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Research

Interactions of DEET and Novel Repellents With Mosquito Odorant Receptors

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Abstract

The carboxamide *N,N*-di-ethyl-*meta*-toluamide (DEET) is the most effective and widely used insect repellent today. However, drawbacks concerning the efficacy and the safety of the repellent have led to efforts to design new classes of insect repellents. Through quantitative structure–activity relationships, chemists have discovered two chemical groups of novel repellents: the acylpiperidines and the carboxamides, with the acylpiperidines generally more potent in biological assays. Although the exact mechanism of action of DEET and other repellents has not yet been thoroughly elucidated, previous research shows that the activity of insect odorant receptors are inhibited in the presence of repellents. The present electrophysiological study employs two-electrode voltage clamp with *Xenopus laevis* oocytes expressing AgOR2/AgOrco and AgOR8/AgOrco receptors to assess the effects of the novel repellents on *Anopheles gambiae* Giles (Insecta: Diptera: Culicidae) mosquito odorant receptors. The novel acylpiperidines and carboxamides reversibly inhibited (12–91%) odorant-evoked currents from both AgOR2/AgOrco and AgOR8/AgOrco receptors in a dose-dependent manner at all tested concentrations (30 μM to 1 mM). Furthermore, all the novel agents were more potent inhibitors of the receptors than DEET, with the acylpiperidines producing on average greater inhibition than the carboxamides. Interestingly, there was a correlation (r^2 = 0.72) between the percentage inhibition of AgOR2/AgOrco receptor currents and protection times of the acylpiperidines. Our results add to existing evidence that the repellency of a compound is linked to its ability to disrupt the insect olfactory system and that the acylpiperidines could represent a class of more effective alternatives to the current gold standard, DEET.

Key words: odorant, receptor, DEET, repellent, oocyte

Mosquito-borne diseases such as malaria, dengue, and yellow fever are responsible for approximately 700,000 deaths annually (WHO 2017). Repellents disrupt interactions between humans and mosquitoes, playing a crucial role in preventing bites and disease transmission (Curtis 1992). *N,N*-Di-ethyl-*meta*-toluamide (DEET), a carboxamide, is currently the most effective and long-lasting commercially available mosquito repellent (Fradin and Day 2002, Katritzky et al. 2010). DEET is the active ingredient in most topically applied consumer products, including pressurized aerosols, pump sprays, creams, liquids, roll-ons, and towelettes, with DEET concentrations in products ranging from 5 to 100% in solution (Gilbert 1966).

Despite DEET's widespread and sustained use, the repellent has several shortcomings including relatively high cost of synthesis, plasticizing effects on polymers, toxicity to nontarget animals, limited efficacy against certain species of insects and ongoing concerns about safety to users (Oliferenko et al. 2013, Slaninova et al. 2014). Therefore, there is need for new classes of insect repellents to overcome the limitations associated with DEET's use (Katritzky et al. 2008). Criteria for an ideal repellent include protection against a broad range of arthropods, high efficacy for >8 h and no systemic toxicity to users (Fradin 1998). It is hypothesized that the chemical characteristics of a compound are important to its repellent activity and so in order to design new repellents, chemists utilized artificial neural networks to quantitatively assess structure–activity relationships (Katritzky et al. 2008). Two classes of novel repellents were revealed: the acylpiperidines and the carboxamides (Katritzky et al. 2008, 2010). Of the 72 novel compounds synthesized, 13 acylpiperidines and 4 carboxamides were chosen for further testing, including repellency assays with human volunteers from which a

rank order of repellent activity was derived using biological protection times (Katritzky et al. 2008, 2010).

Interestingly, despite the successful synthesis of potent insect repellents, the exact mechanism of action of DEET and other known repellents has not yet been thoroughly elucidated (Ditzen et al. 2008, Bohbot and Dickens 2010, DeGennaro 2015). Previous research proposed various molecular interactions of DEET that may correspond with its repellent activity, including acting as a feeding deterrent through gustatory neurons (Lee et al. 2010, Sanford et al. 2013), reducing contact through chemosensory mechanisms in insects' tarsi (Dennis et al. 2019), the inhibition of insect cholinesterase activity (Corbel et al. 2009), detection and direct avoidance through olfactory receptor neurons as well as odorant masking (Syed and Leal 2008, Afify et al. 2019), and most relevant to this study, effects on insect odorant receptors (Ditzen et al. 2008, Bohbot and Dickens 2010). It is postulated and has been shown in *Culex* and *Aedes* mosquitoes that DEET is first sensed in its vapor phase through neurons expressing odorant receptors, which play a vital role in conferring the ability to detect and respond appropriately to environmental chemical cues (Ha and Smith 2008, Syed and Leal 2008, DeGennaro et al. 2013). The genes encoding for insect odorant receptors are typically highly divergent, with an average of ~20% amino-acid identity shared even within a species (Vosshall 2000). The functional insect odorant receptors form a unique heterodimeric nonselective cation channel, comprised of a highly conserved co-receptor subunit, Orco. Orco is required for the assembly, traffic and functionality of the receptor, and combines with a divergent subunit (ORx) that allows for odorant specificity (Larsson et al. 2004, Butterwick et al. 2018). In previous studies, DEET has been shown to potently decrease the responses of odorant receptors cloned from the *Aedes aegypti* and *Anopheles gambiae* Giles (Insecta: Diptera: Culicidae) species of mosquitoes (Ditzen et al. 2008, Bohbot and Dickens 2010). Markedly, these species of mosquitoes among others have evolved to have a particular preference for humans (Besansky et al. 2004). Therefore, in the present study, we investigated the effects of the novel acylpiperidines and carboxamides on odorant receptors from the antennae of *An. gambiae sensu lato* mosquito, one of the most important vectors of malaria on the African continent (Katritzky et al. 2008, 2010).

Repellency assays performed with the novel repellents showed the novel acylpiperidines having on average, longer protection times compared to DEET and the novel carboxamides (Katritzky et al. 2008, 2010). We, therefore, hypothesized that the novel acylpiperidines would inhibit the odorant receptors more potently than the novel carboxamides. Although there are numerous mosquito odorant receptor combinations, due to availability and technical limitations, we assayed the AgOR2/AgOrco and AgOR8/AgOrco receptor combinations, which were among the first odorant receptors to be characterized as molecular targets for DEET (see Ditzen et al. 2008). Using the heterologous expression of the receptors in *Xenopus laevis* oocytes and standard two-electrode voltage clamp technique, we demonstrate a correlation between repellents' level of inhibition of the odorant receptors and protection time in human trials.

Materials and Methods

Xenopus laevis Oocytes Expression System

cDNA encoding for the AgOR2, AgOR8, and AgOrco mosquito odorant receptors were kindly provided by Dr. Laurence J. Zwiebel (Vanderbilt University, Nashville, TN). The receptor subunits were cloned in pSP64T vector and plasmids were linearized using EcoR1 restriction enzyme, with unique sites 3′ to the cloned cDNA. Linearized DNA was used as the template for mRNA synthesis using SP6 RNA polymerase for sense transcription (Thermo Fisher Scientific, Waltham, MA). Synthetic RNAs were treated with DNAaseI, purified with RNeasy kit (Qiagen, Hilden, Germany) and checked for quality by agarose gel electrophoresis.

AgOR2 and AgOR8were individually co-expressed with AgOrco in *Xenopus* oocytes. Ovaries from *Xenopus laevis* frogs were obtained from Xenopus One (Ann Arbor, MI) and oocytes were isolated from the lobe on the day of acquisition. The eggs were enzymatically defolliculated by treatment with 1 mg/ml collagenase A (Sigma–Aldrich, St. Louis, MO) in OR-2 media containing (mM): 82 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES (pH = 7.6) on a rotating platform for 50–80 min at room temperature. Oocytes were washed with and transferred to a solution (ND-96), containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES with 0.5% horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml sodium pyruvate (Life Technologies Corporation, Grand Island, NY), and 0.5 mM theophylline (Acros Organics, NJ). Stage IV and V oocytes were sorted and incubated at 16°C. Between 1 and 3 d after isolation, mRNA was injected into the oocytes using a Nanoject II (Drummond Scientific Co., Broomall, PA). For all eggs, injection volume was 36.8 nl with mRNA concentrations at ~60 ng/µl for all receptor combinations. Injected oocytes were maintained in solution at 16°C and then transferred to 4°C 2–3 d postinjection.

Electrophysiology

Between 1 and 7 d after mRNA injection, the oocytes were screened for currents evoked by 2-methyl phenol (10 µM) for the AgOR2/AgOrco combination and 1-octen-3-ol (10 µM) for the AgOR8/AgOrco combination. This was performed using standard two-electrode voltage clamp technique with an OC-75 C clamp (Warner Instruments Corp., Hamden, CT). Oocytes were individually placed in a small depression within a 100-µl oocyte chamber (Warner Instruments Corp.) and were continually superfused at 5 ml/min with ND-96 (without antibiotics, horse serum, sodium pyruvate, theophylline). Glass microelectrodes (World Precision Instruments, Sarasota, FL) were fabricated using a two-stage pull (Narishige, Tokyo, Japan) and backfilled with 3 M KCl. For all experiments, oocytes were voltage-clamped at –100 mV. Odorants, DEET, and all novel repellent stock solutions were sequentially dissolved in ND-96 immediately prior to use and gravity feed (5 ml/min) with an automated switching device (ALA Scientific Instr., Westbury, NY) used to expose eggs to solutions. All experiments were carried out at ambient room temperature (20–23°C). Evoked currents were digitized at 200 Hz and were recorded and analyzed using LabChart software (ADInstruments, Dunedin, NZ). Solution switches to the cognate ligand (in the presence or absence of repellents) were applied until currents were determined to reach peak amplitude to ensure accuracy in measuring inhibitions. Between solution switches, there was a 3- to 5-min exposure to control recording solution (ND-96) to allow sufficient washout and recovery from receptor desensitization.

Dilutions of DEET and novel repellents (30 µM to 1 mM) were prepared by adding quantities of 1 M stock solutions in dimethyl sulfoxide (DMSO) to the ND-96 recording solution. Reservoirs for control and drug applications contained equivalent DMSO concentrations up to 0.1% (determined to have negligible effects on receptor currents, data not shown). DEET and receptor ligands were purchased from Sigma–Aldrich, while novel repellents were synthesized (Katritzky et al. 2008) and obtained from the United States Department of Agriculture (USDA).

For the AgOR2/AgOrco receptor combination, currents were alternately elicited by an odorant followed by the same odorant in the presence of a repellent. The current elicited by the odorant only, before and after the application of an odorant in the presence of a repellent was used as the control current amplitude. For the AgOR8/AgOrco receptor combination, there was significant current run-down noted, and so after application of two pulses of control odorant at the beginning and end of the experiment, a linear run-down of current was assumed and used to estimate control current amplitude throughout the duration of the recording. For both receptor combinations, the inhibition of a current evoked by an odorant in the presence of a repellent was calculated as a fraction of the respective control amplitude and converted to the percentage values reported throughout the study. Further analyses of acquired currents were carried out using Origin software (OriginLab Corp., Northampton, MA). All collated data are expressed as mean ± SEM, calculated from at least $n = 5$ individual oocytes for each data point unless stated otherwise.

In vivo Protection Assays

Details of the in vivo mosquito protection time assay are reported in Katritzky et al. (2008). In brief, to determine protection times, 2.5 and 25 µl/cm^3 of each repellent was placed in a vial and a 50-cm^2 muslin cloth was inserted into the vial. The vial was stored at –4°C until biological testing. On the first day of testing, the cloth was removed from the vial, mounted unto a card stock (5 × 2.5 cm), dried, and then placed over a human volunteer's arm, allowing exposure to the cloth. For each consecutive day, the arm was placed into a cage of *Aedes aegypti* mosquitoes for 1 min and the number of blood-feeding mosquitoes was counted. The failure point for these experiments was 5 bites or 1% of the cage population obtaining blood. Consequently, if a repellent failed to protect the arm from 5 bites on the 14th day of testing, that repellent's protection time would be noted as 13 d. Protection times for the repellents tested in this study ranged from 1.0 to 13.5 d.

Results

Xenopus laevis oocytes expressing AgOR2/AgOrco and AgOR8/AgOrco receptors were used to investigate the effects of novel acylpiperidines and carboxamides (Fig. 1) on mosquito odorant receptors. Cation currents elicited from the receptors by odorants were reversibly blocked by the application of ND-96 with Na^+ replaced with NMDG (data not shown). Absolute currents elicited by these odorants (10 µM) ranged from 0.01 to >1 µA for both receptor combinations. The carboxamides, DEET and **5g′** as well as **4o′** (an acylpiperidine) inhibited currents evoked by 2-methyl phenol (10 µM, Fig. 2A) from the AgOR2/AgOrco receptor combination and currents evoked by 1-octen-3-ol (10 µM, Fig. 2B) from the AgOR8/AgOrco receptor combination. Currents elicited from AgOR2/AgOrco and AgOR8/AgOrco receptors were dose-dependently blocked by DEET at 30 and 300 µM and by the acylpiperidine, **4o′** and the novel carboxamide, **5g′**. The effects of all repellents were reversible upon washout, evident in the return of the control current following repellent application (although current run down was noted in AgOR8/AgOrco recordings). Among the three repellents,

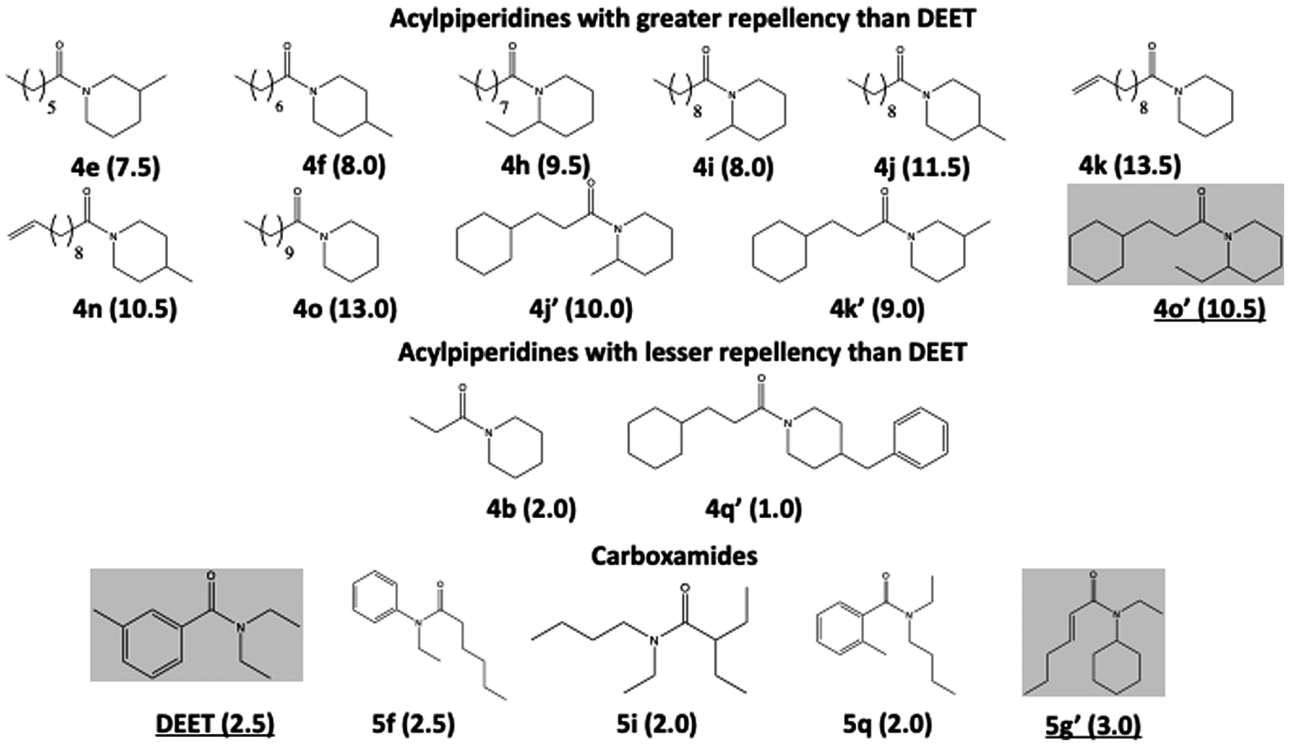

Fig. 1. Molecular structures of the novel repellents and DEET, as well as their respective repellent activity noted in parentheses (protection time in days for compounds applied at 2.5 µM/cm^2, see Katritzky et al. 2008). The chemical names for the repellents are as follows: 4e: 1-heptanoyl-3-methylpiperidine, 4f: 1-octanoyl-4-methylpiperidine, 4h: 1-nonanoyl-2-ethylpiperidine, 4i: 1-decanoyl-2-methylpiperdiine, 4j: 1-decanoyl-4-methylpiperidine, 4k: 1-undec-1-enoylpiperidine, 4n: 1-undec-1-enoyl-4-methylpiperidine, 4o: 1-undecanoylpiperidine, 4b: 1-ethanoylpiperidine, 4j′: 2-cyclohexylethyl-2-methylpiperonamide, 4k′: 2-cyclohexylethyl-3-methylpiperonamide, 4o′: 2-cyclohexylethyl-2-ethylpiperonamide, 4q′: 2-cyclohexylethyl-4-benzylpiperonamide, DEET: *N*,*N*-diethyl-m-toluamide, 5f: *N*-ethyl,*N*-phenyl-1-hexamide, 5i: *N*-butyl,*N*-ethyl-3-oxo-propylamide, 5q: *N*-butyl,*N*-ethyl-*o*-toluamide, and 5g′: *N*-benzyl,*N*-ethyl-1-oxo-hexanamide. The repellents will be referred to as the labels noted in the figure throughout the study. Compounds studied in further detail are denoted by underlined labels.

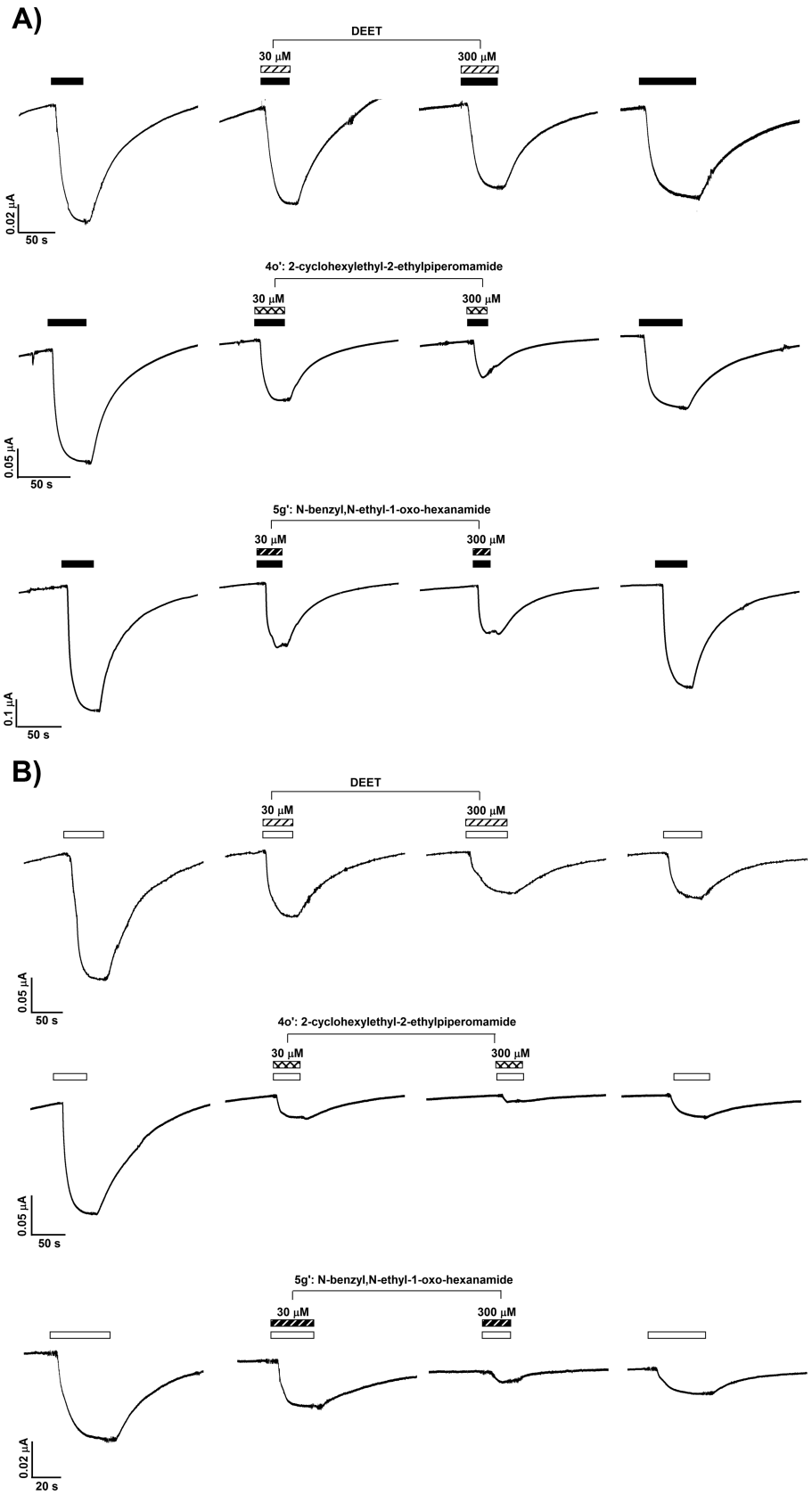

Fig. 2. DEET and novel repellents reversibly inhibit AgOR2/AgOrco and AgOR8/AgOrco. A) Current traces illustrate repellent inhibition of responses evoked by 2-methyl phenol (10 μM) from oocytes expressing the AgOR2/AgOrco receptor combination. Co-application of the ligand (filled bar) with DEET (striped bar), the acylpiperidine, 4o′ (checkered bar), and the carboxamide, 5g′ (filled striped bar) inhibited receptor currents in a reversible manner. B) Current traces illustrate repellent inhibition of responses evoked by 1-octen-3-ol (10 μM) from oocytes expressing AgOR8/AgOrco receptor combination. Co-application of the ligand (open bar) with DEET (striped bar), the acylpiperidine, **4o**′ (checkered bar), an acylpiperidines, and the carboxamide, **5g**′, (filled striped bar) inhibited receptor currents in a reversible manner.

the acylpiperidine, **4o′** was the most potent inhibitor of both receptor combinations at 300 μM. The co-application **4o′** (300 μM) with 10 μM of the cognate ligand, inhibited AgOR2/AgOrco currents by 51.2 ± 0.9% and AgOR8/AgOrco currents by 90.0 ± 4.0%. The effects of DEET, **4o′** and **5g′** on the receptors showed dose-dependency when co-applied with the respective odorant (Fig. 3). The novel repellents (**4o′** and **5g′**) were more potent inhibitors than DEET at concentrations tested (100 μM to 1 mM). AgOR8/AgOrco currents were more sensitive to block by the repellents at all concentrations tested. For example, **5g′** produced a 72.1 ± 2.5% block of AgOR2/AgOrco currents (Fig. 3A) and a 93.6 ± 1.0% block of AgOR8/AgOrco currents (Fig. 3B). DEET, **4o′** and **5g′** elicited negligible directly activated currents (data not shown).

All the novel repellents inhibited AgOR2/AgOrco (Fig. 4A) and AgOR8/AgOrco (Fig. 4B) receptor currents when co-applied at 300 μM with 10 μM 2-methyl phenol for AgOR2/AgOrco

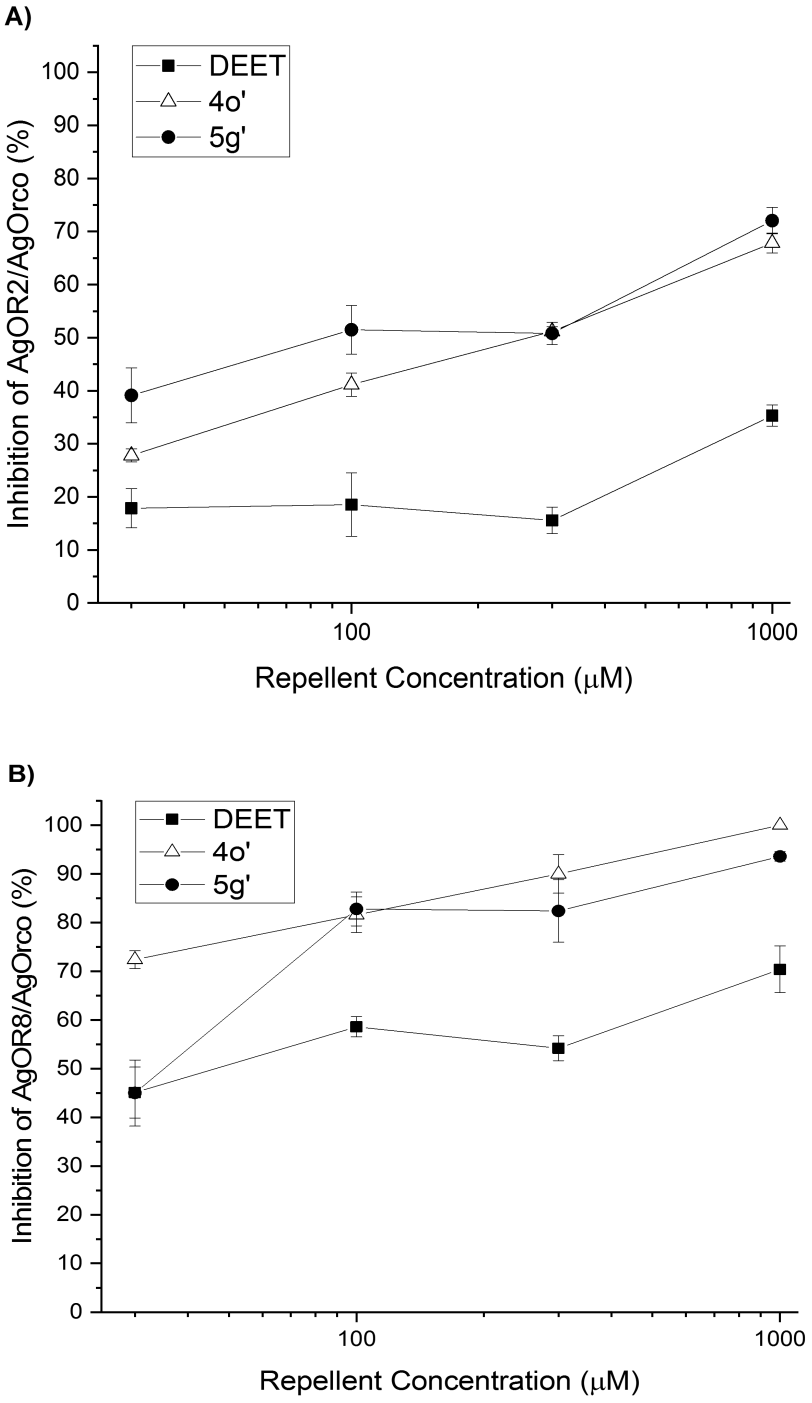

Fig. 3. DEET and novel repellents inhibit AgOR2/AgOrco and AgOR8/AgOrco in a dose-dependent manner. A) Concentration-response for inhibition of currents evoked by 2-methyl phenol (10 μM) in oocytes expressing AgOR2/AgOrco receptors by DEET, **4o′** and **5g′** (30 μM to 1 mM). Data points represent mean inhibition ± SEM. for $n \geq 5$ oocytes. B) Concentration-response for inhibition of currents evoked by 1-octen-3-ol (10 μM) in oocytes expressing AgOR8/AgOrco receptors by DEET, **4o′** and **5g′** (30 μM to 1 mM). Data points represent mean inhibition ± SEM for $n \geq 5$ oocytes.

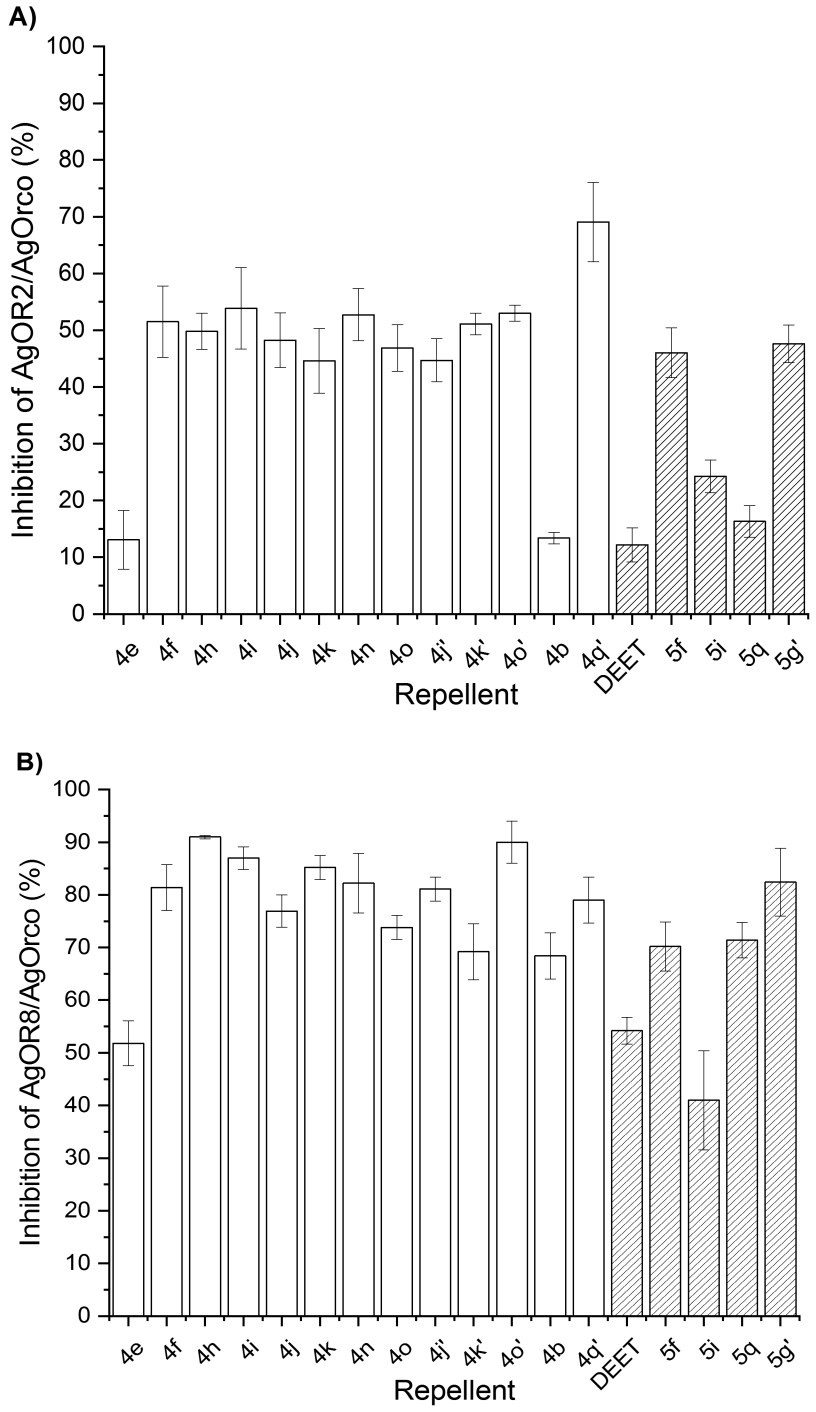

Fig. 4. DEET and all the novel repellents inhibit AgOR2/AgOrco and AgOR8/AgOrco receptor currents. A) Inhibitions of currents evoked by 2-methyl phenol (10 μM) by co-application of novel acylpiperidines (open bars), DEET. and novel carboxamides (striped bars, 300 μM). All repellents inhibited odorant-evoked currents from oocytes expressing AgOR2/AgOrco receptors. Data represent mean inhibition ± SEM for $n \geq 5$ oocytes. B) Inhibitions of currents evoked by 1-octen-3-ol (10 μM) by co-application of novel acylpiperidines (open bars), DEET and novel carboxamides (striped bars, 300 μM). All repellents inhibited odorant-evoked currents from oocytes expressing AgOR8/AgOrco receptors. Data represent mean inhibition ± SEM for $n \geq 5$ oocytes.

receptors or 10 μM 1-octen-3-ol for AgOR8/AgOrco receptors. Acylpiperidines inhibited both receptors more potently, producing mean inhibitions of 45.5 ± 4.3% for AgOR2/AgOrco receptor currents and 78.2 ± 3.0% for AgOR8/AgOrco receptor currents. By comparison, carboxamides inhibited the receptors with mean inhibitions of 29.3 ± 7.4% for AgOR2/AgOrco receptor currents and 63.8 ± 7.3% for AgOR8/AgOrco receptor currents. The acylpiperidine **4q′** was the most potent inhibitor of AgOR2/AgOrco currents, blocking by 69.1 ± 7.0%, whereas the acylpiperidine **4h** was the most potent inhibitor of AgOR8/AgOrco currents, blocking by 91.0 ± 0.3%. There was a significant difference between the percentage inhibitions evoked by all repellents on the AgOR2/AgOrco receptors and on the AgOR8/AgOrco receptors (two-tailed *t*-test with $P < 0.01$) with all

repellents producing more potent blocks of AgOR8/AgOrco compared to AgOR2/AgOrco currents.

Finally, although the acylpiperidines were the more potent inhibitors, there was substantial variation in percentage block, particularly observed for AgOR2/AgOrco receptors. Interestingly, there was a strong correlation between percentage inhibitions of AgOR2/AgOrco currents and the protection times of the repellents (Fig. 5, $r^2 = 0.72$). Notable outliers included the acylpiperidines **4e** and **4q′**, the latter of which has a unique, bulkier structure in comparison to the other repellents (see Fig. 1). In the correlational analysis, it was evident that there was clustering observed for the acylpiperidines and carboxamides given their relative inhibitions and protection times for the individual chemical groups. Furthermore, there was no correlation between the protection time of the repellents and percentage inhibitions of the AgOR8/AgOrco currents ($r^2 = 0.25$, graph not shown).

Discussion

In this study, we investigated the actions of DEET and novel repellents on AgOR2/AgOrco and AgOR8/AgOrco mosquito odorant receptors. Initially, we found that DEET and the novel repellents reversibly inhibit both AgOR2/AgOrco and AgOR8/AgOrco receptor currents. Second, focusing on DEET, one novel acylpiperidine and a novel carboxamide, we found that the repellents inhibited both receptor currents dose dependently with concentrations tested (30 μM to 1 mM). Additionally, we found that almost all the novel agents were more effective than DEET in blocking *Anopheles gambiae* AgOR2/AgOrco and AgOR8/AgOrco receptor currents. In comparing the acylpiperidines and carboxamides, we found that the acylpiperidines were the more potent inhibitors of both receptor combinations. Finally, we found that the percentage inhibition of AgOR2/AgOrco receptor currents correlated with the protection time of the repellents against *Aedes aegypti* mosquitoes ($r^2 = 0.72$).

Affecting the measure of this correlation coefficient, it was apparent that the distribution of carboxamides and acylpiperidines were clustered with the carboxamides having similar protection times and most acylpiperidines exhibiting similar levels of odorant receptor inhibition (Fig. 5). There were some exceptions to the latter, with **4q′** producing the highest levels of inhibition (longest chain acylpiperidine) and with **4b** and **4e** producing minimal receptor inhibition (suggesting a 'cut off' whereby at least six carbons are required in the aliphatic chain in order to confer significant inhibitory activity). Additionally, this study focused on two of multiple *Anopheles gambiae* mosquito odorant receptors and compared their activity with protection times based on in vivo testing with *Aedes aegypti* mosquitoes. Considering the highly divergent nature of mosquito odorant receptors, the correlation analysis may be limited in this respect, although the AgOR2 subunit used in our analyses, has been shown to be highly homologous between both species, sharing 70.5% identity (Bohbot et al. 2007). Furthermore, with the use of a heterologous expression system and repellent compounds that may have varying volatilities, it is difficult to ascertain whether experimental concentrations are equivalent to what receptors would be exposed to in vivo. For instance, it should be noted that a recent in vivo study testing *Anopheles* mosquitoes found that DEET was not a repellent odor to the mosquitoes, and did not inhibit the function of the olfactory receptor neurons (Afify et al. 2019). These results might reflect differences in repellent concentrations and/or assay designs. Finally, there has been evidence from in vivo studies that there may be direct chemical interactions between odorant and repellent molecules (Syed and Leal 2008, Afify et al. 2019), which cannot be ruled out in the current study. Nevertheless, data obtained are similar to other studies in the field, which indicate that repellency may be linked to the ability to disrupt the insect olfactory system (Ditzen et al. 2008, Syed and Leal 2008, DeGennaro et al. 2013.) However, it is likely that there are other molecular targets and sensory systems that influence repellent activity that were not assessed in this

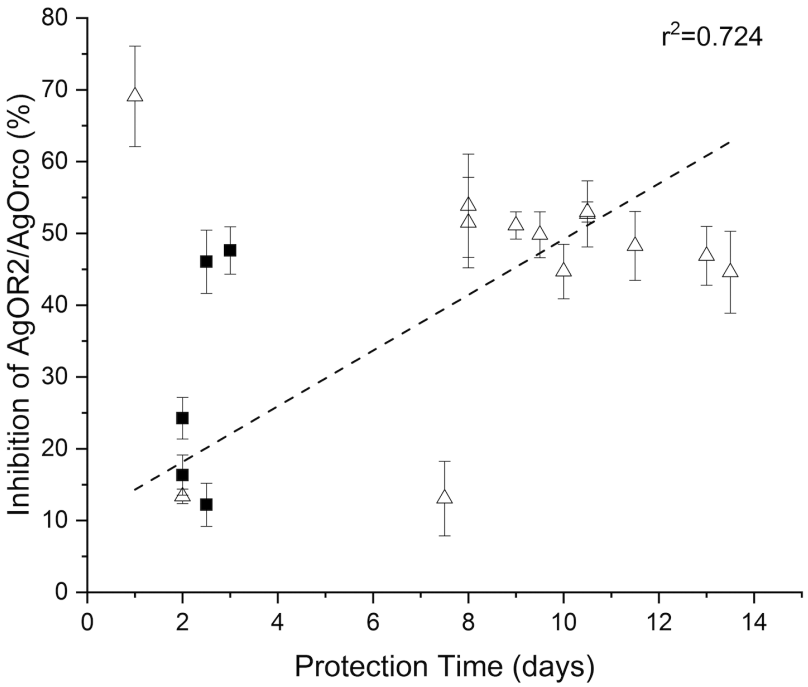

Fig. 5. Correlation between repellent activity of the novel repellents (protection time in days) and the percentage inhibition of currents evoked by 2-methyl phenol (10 μM) in oocytes expressing AgOR2/AgOrco receptors. Closed symbols denote carboxamides (including DEET). while open symbols denote acylpiperidines.

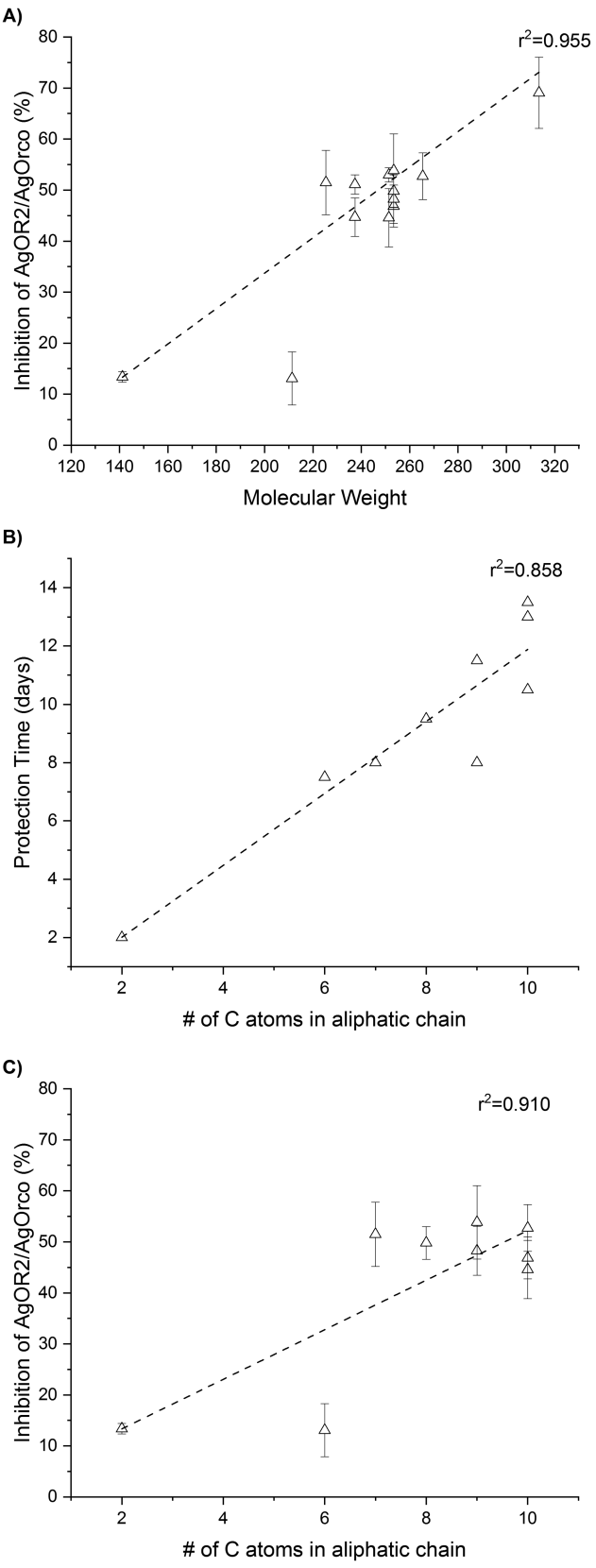

Fig. 6. Percentage inhibitions of AgOR2/AgOrco currents correlate with repellent activity and number of carbon atoms present in aliphatic chains of select acylpiperidines correlates with repellent activity and percentage inhibitions of AgOR2/AgOrco currents. A) Correlation between molecular weight of all acylpiperidines and percentage inhibition of currents evoked by 2-methyl phenol (10 μM) in oocytes expressing AgOR2/AgOrco receptors. B) Correlation between number of carbon atoms present in aliphatic chains of select acylpiperidines (**4e**, **4f**, **4h**, **4i**, **4j**, **4k**, **4n**, **4o**, **4b**) and their repellent activity (protection time in days). C) Correlation between number of carbon atoms present in aliphatic chains of select acylpiperidines (**4e**, **4f**, **4h**, **4i**, **4j**, **4k**, **4n**, **4o**, **4b**) and percentage inhibition of currents evoked by 2-methyl phenol (10 μM) in oocytes expressing AgOR2/AgOrco receptors.

study. For example, while the exact mechanism of action of insect repellents is still the subject of ongoing research, DEET and other repellents have been shown to act as mosquito feeding deterrents through gustatory receptors on the labella of the mouthparts and also repel mosquitoes by way of chemosensory mechanisms on insects' tarsi (Lee et al. 2010, Sanford et al. 2013, Dennis et al. 2019).

In pursuing further correlational analyses on the more potent class of repellents, we found a correlation between the molecular weight of the acylpiperidines and the percentage inhibition of AgOR2/AgOrco receptor currents (r^2 = 0.96, Fig. 6A). We postulate that the acylpiperidines are able to bind and occupy a pocket within the receptor, allowing for the antagonist effects observed. The cryo-electron microscopy structure of the insect odorant receptor Orco subunit shows that within the extracellular leaflet, there are several residues within a pocket that may serve as a binding site for small molecule ligands (Butterwick et al. 2018). Therefore, while the present study measures repellent effects on two receptors combinations, it is possible that other OR variants would bind repellents in an equivalent manner given the highly conserved nature of Orco among receptor variants. In regards to the repellents tested in the current study, there may be an upper-limit whereby an acylpiperidine is too large to bind within the receptor pocket although this limit was not reached in this study, with the largest acylpiperidine tested, **4q′** (molecular weight = 313.48) being the most potent inhibitor of AgOR2/AgOrco receptor currents. Aside from insect repellents, there are other known potent inhibitors of *Anopheles* odorant receptors, including VU0183254, which were specially designed as receptor antagonists (Jones et al. 2012). Interestingly, VU0183254, DEET and all the novel repellents evaluated in this study, possess an amide carbonyl group, which is hypothesized to play a role in ligand–receptor noncovalent bonding as well as repellent activity (Katritzky et al. 2008, Jones et al. 2012).

We also performed correlational analyses on a subset of acylpiperidines (**4e**, **4f**, **4h**, **4i**, **4j**, **4k**, **4n**, **4o**, **4b**) that were characterized by similar molecular structures but varying lengths of aliphatic chain (see Fig. 1). We found strong correlations between the number of carbon atoms in the aliphatic chain and the protection time of the compounds (r^2 = 0.86, Fig. 6B) as well as the percentage inhibition of AgOR2/AgOrco receptor currents (r^2 = 0.91, Fig. 6C). It was evident that the acylpiperidines need at least six carbon atoms in their aliphatic chain in order to be potent inhibitors (Fig. 6C). These analyses are in agreement with the correlation between molecular weight and percentage inhibition of AgOR2/AgOrco receptors. Notably, while the repellents tested produced more potent inhibitions of AgOR8/AgOrco receptor currents compared with AgOR2/AgOrco receptor currents, there was no correlation found between percentage inhibition of AgOR8/AgOrco receptor currents and protection time of the repellents. We attribute this to the fact that most repellents produced blocks >70%, and with such high levels of inhibition, there was less of a dynamic range to assess relative potency for inhibition.

Additionally, while there was a strong correlation between molecular weight and percentage inhibition of AgOR2/AgOrco receptor currents, there was no correlation found between molecular weight and the protection time of the repellents (r^2 = 0.17, data not

shown). In addition to molecular targets, properties of a chemical including the size of the molecule, volatility, lability, and cloth or dermal absorption may contribute to the protection time for any given repellent.

From this and previous studies (Ditzen et al. 2008, DeGennaro et al. 2013), we conclude that AgOR2/AgOrco and AgOR8/AgOrco receptor responses to odors can be inhibited by DEET and novel repellents, offering a mechanism of odor-directed repellency. Furthermore, there was a correlation between the molecular structure of the more potent class of repellents, the acylpiperidines, and their repellent activity. This study can inform future research aimed at deriving compounds that can act as alternatives to the current gold standard of DEET. Specifically, chemical properties such as molecular weight and the length of an aliphatic chain can be used as predictors of odorant receptor activity and protection times to design future novel agents.

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