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Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase lower fecundity in the German cockroach: correlation between the effects on fecundity *in vivo* with the inhibition of enzymatic activity in embryo cells

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Abstract: The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is crucial to insect development and reproduction, as revealed by the sterilising properties of some specific inhibitors of it. In the present paper, we study the sterilising effects of a number of HMG-CoA reductase inhibitors on the German cockroach, *Blattella germanica* (L). The inhibitors tested were naringenin, lovastatin, mevastatin, simvastatin, atorvastatin and fluvastatin. The first two compounds were ineffective or scarcely effective as HMG-CoA reductase inhibitors. The most active compounds *in vivo* were fluvastatin and atorvastatin, followed by simvastatin and mevastatin. They were equally ranked when tested as HMG-CoA reductase inhibitors in the *B germanica* embryonic derived cell line UM-BGE-1. This suggests that this cell line may be an appropriate tool for testing HMG-CoA reductase inhibitors and so to predict their properties as insect sterilising agents with insecticide potential.

Keywords: *Blattella*; German cockroach; HMG-CoA reductase; statins; enzymatic activity; fecundity

1 INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase has been described in vertebrates and insects as the rate-limiting enzyme of the mevalonate pathway.[1] The most distinctive final compound of the mevalonate pathway in vertebrates is cholesterol, but insects cannot synthesise cholesterol *de novo*,[2] and the mevalonate pathway leads to final products like juvenile hormone, ubiquinone and dolichol, which play crucial roles in developmental and reproductive processes. In insects, HMG-CoA reductase has been cloned in the fruitfly *Drosophila melanogaster* Meigen,[3] the German cockroach *Blattella germanica* (L)[4] and the moth *Agrotis ipsilon* (Hufnagel).[5] In addition, a partial sequence of the enzyme has been described in the bark beetle *Ips paraconfusus* Lanier.[6]

In *B germanica*, information derived from HMG-CoA reductase cloning has enabled the description of enzyme expression patterns and functional studies. In the fat body, HMG-CoA reductase is involved in glycosylation and export of vitellogenin.[7–10] In the ovary, HMG-CoA reductase levels increase during the first reproductive cycle,[11,12] are also present in the eggs and embryo, and peak in early embryogenesis.[12] It has also been reported that fluvastatin, a well-known HMG-CoA reductase inhibitor not only in vertebrates[13] but also in insects,[10,14] inhibits HMG-CoA reductase in the ovary and in the embryo of *B germanica* and decreases the fecundity of the treated female.[12] This is related to the key role of HMG-CoA reductase in insect embryogenesis, as demonstrated in mutants of *D melanogaster* that do not express this enzyme.[15]

Given the effect of fluvastatin on *B germanica*, HMG-CoA reductase inhibitors may be considered as prototypes of insecticides with sterilising properties. Therefore, we explored these sterilising properties in a number of HMG-CoA reductase inhibitors that had been developed as hypocholesterolemic agents for vertebrates. These included natural statins

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obtained directly from fermentation, like mevastatin and lovastatin, semi-synthetic statins, like simvastatin, and fully synthetic compounds, like atorvastatin and fluvastatin[13] (Fig 1). The latter was used again as a reference in the present study.

Statins were discovered in extracts from filamentous fungi, and the members of the group are very similar in chemical structure. They have a common polyketide portion and a hydroxyhexahydronaphthalene ring system to which different side chains are linked at C8 (R_1) and C6 (R_2) (Fig 1). Mevastatin (= compactin), obtained from *Penicillium citrinum* Thom, contains a methylbutyric side chain (R_1). Lovastatin (= mevinolin), obtained from *Aspergillus terreus* Thom, contains an additional 6α methyl group (R_2). Simvastatin is a synthetic derivative of lovastatin containing a methyl group in the 2′ position of the side chain (R_1).[13] The structures of the fully synthetic inhibitors fluvastatin and atorvastatin are dissimilar, and quite different from those described above (Fig 1). Only the HMG-CoA-like moiety, responsible for HMG-CoA reductase inhibition, is common to both natural and synthetic compounds. Fluvastatin derives from mevalolactone and atorvastatin from pyridine, and both are obtained in hydroxy acid form.[13] Finally, the citrus bioflavonoid naringenin (Fig 1), which has been shown to lower HMG-CoA levels in vertebrates,[16] was also examined. Mevastatin[17,18] and fluvastatin[14] had been previously reported as inhibitors of juvenile hormone biosynthesis in the cockroaches *Periplaneta americana* (L)[17] and *B germanica*,[18] and in the locust *Locusta migratoria* L.[14]

We tested the inhibitors *in vivo* in *B germanica*, and studied their action on HMG-CoA reductase activity in the ovary and embryos, as well as the derived effects of fecundity decrease. In addition, these compounds were examined in parallel as inhibitors of HMG-CoA reductase activity in the *B germanica* embryonic cell line UM-BGE-1. This cell line derives from 5-day-old embryos of *B germanica*[19] and has been used elsewhere to test the effect of mevastatin on HMG-CoA reductase activity.[20] Given the relative simplicity of the manipulation of UM-BGE-1 cells, we aimed to develop an easier method to test HMG-CoA reductase inhibitors in insect systems.

2 EXPERIMENTAL

2.1 Insects

Adult *B germanica* (Dictyoptera, Blattellidae) females were obtained from a colony reared in the dark at 30 (±1) °C and 60–70% RH.

2.2 Reagents and bioactive compounds

DL-[*glutaryl*-3-^{14}C]-3-Hydroxy-3-methylglutaryl coenzyme A, was obtained from American Radiolabelled Chemicals (St Louis, MO, USA), and [^{32}Pd]CTP from Amersham (Uppsala, Sweden). Fluvastatin was a gift from Novartis Pharma (Bern, Switzerland). Mevastatin and naringenin were purchased from Sigma (St

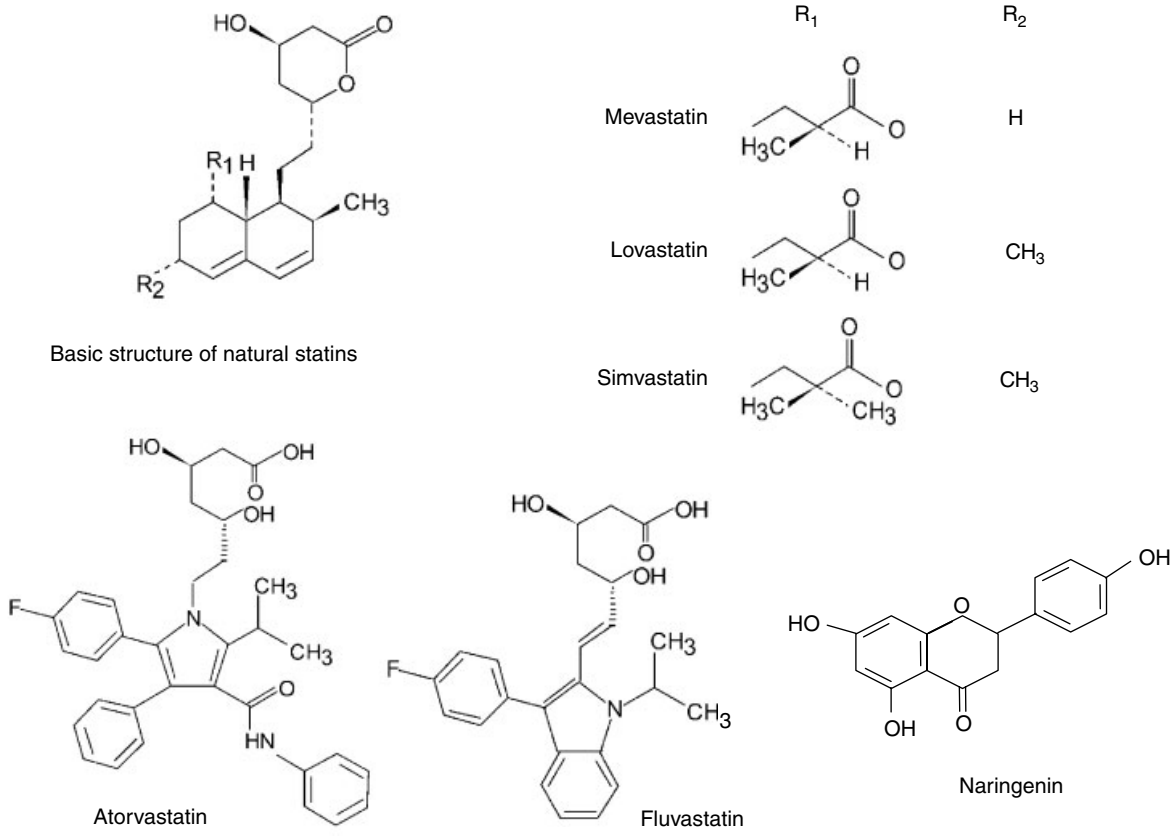

Figure 1. Chemical structure of HMG-CoA reductase inhibitors used in the present study.

Louis, MO, USA). Lovastatin and simvastatin were obtained from Merck Sharp & Dohme (Whitehouse Station, NJ, USA). Atorvastatin was a gift from Parke Davis (Ann Arbor, MI, USA). The chemical structures of these HMG-CoA reductase inhibitors are shown in Fig 1.

2.3 Dissections and treatments *in vivo*

The inhibitors of HMG-CoA reductase were applied topically to the dorsal part of the abdomen, in acetone solution. The maximal volume used was 2 µl, and the same volume of acetone was applied to controls. To study the effects of the inhibitors on the ovary, virgin females were treated on day 5 of adult life and ovaries were dissected 24 h later. To study the delayed effects on embryogenesis, females were treated on day 5 of adult life and the embryos in the ootheca were studied on day 3 after the formation of the ootheca. Ovaries and embryos in the ootheca were obtained from carbon-dioxide-anaesthetized specimens and preserved at −70 °C until the analyses. To study ootheca formation and larval production, 5-day-old virgin females were treated with the inhibitor, placed in the presence of adult males and left until the eggs of the first ootheca had hatched.

2.4 Experiments with UM-BGE-1 cells

Blattella germanica embryonic cells UM-BGE-1 were grown in suspension at 25 °C in L15 medium (Sigma), modified as recommended by Munderloh and Kurtti.[21] Briefly, the pH was adjusted to 6.5 with sodium hydroxide and the medium was complemented with 5% foetal bovine serum (GIBCO, Grand Island, NY, USA), 1% lipoprotein-cholesterol concentrate (Sigma) and 50 U ml^{-1} penicillin/50 µg ml^{-1} streptomycin (GIBCO). The cells were collected by centrifugation, washed twice in PBS and re-suspended in fresh cell culture medium at a density of 10^6 cells ml^{-1}. Two hours after transferring cells to the plate, the inhibitor was added and HMG-CoA reductase activity was measured 24 h later. Inhibitors were added in acetone solution. The maximal volume used in these treatments was 10 µl and the same volume of acetone was added to controls.

2.5 Enzymatic activity measurements

To measure HMG-CoA reductase activity, an ovary pair or an ootheca (with the eggs inside) was homogenised with a plastic pestle in 200 µl of buffer A (sucrose 100 mM, K_2HPO_4 pH 7.2 40 mM, EDTA 30 mM, KCl 50 mM, PMSF 0.5 mM, Triton X-100 2.5 ml litre^{-1} and DTT 10 mM). Cells from the UM-BGE-1 line were re-suspended in buffer A and lysed for 5 min in an ultrasonic bath. Protein contents in homogenates were measured following Bradford.[22] Two aliquots were assayed in parallel for each determination of enzymatic activity as described elsewhere.[23] The assay was performed with 100 µg of total ovary, ootheca or UM-BGE-1 cell protein for 40 min. In these conditions, the assay was linear and the amount of substrate consumed was less than 5%.

2.6 Western blot analysis of UM-BGE-1 cells

For protein extraction, the cells were homogenised as described above and the protein contents of homogenates were measured following Bradford.[22] Aliquots of 60 µg of total proteins were electrophoresed in 7.5% SDS-PAGE gels, transferred to a nitrocellulose membrane (Amersham) and processed for Enhanced Chemiluminiscence (ECL) Western blotting, using a kit from Amersham and following the manufacturer's guidelines. The antibody used, which has been described elsewhere,[12] immunodetects two bands of molecular masses of 58 kD and 66 kD, which represent proteolytic fragments of *B germanica* HMG-CoA reductase.[12] In the present experiments, the antibody was used at 1:400.

3 RESULTS AND DISCUSSION

3.1 Effects on HMG-CoA reductase activity in the ovary

As HMG-CoA reductase activity in the ovary of *B germanica* peaks on day 5,[12] the short-term effects of inhibitors were studied on females treated (1 µg, 10 µg and 50 µg) on day 5 and analysed 24 h later. Naringenin did not inhibit HMG-CoA reductase activity even at the 50-µg dose (Fig 2). At the same dose, mevastatin and lovastatin inhibited HMG-CoA reductase activity by 40%. Simvastatin was also active at the 50-µg dose (70% inhibition), but did not inhibit HMG-CoA reductase activity at lower doses. The most powerful inhibitors were atorvastatin and fluvastatin (around 50% inhibition at 1 µg). Inhibitors directly derived from fungal metabolism and not chemically modified, like mevastatin and lovastatin, induced a modest inhibition when tested at the 50-µg dose. Simvastatin, a synthetic derivative of lovastatin but methylated in the 2′ position of the side chain,

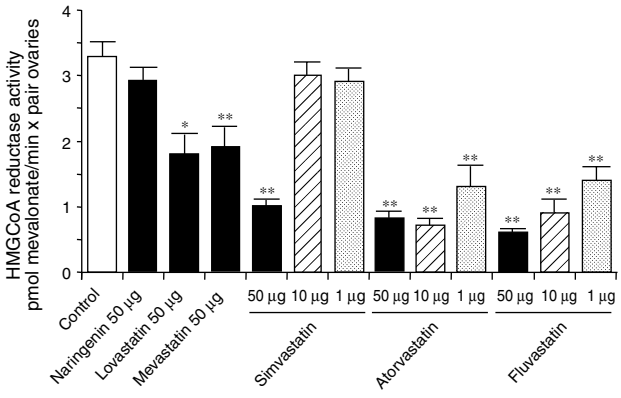

Figure 2. Effect of various inhibitors on HMG-CoA reductase activity in the ovary of *Blattella germanica*. Females were treated with the indicated doses of each inhibitor on day 5 of adult life and enzymatic activity was studied 24 h later. Values are expressed as the mean (± SEM) ($n = 4–10$) and the asterisks indicate significant differences with respect to control (*t* test, $^*P < 0.05$, $^{**}P < 0.01$).

showed a potent inhibitory activity at 50 μg, but became inactive at 10 μg. Atorvastatin and fluvastatin, which are obtained entirely by chemical synthesis, proved to be the most powerful inhibitors assayed, since they were still active at a dose of 1 μg. These compounds were equally ranked in tests of HMG-CoA reductase inhibition in vertebrates.[24] The inhibitory activity induced by fluvastatin was similar to that previously reported.[12]

3.2 Effects on HMG-CoA reductase activity in embryos

The enzyme HMG-CoA reductase is present in considerable amounts in early embryos and fluvastatin decreases fecundity of vitellogenic females,[12] suggesting that fluvastatin has a delayed effect on embryo viability. In the present experiments, we compared the effect of fluvastatin with that of other HMG-CoA reductase inhibitors. We used all the inhibitors tested in the ovaries except naringenin, which had proved inactive, and lovastatin, which had given a modest and scarcely significant inhibition at 50 μg. Given that the highest levels of HMG-CoA reductase enzymatic activity were observed on day 3 after the formation of the ootheca,[12] inhibitors were administered to 5-day-old vitellogenic females, and effects were studied in the embryos on day 3 after ootheca formation. At 50 μg, mevastatin was inactive, whereas simvastatin halved HMG-CoA reductase activity (Fig 3). In contrast, at the same dose, atorvastatin and fluvastatin inhibited the enzymatic activity by 70% and were still significantly active (50% inhibition) at 1 μg. These results point again to atorvastatin and fluvastatin as the most powerful inhibitors among those tested. In vertebrate systems, atorvastatin and fluvastatin interact very efficiently with HMG-CoA reductase and undergo a relatively slow degradation.[25] The same reasons may also account for the high inhibitory capabilities of these two compounds in insects. Results obtained with fluvastatin were comparable to those previously described.[12]

3.3 Effects on larval production

Fluvastatin applied to vitellogenic females decreases larval production, probably through the incorporation of the compound into the oocyte and the subsequent inhibitory effect on the embryo.[12] In the present

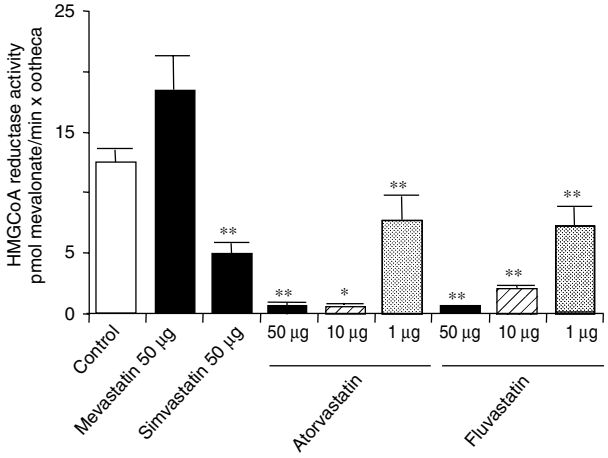

Figure 3. Effect of various inhibitors on HMG-CoA reductase activity in the embryos of *Blattella germanica*. Females were treated with the indicated doses of each inhibitor on day 5 of adult life and enzymatic activity was studied in the embryos 3 days after ootheca formation. Values are expressed as the mean (± SEM) ($n = 4–14$) and the asterisks indicate significant differences with respect to control (*t* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Table 1. Effect of HMG-CoA reductase inhibitors on ootheca formation and larval production in *Blattella germanica*. Adult females were treated with selected doses of the compounds or with acetone (control) on 5-day-old vitellogenic females. Values are expressed in absolute terms, as a percentage, or as the mean ± SEM. The asterisks indicate significant differences with respect to control (*t* test, $^{*}P < 0.05$,$^{**}P < 0.01$)

	Control	Compactin (50 μg)	Simvastatin (50 μg)	Atorvastatin (50 μg)	Atorvastatin (10 μg)	Fluvastatin (50 μg)	Fluvastatin (10 μg)	Fluvastatin (1 μg)
Number of females used	79	10	10	8	8	40	43	19
Females that died	1	0	1	0	0	14	4	0
Days until the formation of first ootheca	7.5 (± 0.1)	7.2 (± 0.2)	7.8 (± 0.1)	7.4 (± 0.3)	7.1 (± 0.1)	7.8 (± 0.2)	7.5 (± 0.1)	7.3 (± 0.1)
Number of first ootheca formed	75	9	9	8	7	38	41	19
Number of viable first ootheca	73	8	3	1	5	0	0	19
Duration of embryogenesis (days)	15.6 (± 0.1)	15.0 (± 0.0)	16.5 (± 0.0)	15	15.6 (± 0.4)	—	—	15.8 (± 0.1)
Number of larvae emerging from the first viable ootheca	37.5 (± 0.9)	32.6 (± 1.1)	39 (±0.6)	25	22.2 (± 4.8)*	—	—	29.6 (± 2.2)**
Average of larvae obtained per survivor	35.1	26.1	13.0	3.1	13.8	0.0	0.0	30.0
Total reduction of fertility (with respect to larvae obtained per survivor)	0%	26%	63%	91%	61%	100%	100%	15%

study we have investigated the same phenomenon by comparing the effect of the four inhibitors studied in embryos. The compounds were applied on 5-day-old vitellogenic females, and larval production of the first ootheca was recorded. At 50 μg, mevastatin did not significantly decrease ootheca formation or larval production (Table 1). Simvastatin decreased the number of viable ootheca by 67%, but did not affect the number of emerging larvae. At 50 μg of atorvastatin, only one of eight ootheca was viable and produced 25 larvae (whereas control specimens produced an average of 37 larvae per ootheca); at 10 μg the same compound decreased viable ootheca and the number of emerging larvae per ootheca by 29% and 40%, respectively. The most active compound was fluvastatin, which completely inhibited the emergence of larvae from oothecae at 50 and 10 μg. Even at 1 μg it significantly decreased (21%) the number of larvae emerging from viable oothecae. In addition, it caused 35% mortality at 50 μg. These results are consistent with those previously obtained.[12] As expected, the results of inhibition of HMG-CoA reductase activity in 3-day-old embryos (Fig 3) correlated with the decrease of fecundity described here (Table 1), fluvastatin being the most active compound, followed by atorvastatin, simvastatin and mevastatin. In *D melanogaster*, maternal HMG-CoA reductase is required for early embryonic development[26] and to guide primordial germ cells to the somatic gonad,[16] which makes mutant embryos that do not express this enzyme inviable. The decrease of fecundity observed in females treated with the above compounds seems to be related with these key roles of HMG-CoA reductase.

3.4 Effects on HMG-CoA reductase activity in UM-BGE-1 cells

The compounds used in the experiments of the decrease of fecundity were studied in parallel as inhibitors of HMG-CoA reductase activity in UM-BGE-1 cells. All compounds tend to increase HMG-CoA reductase activity at low doses, whereas they inhibit it at higher doses (Fig 4). Although the pattern of dose–activity was similar for all compounds, there were quantitative differences between them. For example, atorvastatin and fluvastatin caused moderate increases in HMG-CoA reductase activity at 0.002 and 0.02 μM, but began to inhibit it at 0.2 μM. In contrast, mevastatin and simvastatin significantly stimulated HMG-CoA reductase activity at 0.002 and 0.02 μM, respectively, and still tended to be stimulatory at 0.2 μM. These data are in agreement with a previous report[20] that 0.02 μM of mevastatin increases HMG-CoA reductase activity in UM-BGE-1 cells. Similar results have been reported in *D melanogaster* Kc cells,[27] where 1 μM mevastatin enhances HMG-CoA reductase activity. Stimulation at low doses of the inhibitor may be due to the rebound effect described in the case of mevastatin in vertebrate systems, where the compound increases the mRNA and protein levels

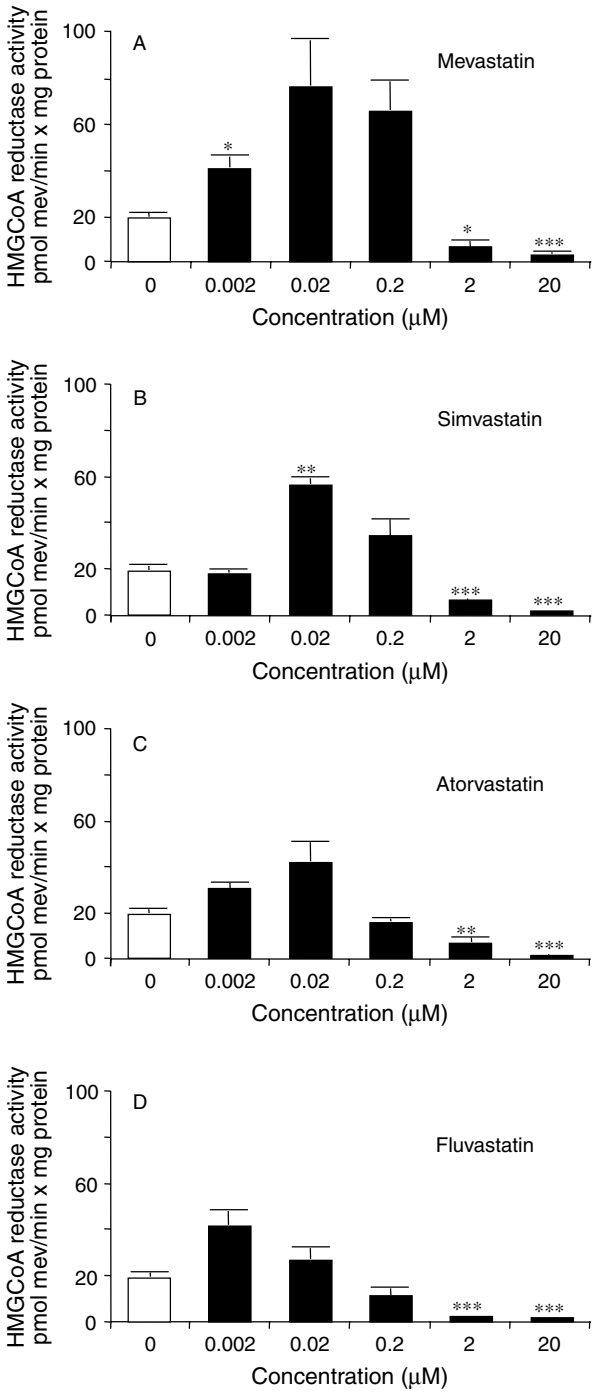

Figure 4. Effect of various inhibitors on HMG-CoA reductase activity in UM-BGE-1 cells. UM-BGE-1 cells (10^6) were incubated with the indicated concentrations of each inhibitor during 24 h, and then assayed for HMG-CoA reductase activity. Values are expressed as the mean (± SEM) ($n = 3–4$) and the asterisks indicate significant differences with respect to control (*t* test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

of HMG-CoA reductase.[28] Thus, we studied HMG-CoA reductase levels in cells treated with mevastatin and fluvastatin at the doses used in the experiments of inhibition of enzymatic activity. Neither compound increased or decreased HMG-CoA reductase levels at any of the doses studied (Fig 5). Therefore, the fluctuations of enzymatic activity caused by the various doses of the inhibitors seem rather to be related to

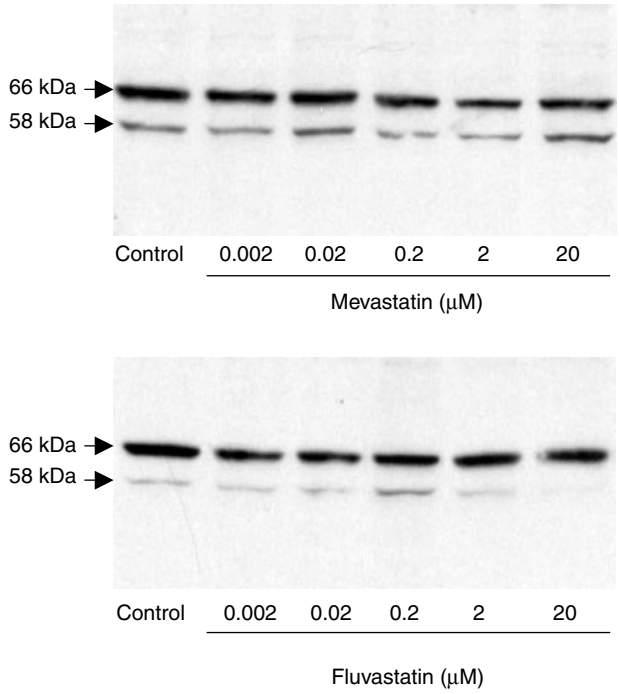

Figure 5. Western blot analysis of HMG-CoA reductase levels in UM-BGE-1 cells incubated for 24 h in the presence of the indicated doses of mevastatin or fluvastatin. The antibody immunodetects two bands of molecular masses 58 kD and 66 kD, which represent proteolytic fragments of *Blattella germanica* HMG-CoA reductase. The blots are representative of three replicates.

post-translational modifications, like phosphorylation and dephosphorylation, which would inactivate and activate the enzyme, respectively.

Finally, we wondered whether the high inhibitory activity found at maximal doses of the compounds was due to an unspecific and irreversible toxic effect. Therefore, we tested fluvastatin at the 20-μM dose again, and measured the HMG-CoA reductase activity after 24 h of incubation, whereas in a parallel batch of cells we did the same treatment, but after 24 h of incubation we washed the cells twice in PBS 100 mM and incubated them in fresh cell culture medium for a supplementary period of 24 h. The cells incubated for 24 h in the presence of fluvastatin showed a significantly reduced HMG-CoA reductase activity, as expected, whereas HMG-CoA reductase activity levels in those similarly treated but washed and re-incubated for 24 h in fresh medium were similar to controls (Fig 6). This suggests that the inhibitor did not produce irreversible damage to the cells.

4 CONCLUSIONS

Inhibitors of HMG-CoA reductase applied to vitellogenic females of the German cockroach, *B germanica*, decrease the activity of the enzyme in the ovary and in the embryo, and lower larval production. The comparison of results obtained with the compounds studied indicates that the natural fungal metabolites mevastatin and lovastatin are the least active, closely followed by simvastatin, a synthetic derivative of lovastatin methylated at the 2′ position of the side chain.

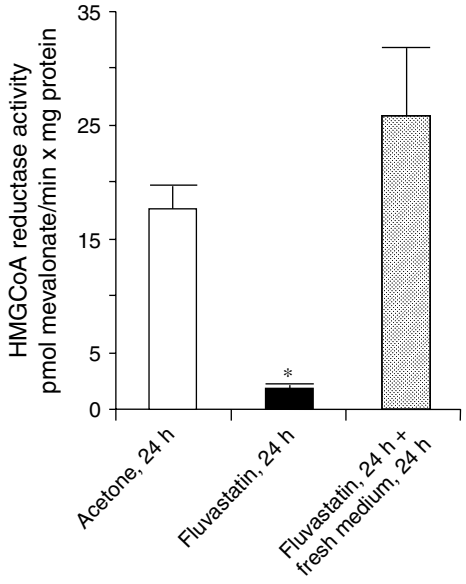

Figure 6. HMG-CoA reductase activity in UM-BGE-1 cells incubated for 24 h with 20 μM fluvastatin or incubated for 24 h with 20 μM fluvastatin and then washed and incubated again for 24 h in fresh medium. Results are expressed as the mean (± SEM) ($n = 4$) and the asterisks indicate significant differences with respect to control (*t* test, $^*P < 0.001$).

All these natural or semi-synthetic compounds share a common polyketide portion and a hydroxyhexahydronaphthalene ring system. However, the most active compounds were those entirely synthetic, ie the mevalolactone derivative fluvastatin and the pyrrole derivative atorvastatin. Of these, fluvastatin was the most active compound tested and caused total sterility *in vivo* in the first reproductive cycle at a dose of 10 μg. These compounds were equally ranked when assayed as HMG-CoA reductase inhibitors in UM-BGE-1 cells. This suggests that this cell line is a suitable experimental system to screen HMG-CoA reductase inhibitors and thus predict their properties as insect sterilising agents with insecticide potential.

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