas it also can weaken the binding affinities with other

254 species.

3.4. Effects of HMGR inhibitors on JH biosynthesis *in vitro and in vivo*

To validate the results of sequence alignment and structure comparison, three commercial

human HMGR inhibitors (statins) were used for the assay of JH biosynthesis *in vitro* and *in vivo*

in *M. sexta*, *A. mellifera* and *D. punctata*. Lovastatin is a type I statin. Fluvastatin and

pitavastatin are type II statins. The IC_{50} value of each compound is shown in Table 1. For the

lepidopteran pest *M. sexta*, all the compounds have potent inhibitory activity on JH biosynthesis.

However, these compounds have little or no effect on *A. mellifera*, which suggests that HMGR

inhibitors tested in the present work are safe for honeybees. Similarly, the inhibitory effects on

D. punctata were much lower than on *M. sexta*. The above results suggest that insect HMGR (in

particular, lepidopteran HMGR) might be an eco-friendly insecticide target.

Type II statins have a greater effect than type I for the inhibition of JH biosynthesis in *M.*

sexta. The IC_{50} value of lovastatin was 99.4 nM, which is much higher than that of fluvastatin or

pitavastatin (their IC_{50} values are 5.1 nM and 5.2 nM, respectively). These results suggest that

type II statins should be good lead compounds for new insecticide design. In addition to their

effects *in vitro*, the statins also showed significant effects on JH production by *M. sexta*

following treatment *in vivo*.

3.4.1. Injection

Following injection of the statin into newly molted fifth larval instar *M. sexta*, JH

biosynthesis was assayed after 3 hours with significant inhibitory effects apparent. After 3 hours,

the inhibition of fluvastatin, pitavastatin and lovastatin was 64.1 ± 5.2%, 61.4 ± 5.8% and 60.6 ±

275   5.8%, respectively.

276   *3.4.2. Effects on JH biosynthesis following oral administration*

277   In addition to the effects in the injection bioassays, the statins also showed a significant

278   effect on JH production following oral administration (Figure 3A). In bioassays at 1 μM,

279   inhibition of JH biosynthesis by fluvastatin, pitavastatin and lovastatin was $58.9 \pm 8.9\%$, $54.4 \pm$

280   3.1% and $62.6 \pm 3.4\%$, respectively. This suggests that HMGR inhibitors can also inhibit JH

281   biosynthesis following oral administration.

282   *3.4.3. Topical application*

283   In topical cuticular assays, no compound demonstrated any effect on JH biosynthesis

284   (Figure 3B); this might be attributable to the poor cuticular penetration of the reagents, and

285   indicates that HMGR inhibitors are unlikely to be contact insecticides.

286   **3.5. Pest control application**

287   *3.5.1. Ovicidal effects*

288   The three compounds also demonstrated significant activity on viability of *M. sexta* eggs

289   (Table 2). At a concentration (100 μM), the mortality of eggs following treatment with

290   fluvastatin, pitavastatin and lovastatin was 76.1%, 100% and 100%, respectively. A

291   concentration of 50 μM of these compounds gave about 50% inhibition.

292   *3.5.2. Larvicidal effects following oral administration of statins*

293   The experiment in section 3.4.2 demonstrated that the statins have a significant effect on JH

294   biosynthesis following oral administration. Accordingly, a stomach toxicity test was performed.

295   We first determined which instars of *M. sexta* were most sensitive to the statins following

296   feeding at high concentration (1000 μM) in 1st, 2nd and 3rd instars, respectively, and recorded the

297   mortality in Table 3. All larvae that commenced feeding from the 1st stadium died prior to

298   pupation, and most died in the 1st stadium. Larvae fed from the 2nd stadium were also sensitive to

299   the statins with a high mortality (above 85%). However, when the treatment commenced from

300   the 3rd stadium, the mortality before the next molt was less than 10%, and mortality just prior to

301   pupation was less than 40%. For all the inhibitors, the earlier instars were more sensitive than the

302   later instars.

303   We then topically treated larvae with different concentrations of the statins, commencing

304   with 1st instars, and recorded larval mortality (Table 4). The statins showed significant larvicidal

305   activity at 100 μM. The IC_{50} values of fluvastatin, pitavastatin and lovastatin were 2101 μM,

306   63.0 μM and 298.3 μM, respectively.

307   In the dead larvae, the most striking characteristic was the darkening of the cuticle in some

308   animals as well as molting disturbances (Figure 4). This is consistent with the phenomenon of

309   the inhibition of JH biosynthesis (*Monger et al., 1982*).

310   *3.6.3. Growth regulation*

311   A long-term feeding study with low concentration of inhibitors was performed to identify

312   their effects on growth. We fed larvae with inhibitors starting with 1st instars, and recorded the

313   number of days from hatching to the 5th stadium (larvae which died before the 5th stadium were

314   not recorded). For fluvastatin, the number of days from newly hatched larvae to the 5th stadium

315   at 100 μM, 10 μM and 1 μM was 17.3, 13.6 and 13.5 days, respectively. In the control group

316   (not fed inhibitors), the interval was 11.9 days. It appears that the HMGR inhibitors significantly

317   slowed the growth rate of *M. sexta*. Figure 5A shows the difference in growth more clearly.

318   Three larval groups hatched on the same day; one was fed normal food as control, one was

319   treated with 100 μM of fluvastatin, and the other was treated with 1 μM of fluvastatin. The

320   difference in size was readily apparent by comparison with the control 4th instars. The other

321   statins also showed the same growth effect as fluvastatin (Figure S2).

322   Following treatment with a low concentration (1μM) of the statins, we observed that

323   pupation of these larvae was not normal. Most larvae died in the process of pupation as a result

324   of malformation (Figure 5B). This suggests that the statins can be potent insect growth regulators.

325   **4. Discussion**

326   Currently, there is an on-going need for the discovery and development of new insecticides

327   to combat growing problems associated with resistance, environmental pollution, accumulation

328   of pesticide residues in the food chain and detrimental effects on non-target organisms. Hence,

329   the need for eco-friendly insecticides with safe and novel modes of action or targets is becoming

330   increasingly important. In this study, we have not only focused on elucidating new eco-friendly

331   insecticide targets and lead compounds, but also attempted to provide an empirical method for

332   eco-friendly insecticide discovery.

333   Insect JHs are a group of structurally related sesquiterpenoids that regulate a number of

334   physiological processes including embryogenesis, larval and adult development, metamorphosis,

335   reproduction, pheromone biosynthesis, diapause, migration, polymorphism, and metabolism

336   (*Nijhout, 1994*; *Kerkut & Gilbert, 1985*; *Gilbert, Granger & Roe, 2000*). To our knowledge, the

337   occurrence of JHs and related sesquiterpenoids such as methyl farnesoate is confined to animals

338   in the Arthropoda. It has been demonstrated that the design of JH mimics or anti-JH agents is an

339   effective strategy for insecticide discovery. Screening new targets involved in JH biosynthesis

340   has been a subject of study for two decades (*Bede et al., 2001*). As HMGR has been postulated to

341   be a key enzyme in the regulation of the MVA pathway in insects, some HMGR inhibitors

342   (statins) have been used to investigate their effects on JH biosynthesis. Compactin, mevinolin

343   and fluvastatin have been demonstrated to be potent inhibitors of JH biosynthesis *in vitro*,

344   whereas studies of their effects *in vivo* are incomplete (*Monger et al., 1982*; *Couillaud, 1991*).

345   Thus, to date, HMGR has not been used as a potential insecticide target. Furthermore, whether

346   HMGR inhibitors have detrimental effects on non-target organisms remains unknown.

347   We predicted and evaluated the ecological safety of HMGR by using sequence alignment

348   and structural comparison. Sequence analysis showed that the Lepidoptera differ from other

349   organisms. Zapata et al. tested the effects of two HMGR inhibitors, fluvastatin and compactin, on

350   HMGR activity of *Blattella germanica*. Both compounds significantly inhibited the enzymatic

351   activity at a high concentration (50 μg per animal) by approximately 25% *in vivo* (*Zapata et al.,*

352   *2002*). The inhibition by fluvastatin on *M. sexta* HMGR was approximately 60% at a low

353   concentration (0.8 ng per animal in an injection assay and 2 ng per animal following oral

354   administration, respectively) in our study. BLAST showed that the identity value between *B.*

355   *germanica* HMGR and *M. sexta* HMGR was 47%. Bacterial HMGR has a low sequence identity

356   value compared with *M. sexta* HMGR. Lovastatin inhibited *Pseudomonas mevalonii* at a high

357   concentration (K_i value =0.53 mM) (*Hedl & Rodwell, 2004*). However, the inhibitory effect of

358   lovastatin on *M. sexta* was much greater than on *P. mevalonii* (IC_{50} value 99 nM). The identity

359   value between *P. mevalonii* HMGR and *M. sexta* HMGR was 23%. In our experiment, the

360   identity value of *A. mellifera* and *D. punctata* versus *M. sexta* was 48% and 50%. Three HMGR

361   inhibitors have no or little effect on *A. mellifera* and *D. punctata*; however these compounds are

362   potent inhibitors in *M. sexta*. This suggests that there might be a link between the sequence

363   alignment data and inhibition.

364   The sequence and 3-D structure (in particular, the molecular potential surface properties) of

365   lepidopteran HMGR differs from other organisms (*A. mellifera* and *D. punctata*) in this study.

366   Assays of JH biosynthesis in the presence of HMGR inhibitors in different insect species showed

367   that those inhibitors have potent effects on the lepidopteran pest *M. sexta*, but are much less

368   effective on *A. mellifera* (Hymenoptera) and *D. punctata* (Blattodea). This confirms our

369   suggestion regarding the value and assessment of the ecological safety of HMGR as an

370   insecticide target candidate.

371   The applicability to pest control is crucial in the evaluation of the potential of a chemical to

372   act as an insecticide. Previous studies indicated that HMGR was the control point in JH

373   biosynthesis in *M. sexta* (*Monger et al., 1982*). As a consequence of limited experimental data on

374   the application of HMGR inhibitors for pest control, we tested the effects *in vivo* of three HMGR

375   inhibitors on *M. sexta*. Our present study revealed that HMGR inhibitors can be potential

376   insecticide candidates with excellent ovicidal activity, larvicidal activity and growth regulatory

377   effects. In the fat body, HMGR was crucial to vitellogenesis and reproduction. Short-term assays

378   showed that HMGR inhibitors reduce the protein levels and enzymatic activity of HMGR, and

379   long-term experiments revealed that fluvastatin impairs embryo development (*Zapata et al.,*

380   *2002*). Our work clearly indicates that HMGR is a key enzyme in embryogenesis, larval and

381   adult development and metamorphosis. In *Agrotis ipsilon*, fluvastatin also disrupted normal

382   spermatophore transfer (*Duportets et al., 1998*). It suggested that insect HMGR can be an

383   insecticide target and its inhibitors could be insecticide lead compounds.

384   We conclude that an empirical method of discovery of eco-friendly insecticides

385   encompasses the prediction of ecological safety of insecticide target candidates and the

386   probability of the application for pest control. The steps for ecological safety prediction are as

387   follows:

388   (1) Collect sequence data of insecticide target candidates from all species of interest.

389   (2) Perform sequence alignment of each species and compare to the selected target pest.

390   Statistically analyze the identity values from sequence alignments. If there is no

391   difference between pest and non-target organisms, this candidate is a likely to be an eco-

392   toxic insecticide target. If not, go to the next step.

393   (3) Perform structural comparisons and docking studies with ligands of pests and other non-

394   target organisms. If there is no difference between their structures (especially the

395   binding pockets) or binding affinities, this candidate is a possible eco-toxic insecticide

396   target. If not, it could be an eco-friendly insecticide target. New eco-friendly

397   insecticides can be designed based on structural differences.

398   Although this method of prediction cannot replace the requisite toxicity tests, it can

399   avoid unnecessary waste, save manpower, material and time in the discovery of new eco-

400   friendly insecticides.

401 **5. Conclusion**

402   We have demonstrated that insect HMGR can be a potential selective insecticide target, and

403 its inhibitors can be potential selective insecticides. Our research should be helpful for designing

404 new selective insecticides. Furthermore, we have demonstrated that sequence alignment,

405 homology modeling and structural comparison can be used to determine which enzymes or

406 receptors could be selective pesticide targets. Pest control applications have shown that the

407 HMGR inhibitors are potential insect growth regulators, especially for lepidopteran pest control.

409 **Acknowledgments**

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411 bioassay equipment.

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497   German cockroach. *Archives of Insect Biochemistry and Physiology* **49**:177–186 DOI

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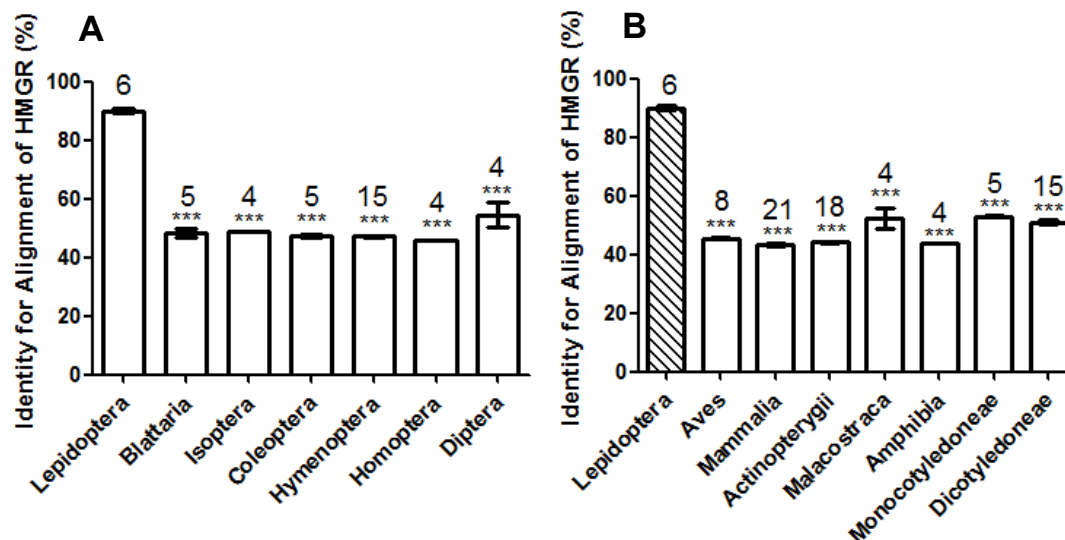
Figure 1. Comparison of the identity value of per species HMGR sequence relative to

Manduca sexta. (**A**) Asterisks indicate significant differences between Lepidoptera and other

orders of Insecta as determined by Dunnett's multiple comparison test following one-way

ANOVA: ***, $P<0.001$. (**B**) Asterisks indicate significant differences between Lepidoptera

and other organisms other than Insecta as determined by Dunnett's multiple comparison test

following one-way ANOVA: ***, $P<0.001$.

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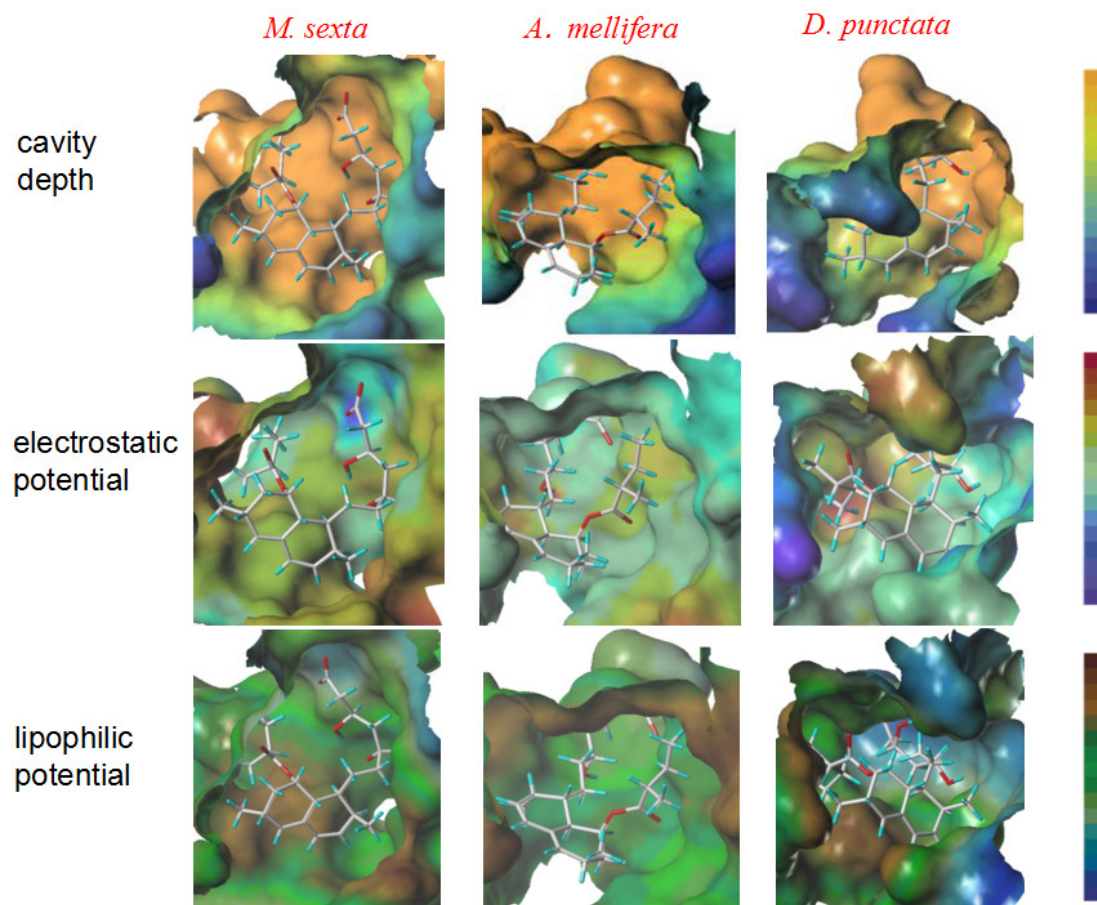


Figure 2. Structure comparison of binding pockets of *M. sexta*, *A. mellifera* and *D. punctata*

HMGR. The molecule in the pocket is lovastatin. In the presentation of cavity depth, the deep

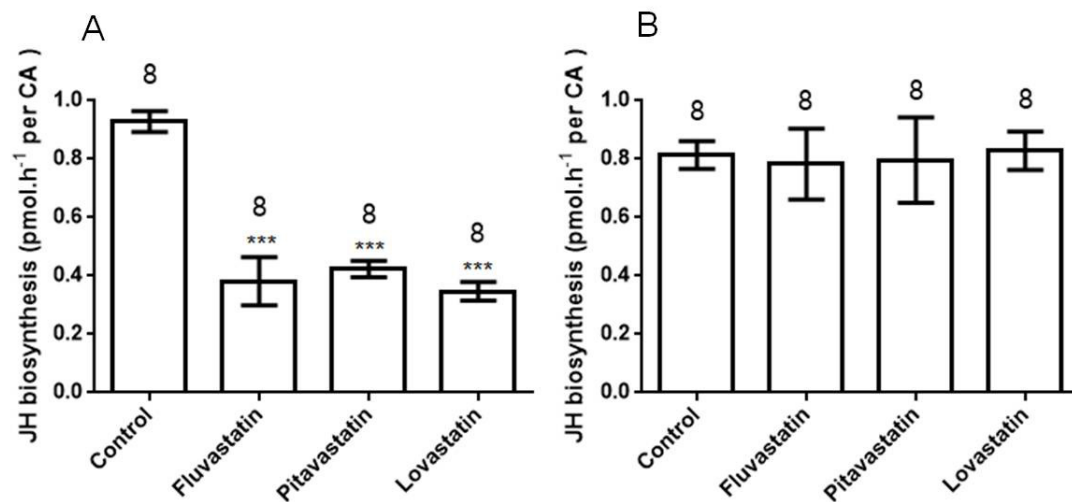
blue colour represents the outermost surface of the structure, whereas the orange colour

represents the deepest part of the cavity. In the presentation of molecular electrostatic potential,

the deep blue colour represents the most negative potential, whereas the deep red colour

represents the most positive potential. In the presentation of the molecular lipophilic potential,

the deep blue colour represents the most hydrophilic parts of the surface, whereas the deep

brown colour represents the most lipophilic parts of the surface.

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518 **Figure 3.** JH biosynthesis following oral treatment of various inhibitors (statins) (A) and topical

519 cuticular application of the same inhibitors (B). Each bar represents the mean ± SEM. Asterisks

520 indicate significant differences between inhibitor- and water-fed groups of animals as determined

521 by Dunnett's multiple comparison test following one-way ANOVA: ***, $P<0.001$.

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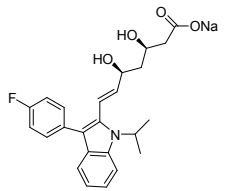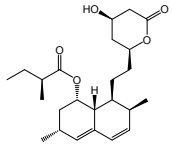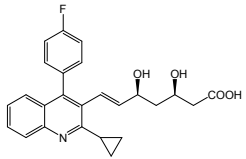
524 **Figure 4.** Dead larvae following feeding with 1000 μM of fluvastatin as 1ˢᵗ instars. Twenty

525 animals were used in this treatment.

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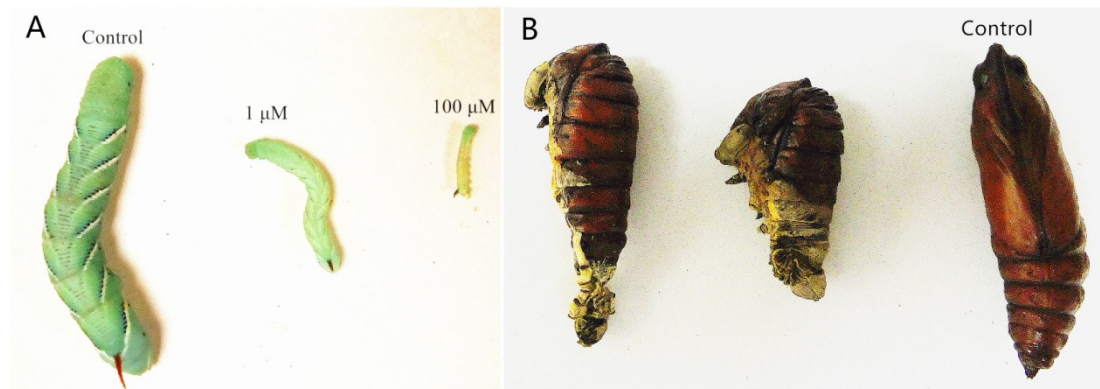
528 **Figure 5.** Developmental arrest and growth retardation in *M. sexta* following fluvastatin

529 treatment. There were twenty animals for each group. (A) Three larval groups hatched on the

530 same day; one was fed normal food as the control; the others were treated with 1 µM and 100

531 µM of fluvastatin, respectively. The difference in size is readily apparent in the control 4th instars

532 (10 days after feeding). (B) Newly hatched larvae were treated with 1 µM fluvastatin, most died

533 in the process of pupation as a result of malformation.

535 **Table 1.** The IC$_{50}$ values of HMGR inhibition of JH biosynthesis *in vitro*.

Compound	Structure	*M. sexta*, IC$_{50}$ value (nM)	*A. mellifera*, IC$_{50}$ value (nM)	*D. punctata*, IC$_{50}$ value (nM)
Fluvastatin		5.11	18100	150.0
Lovastatin		99.45	No effect	884.7
Pitavastatin		5.23	157500	395.2

536

538   **Table 2**. Ovicidal effect of HMGR inhibitors on *M. sexta* eggs at different concentrations.

539

Compound	Concentration (μM)	Number of eggs	Mortality (%)
Fluvastatin	1000	60	100
	100	110	76.1
	10	86	11.9
	1	107	9.5
Pitavastatin	2000	69	100
	200	90	100
	20	90	40.3
	2	70	38.3
Lovastatin	1000	66	100
	100	99	99.99
	10	96	30.9
	1	88	37.8
Control	0	100	0

540

542 **Table 3**. Mortality following feeding with statins at 1000 μM during the first three stadia.

Compound	Feeding treatment	Mortality before next molt (%)	Mortality before pupation (%)
Fluvastatin	From 1st instar	100	100
	From 2nd instar	51	92
	From 3rd instar	6	13
Pitavastatin	From 1st instar	82	100
	From 2nd instar	80	85
	From 3rd instar	10	40
Lovastatin	From 1st instar	100	100
	From 2nd instar	90	100
	From 3rd instar	20	90

543

545 **Table 4**. Larval mortality following treatment of 1st instars

Compound	Concentration (μM)	Mortality before 3rd instar (%)	Larval mortality (%)
Fluvastatin	1000	100	100
	100	25	25
	10	8.3	8.3
	1	16.7	16.7
Pitavastatin	1000	100	100
	100	100	100
	10	41.7	41.7
	1	25	41.7
Lovastatin	1000	100	100
	100	33.3	33.3
	10	0	8.3
	1	0	0

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