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Abstract

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Keywords

monoterpenoid, carvacrol, nAChR, nicotine, neonicotinoid

Disciplines

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Comments

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The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor

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Abstract

BACKGROUND: The phenolic monoterpenoid carvacrol, which is found in many plant essential oils (thyme, oregano and Alaska yellow cedar), is highly active against pest arthropods, but its mechanisms of action are not fully understood. Here, carvacrol is shown to bind in a membrane preparation containing insect nicotinic acetylcholine receptors (nAChRs). [^{14}C]-Nicotine binding assays with *Musca domestica* (housefly) nAChRs were used in this study to demonstrate carvacrol's binding to nAChRs, thereby acting as a modulator of the receptors.

RESULTS: Carvacrol showed a concentration-dependent inhibition of [^{14}C]-nicotine binding in a membrane preparation of housefly heads containing nAChRs, with IC_{50} = 1.4 μM, in a non-competitive pattern. Binding studies with neonicotinoid insecticides revealed that imidacloprid and thiamethoxam did not inhibit the binding of [^{14}C]-nicotine, while dinotefuran, from the guanidine subclass of neonicotinoids, inhibited nicotine binding like carvacrol.

CONCLUSIONS: Carvacrol binds to housefly nAChRs at a binding site distinct from nicotine and acetylcholine, and the nAChRs are a possible target of carvacrol for its insecticidal activity.

Keywords: monoterpenoid; carvacrol; nAChR; nicotine; neonicotinoid

1 INTRODUCTION

Carvacrol is a phenolic monoterpenoid present in many plant essential oils such as those obtained from thyme (*Thymus vulgaris*), pepperwort (*Lepidium* sp.), Alaska yellow cedar (*Callitropsis nootkatensis*) and oregano (*Origanum vulgare*). Carvacrol has a pleasant aroma of oregano and antimicrobial properties, and it is widely used as a food additive.[1–3] Recent studies illustrate that carvacrol and many other monoterpenoids are acutely toxic to various invertebrate pests, including insects, acarines and nematodes.[4–10] Moreover, these naturally occurring compounds biodegrade or otherwise dissipate rapidly in the environment and have low toxicity to mammals (acute and chronic), fish and other non-target organisms.[11] All these advantages make monoterpenoids a good choice as alternatives to synthetic chemicals for pest management.

The mechanism of action of monoterpenoid insecticides in insects is not fully understood, although some possible targets suggested and investigated include a γ-aminobutyric acid (GABA) receptor,[12–15] an octopamine receptor,[16–18] a tyramine receptor[19] and inhibition of acetylcholinesterase (AChE).[20,21] The present study addresses the potential for carvacrol to exert its insecticidal effect by binding to the nicotinic acetylcholine receptor (nAChR) in the housefly central nervous system.

The nAChR is a cholinergic receptor that forms ligand-gated ion channels in the plasma membranes of certain neurons in the insect central nervous system. The binding of acetylcholine (ACh), an excitatory neurotransmitter, activates the nAChR and opens the cation channels on the post-synaptic membrane. The diffusion of Na^+ and K^+ across the receptor causes depolarization, which opens voltage-gated sodium channels, resulting in firing of the action potential.[22,23] In insects, nAChR is a major target for many insecticides. Nicotine and neonicotinoids act as receptor agonists, which mimic the function of acetylcholine. The binding of neonicotinoids to the nAChR can cause excitatory effects on the insect nervous system.[24–26] Another type of insecticide, spinosad, binds to the nAChR and enhances the sensitivity of the nAChR to the endogenous neurotransmitter acetylcholine.[27,28] In mammalian models, some monoterpenoids, such as borneol and camphor, have been found to inhibit the nAChR-mediated effects non-competitively.[29,30] However, no evidence has been shown that monoterpenoids can modulate the function of the nAChR in insects. The purpose of this study was to investigate the binding of carvacrol to a native nAChR in the housefly and compare the binding of nicotinoids along with two subclasses of neonicotinoids.

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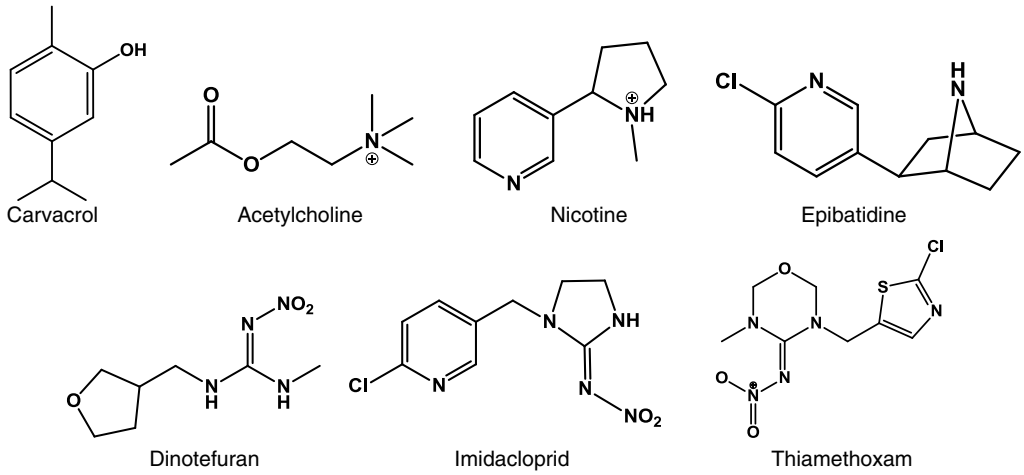

Figure 1. Chemical structures of carvacrol, acetylcholine and the nicotinoids and neonicotinoids used in this study.

2 MATERIALS AND METHODS

2.1 Materials

Carvacrol (5-isopropyl-2-methylphenol) (98%), epibatidine (99%), dinotefuran (99%), thiamethoxam (99%) and imidacloprid (99.9%) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Unlabeled nicotine was purchased from Acros Organics (Geel, Belgium). The [^{14}C]-nicotine was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). Structures of carvacrol, epibatidine, nicotine, dinotefuran, thiamethoxam and imidacloprid are shown in Fig. 1.

2.2 Tissue preparation

Housefly heads (0.8 g) were homogenized in 5 mL of 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M of sucrose (buffer A) with a glass homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 700 × *g* for 10 min. The pellet was discarded, and the supernatant was recentrifuged at 125 000 × *g* for 60 min. The pellet was suspended in 10 mM phosphate buffer (pH 7.4) containing 50 mM of NaCl (buffer B) and used directly for the assays or stored at −80 °C and used within a week. The Lowry protein assay was used to determine a final concentration of total protein.[31]

2.3 [^{14}C]-Nicotine binding assay

Membrane preparation containing 200 µg of protein was incubated for 70 min at room temperature with 2 µM of [^{14}C]-nicotine (specific radioactivity 55 mCi mmol^{-1}), different amounts of candidate ligands and buffer B. The total assay buffer volume was 200 µL. After incubation, samples were immediately filtered on Millipore glass-fiber filters (pore size 1 µm) and washed 3 times. Washing was performed by placing filters in 10 mL of ice-cold buffer B and shaking at 300 rpm. After washing, filters were vacuum dried and put into 10 mL of scintillation cocktail (Ultima Gold, PerkinElmer) overnight. Radioactivity was measured on a Beckman LC5000 CE liquid scintillation counter. Specific binding was calculated as the difference between the total ^{14}C bound and non-specific ^{14}C bound with 1 mM of cold nicotine in buffer B solution. The specific binding was 50–60% of the total binding. For the saturation binding assay, 40 nM, 120 nM, 200 nM, 400 nM, 1.2 µM, 2 µM and 5 µM concentrations of [^{14}C]-nicotine, with or without 100 µM of carvacrol, were used to test the dissociation constant K_d and the maximal binding capacity B_{max}.[26,32,33]

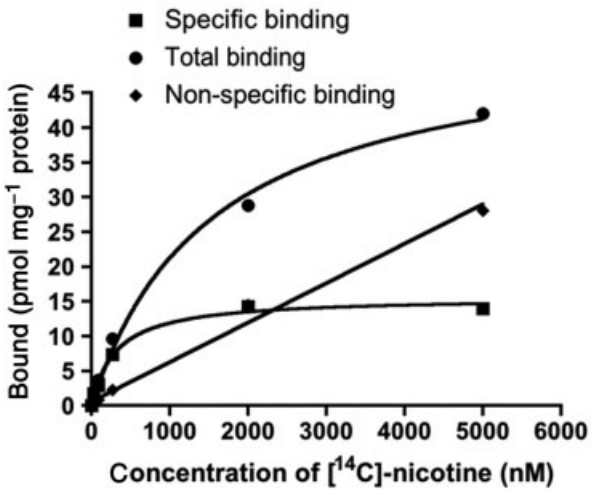

Figure 2. Saturation binding curve for [^{14}C]-nicotine binding to membrane preparation of housefly heads. Displayed is the total binding along with the non-specific (determined with 1 mM of unlabeled nicotine) to generate specific binding to housefly homogenate.

2.4 Data analysis

Results are shown as mean ± standard error of mean (SEM) and were analyzed using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA). The K_d value is the dissociation constant indicating the affinity between ligands and the nAChR. The B_{max} value is the maximal binding capacity for ligand binding to the nAChR. The IC_{50} value is the concentration for half maximal inhibition. The two-tailed Student's *t*-test was used to compare data. A *P*-value of less than 0.05 was considered to be statistically significant.

3 RESULTS

3.1 [^{14}C]-Nicotine saturation binding assay

Nicotine is an agonist for the housefly nAChR. It binds to the same binding site as the endogenous ligand acetylcholine.[32,34] Figure 2 shows the [^{14}C]-nicotine saturation binding assay, accounting for the total binding and non-specific binding. The dissociation constant K_d for [^{14}C]-nicotine binding to the membrane homogenate of housefly head containing nAChR is

308 ± 26 nM, with 50–60% of specific binding out of the total binding. The maximal binding capacity B_{max} for the binding is 15.6 ± 0.3 (pmol mg^{-1} protein).

3.2 Displacement binding of [^{14}C]-nicotine by nicotine, epibatidine, thiamethoxam, dinotefuran and imidacloprid

Imidacloprid, nicotine, epibatidine, thiamethoxam and dinotefuran have previously been demonstrated to be agonists at insect nAChRs.[24–26,35,36] The effects of these five compounds on [^{14}C]-nicotine binding were compared and contrasted with the effect of carvacrol. As demonstrated in Fig. 3A, unlabeled nicotine resulted in a concentration-dependent inhibition of [^{14}C]-nicotine binding, with $IC_{50} = 66 \pm 2\ \mu M$. Dinotefuran, a guanidine neonicotinoid, inhibited the binding of [^{14}C]-nicotine to the receptor, with $IC_{50} = 0.124 \pm 0.054\ \mu M$ (Fig. 3B). Epibatidine, which is a natural nicotinoid, also inhibited the binding of [^{14}C]-nicotine to the receptor, with $IC_{50} = 77 \pm 7\ \mu M$ (Fig. 3C). The nAChR agonists imidacloprid and thiamethoxam showed no effect on [^{14}C]-nicotine binding to the housefly head membrane preparation in this assay (Figs 4A and B). These findings suggest that nicotine, epibatidine and dinotefuran caused a concentration-dependent decrease in the binding of [^{14}C]-nicotine at a native nAChR. It also shows that the binding site(s) of these compounds are distinct from the binding site for imidacloprid and thiamethoxam.

3.3 Inhibitory binding of [^{14}C]-nicotine by carvacrol

In this assay, carvacrol induced a concentration-dependent inhibitory effect on the binding of [^{14}C]-nicotine to the membrane homogenate, with $IC_{50} = 1.4 \pm 0.45\ \mu M$ (Fig. 5), which suggested that carvacrol binds to the housefly nAChR and inhibits the binding of [^{14}C]-nicotine. From the [^{14}C]-nicotine saturation binding assay in the presence of 100 μM of carvacrol, it was determined that the binding of carvacrol increased the dissociation constant K_d for [^{14}C]-nicotine binding to the housefly nAChR preparation from 308 nM to 2879 ± 167 nM. Carvacrol also decreased the maximal binding capacity B_{max} for the binding from 15.6 ± 0.3 (pmol mg^{-1} protein) to 10.5 ± 0.3 (pmol mg^{-1} protein) (Fig. 6). The higher K_d value and lower B_{max} value indicate that carvacrol may non-competitively inhibit [^{14}C]-nicotine binding through its binding to the nAChR at an allosteric site.

4 DISCUSSION

The [^{14}C]-nicotine housefly nAChR inhibition binding assay and saturation binding assay with carvacrol demonstrated that carvacrol can bind to a housefly nAChR and may function as a modulator to the receptor. However, unlike acetylcholine, nicotinoids (e.g. nicotine and epibatidine) and neonicotinoids (e.g. imidacloprid, dinotefuran and thiamethoxam), carvacrol is not considered to be an agonist of the housefly nAChR for two reasons. Firstly, in structure, carvacrol has neither an easily protonated atom (like the quaternary nitrogen in acetylcholine and nicotine) at a physiological pH, nor an electronegative moiety (like the nitro group in imidacloprid) (Fig. 1), one of which is necessary for the binding of nAChR agonists to the tryptophan residue (for acetylcholine and nicotine binding) or lysine/arginine/histidine residues (for imidacloprid binding) at the nAChR binding pocket.[37,38] Secondly, the [^{14}C]-nicotine binding assay in the present study demonstrated that carvacrol binds to nAChRs in housefly head and inhibits the binding of [^{14}C]-nicotine to housefly nAChRs non-competitively, with $IC_{50} = 1.4 \pm 0.45\ \mu M$.

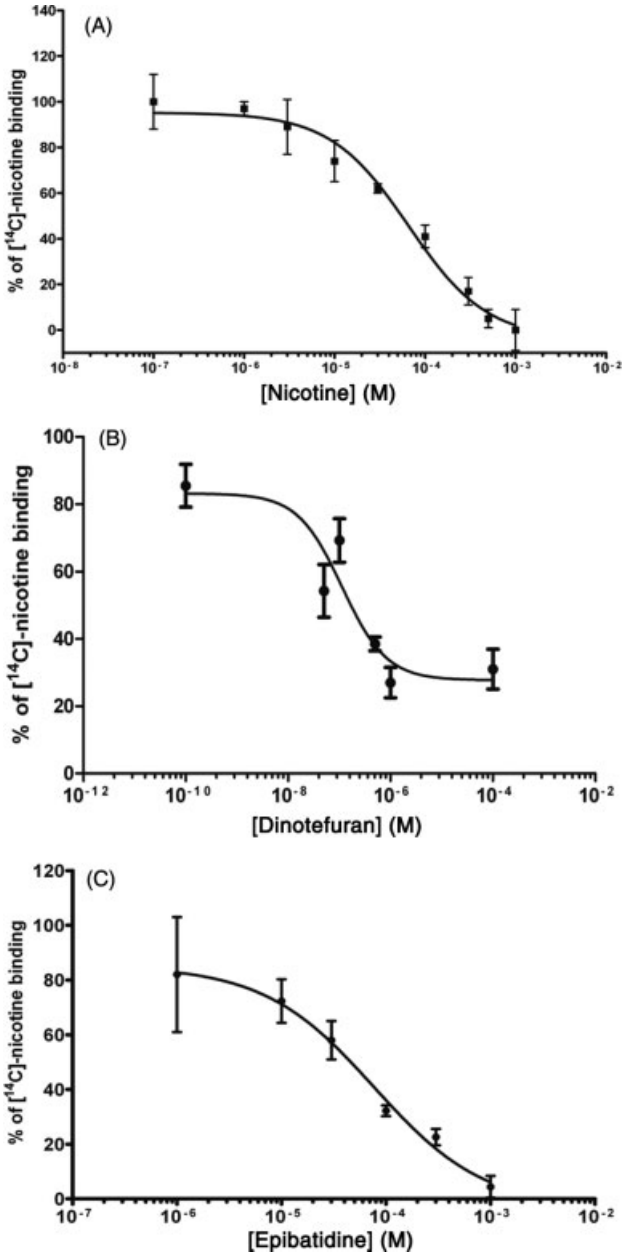

Figure 3. Binding of [^{14}C]-nicotine to membrane homogenates prepared from housefly heads. The nAChR agonists nicotine (A), dinotefuran (B) and epibatidine (C) were able to displace [^{14}C]-nicotine. Results are expressed as percentages of the [^{14}C]-nicotine binding that occurs with various concentrations of an agonist, compared with [^{14}C]-nicotine binding in the absence of an agonist (set to 100%). Each point represents the mean ± SEM, $n = 3$ (three unique membrane preparations).

The non-competitive inhibition of [^{14}C]-nicotine binding to the nAChR by carvacrol indicated that carvacrol may bind to the nAChR in the housefly at a different binding site from nicotine and the endogenous ligand acetylcholine and affect the function of the nAChR system. However, from this radioligand binding assay alone, it cannot be confirmed whether carvacrol can interfere with cation movement stimulated by nAChR agonists. Further evidence is required to verify the hypothesis that carvacrol acts as a non-competitive inhibitor of insect nAChRs.

It has been demonstrated that imidacloprid and thiamethoxam do not compete with [^{14}C]-nicotine binding to native nAChR from

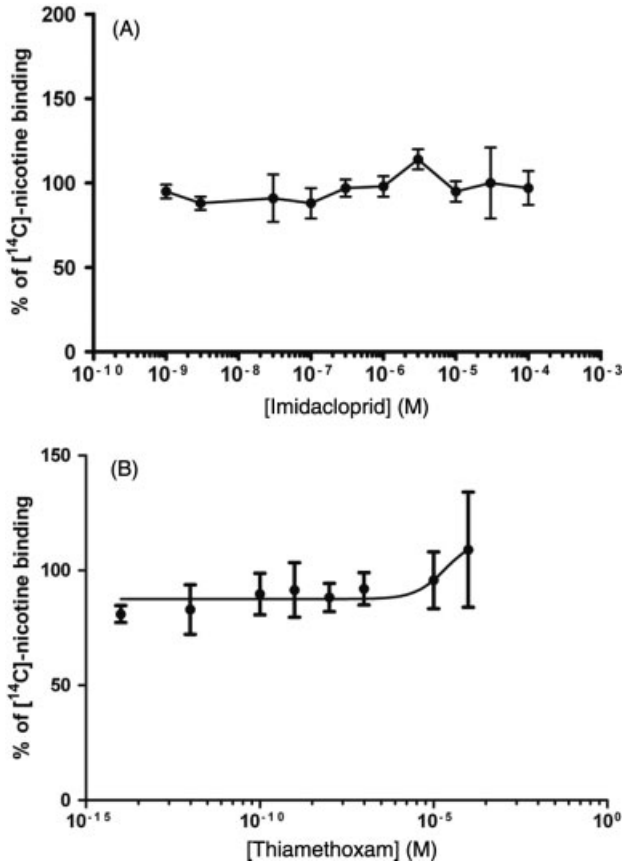

Figure 4. Binding of [^{14}-C]-nicotine to membrane homogenates prepared from housefly heads. The nAChR agonists imidacloprid (A) and thiamethoxam (B) were unable to displace [^{14}-C]-nicotine. Results are expressed as percentages of the [^{14}C]-nicotine binding that occurs with various concentrations of an agonist, compared with [^{14}C]-nicotine binding in the absence of an agonist (set to 100%). Each point represents the mean ± SEM, $n = 3$ (three unique membrane preparations).

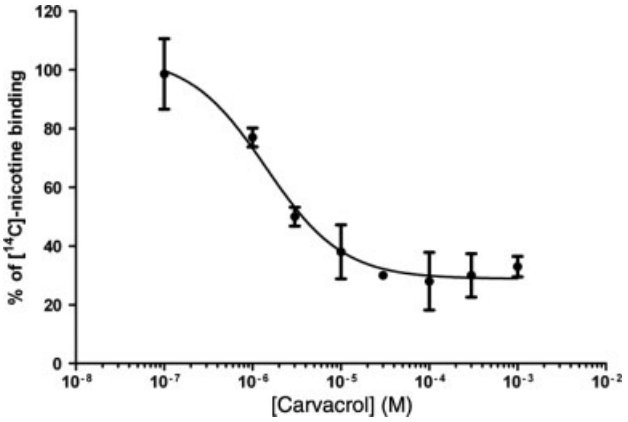

Figure 5. Inhibitory effects of carvacrol on [^{14}C]-nicotine binding in membrane preparations of housefly heads. The [^{14}C]-nicotine binding in the absence of carvacrol is expressed as 100%. Each point represents the mean ± SEM, $n = 3$ (three unique membrane preparations).

a housefly head preparation. This indicates that these structurally similar neonicotinoids (Fig. 1) possibly bind to a different location or different receptor from nicotine, and may possibly not share the same binding site with carvacrol at nAChRs; however, this needs further study. Epibatidine has been reported to have $IC_{50} = 30.8$ nM when [^{3} H]-epibatidine is the radioligand, and 0.243 μM when [^{3} H]-α-bungarotoxin is the radioligand in American cockroach nerve cord.[39] Here it is reported that epibatadine competes with [^{14}C]-nicotine but at a higher concentration (77 μM). Dinotefuran has been shown to compete with [^{3} H]-epibatidine and [^{3} H]-α-bungarotoxin, with IC_{50} values of 890 nM and 36.1 μM in American cockroach nerve cord respectively.[39] This shows that dinotefuran has a stronger effect on the binding of [^{14}C]-nicotine in housefly head preparation than [^{3} H]-epibatidine and [^{3} H]-α-bungarotoxin in American cockroach nerve cord preparation.

It has been previously reported that imidacloprid displaced [^{3} H]-nicotine from tick larval homogenates at high concentrations.[40] Here it is shown that imidacloprid does not displace [^{14}C]-nicotine binding. This difference may be a result of the tissue preparation being obtained from unrelated arthropods (housefly versus tick). Further, Turberg *et al.*[40] used a whole-tick preparation, and binding curves were produced with very low disintegrations per minute (<100 DPM). The present assay utilized less non-nervous tissue by using only flyheads, and employed higher radioactivity in each binding assay. Also, it has been suggested that this decrease in imidacloprid binding may explain why ticks are not as susceptible to imidacloprid when compared with insects.[41] Thiamethoxam has been reported to be a poor agonist of nAChR,[35] and it may bind to a mixed nicotinic/muscarinic acetylcholine receptor, which is a unique target of all neonicotinoid insecticides. This may be a reason why thiamethoxam does not inhibit [^{14}C]-nicotine binding to the housefly nAChR. In this study, both epibatidine and dinotefuran also show inhibitory effects on the binding of [^{14}C]-nicotine at a native nAChR, with $IC_{50} = 77$ μM and 0.124 μM respectively. However, although they both show inhibitory effects on [^{14}C]-nicotine binding, they likely bind to a different site at the nAChR from carvacrol. According to previous studies, epibatidine and dinotefuran were both agonists of nAChR in insects, and they share the same binding site with other acetylcholine agonists.[36,39] The present [^{14}C]-nicotine binding assays suggest that carvacrol binds to housefly nAChR at a binding site distinct from nicotine, and inhibits the [^{14}C]-nicotine binding non-competitively.

Some monoterpenoids, such as borneol and camphor, have been demonstrated to be non-competitive inhibitors of mammalian nAChRs. Park *et al.*[29,30] reported that, in bovine adrenal cells, both borneol and camphor inhibited the increase in intracellular calcium and sodium level induced by an nAChR agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), but did not change the binding of [^{14}C]-nicotine to the nAChR.

Besides binding to insect nAChRs, there may be other possible targets for carvacrol activity, but current evidence indicates that they are secondary targets to nAChRs. Previous studies in the authors' laboratory have demonstrated that carvacrol can inhibit the activity of acetylcholinesterase (AChE) in housefly, with $IC_{50} = 144$ μM. The authors do not believe that inhibition of AChE is the primary action of carvacrol because of the much higher concentration needed.[20] Carvacrol and two other monoterpenoids, thymol and pulegone, enhanced the binding of [^{3} H]-TBOB to GABA receptors in a preparation from housefly heads, and also increased the chloride uptake activated by GABA in preparations of ventral nerve cord of American cockroach. These findings suggest that carvacrol is a positive allosteric modulator for insect GABA receptors and can thereby cause an inhibitory effect on the insect nervous system. However, the effective concentration (EC_{50}) of [^{3} H]-TBOB binding was 48 μM. Increase in chloride uptake in American cockroach microsacs was observed at 500 μM and 1 mM.[15]

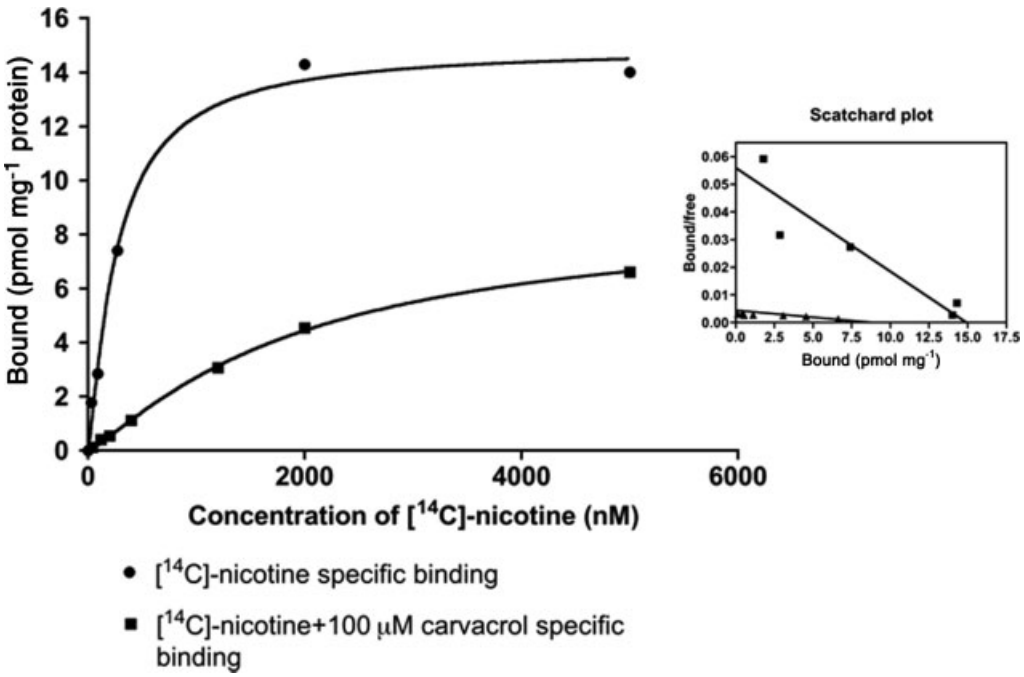

Figure 6. Saturation binding curve for [^{14}C]-nicotine binding to membrane preparation of housefly heads, along with the saturation binding curve of [^{14}C]-nicotine in the presence of 100 μM of carvacrol.

Enan reported that carvacrol was an agonist of the octopamine receptor in American cockroach, with a high sensitivity (254% of control), based on cAMP production, heartbeat and [^{3} H]-octopamine binding to the receptor; however, dose–response relationships were not obtained.[16,17] Carvacrol and its isomer, thymol, were also demonstrated in *Drosophila melanogaster* and two nematode species, *Caenorhabditis elegans* and *Ascaris suum*, to interact with a tyramine receptor, which is a G-protein-associated receptor and negatively coupled to adenylate cyclase through interaction with the G_i-protein.[19] Carvacrol was shown to inhibit [^{3} H]-tyramine binding to the tyramine receptor expressed from S2 cells, and to mimic the function of tyramine to decrease the cAMP level and enhance the intracellular calcium levels in S2 cells expressing tyramine receptors; however, dose–response relationships were not obtained.[19] Ionotropic receptors, such as GABA receptors and nAChRs, can have an immediate and localized response to ligand activation, whereas activation of metabotropic receptors, octopamine or tyramine receptors, may have a slow but sustained and widespread response to ligand activation. Therefore, ionotropic receptors may contribute to faster knockdown and mortality of insects, because both GABA receptors and nAChRs are found in the neuromuscular junction in insects. In comparison with the ionotrophic receptors (nAChRs and GABA receptors) that have been studied to date, carvacrol binds more strongly to the housefly nAChR (1.4 μM) than the GABA receptor(48 μM), which indicates that nAChR is the primary target of carvacrol. Future evaluation of analogs of carvacrol and other terpenoids to determine their lethality and nAChR activity will allow correlations to be developed to determine the contributions of multiple mechanisms of action.

In addition to targeting these receptors of neurotransmitters, carvacrol was also identified as an activator of fruit fly thermotransient receptor potential (thermoTRP) channels, which control hot or cold sensation in insects.[42,43] Carvacrol and many other monoterpenoids were also observed to be inhibitors of non-thermoTRP channels, which respond to various environmental signals, including natural chemicals and mechanical stimuli, in *Drosophila* by using the whole-cell recording patch-clamp method from Schneider 2 and *Drosophila* photoreceptor cells.[44] It is not known whether the effects of monoterpenoids on TRP channels are related to their toxicity to insects.

In summary, the phenolic monoterpenoid insecticide carvacrol can bind to housefly nAChRs at a novel binding site. The binding of carvacrol to this binding site inhibits the binding of [^{14}C]-nicotine non-competitively. This finding provides promising evidence that the insect nAChR could serve as a novel target for monoterpenoid insecticides.

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