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Interaction of plant essential oil terpenoids with the southern cattle tick tyramine receptor: A potential biopesticide target

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ABSTRACT

An outbreak of the southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, (Canestrini), in the United States would have devastating consequences on the cattle industry. Tick populations have developed resistance to current acaricides, highlighting the need to identify new biochemical targets along with new chemistry. Furthermore, acaricide resistance could further hamper control of tick populations during an outbreak. Botanically-based compounds may provide a safe alternative for efficacious control of the southern cattle tick. We have developed a heterologous expression system that stably expresses the cattle tick's tyramine receptor with a G-protein chimera, producing a system that is amenable to high-throughput screening. Screening an in-house terpenoid library, at two screening concentrations (10 μM and 100 μM), has identified four terpenoids (piperonyl alcohol, 1,4-cineole, carvacrol and iso-eugenol) that we believe are positive modulators of the southern cattle tick's tyramine receptor.

1. Introduction

Rhipicephalus (*Boophilus*) *microplus* (Canestrini), hereafter referred to as the southern cattle tick, is an external parasite that is capable of transmitting the causative agents (*Babesia* spp.) that result in bovine babesiosis, also referred to as cattle fever or red water fever. The United States has essentially eradicated the southern cattle tick with the exception of a permanent quarantine zone in southern Texas. Coumaphos, an organophosphate insecticide/acaricide, has been the primary tool used by the Cattle Fever Tick Eradication Program (CFTEP) in the United States. However, historically the control of the southern cattle tick has been achieved by using other chemical insecticides with various mechanisms of action, both within and outside of the United States. As a result of the use of chemical insecticides, some having a similar biochemical mechanisms of action, widespread acaricide resistance has been reported [6,7,18,19,23,24,27–30,41,44,48]. What is most concerning, with regards to acaricide resistance, is the report of a strain of "super tick" that has become resistant, probably through high selective pressure, to multiple acaricides with different biochemical mechanisms of action [13].

The increase of acaricide resistance in the southern cattle tick has resulted in an urgent need to find alternatives, including new chemistry, to be used in an integrative approach to control the southern cattle tick. Recently, there has been a growing interest in the use of botanical acaricides, particularly essential oils, to control *R. microplus* and *R. annulatus* [8,22,26,36,37,39,40,46]. For instance, the essential oil from cumin seeds (*Cuminum cyuminum*) and allspice berries (*Pimenta dioica*) resulted in 100% mortality at concentrations between 2.5% and 20% (v/v). However, not all essential oils are toxic to the southern cattle tick; the essential oil from basil leaves did not display any toxicity at concentrations up to 20% (v/v) [26]. Prates et al. investigated the toxicity of components from molasses grass (*Melinis minutiflora*), and found that some individual components (*e.g.* 1,8-cineole) were able to result in 100%

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mortality alone when compared to the essential oil toxicity [37]. Additionally, it was suggested that essential oils have a synergistic activity to the benzopyran precocene II, which is found in *Calea serrate* essential oil [39]. While toxicity and repellency of plant essential oils and their terpenoid constituents have been recognized for some time, the precise biochemical mechanism of action has yet to be fully understood, especially in ticks. In fact, several mechanisms of toxic action have been proposed, which include the inhibition of acetylcholinesterase [14,25,31,32,35,47], activity at the γ-aminobutyric acid ($GABA_A$) receptor [38,50,51], the nicotinic acetylcholine receptor [52], inhibition of the transient receptor potential (TRP) channels [34], octopamine receptors [9,11,17,21], and tyramine receptors [10]. While essential oils are less toxic than conventional synthetic acaricides, they can still be effective at controlling the southern cattle tick. Additionally, essential oils may provide an increased level of safety for cattle, non-target organisms, and the environment, when compared to conventional synthetic acaricides.

Tyramine is a biogenic monoamine that has been found in a variety of invertebrates, including insects. Tyramine was initially thought to be only important in the synthesis of octopamine; however, tyramine has been shown to be biologically active independent of octopamine [12,42,43,53]. A putative octopamine receptor [1,5], now re-classified as a tyramine receptor [15], has been identified in *R. microplus*. Heterologous expression of this tyramine receptor indicated it couples to the inositol pathway when expressed with a $G\alpha_{qi(5)}$-protein chimeria [15]. In addition, tyramine receptors have been identified in a variety of organisms including *Drosophila melanogaster* [2,4], *Bombyx mori* [20,33], *Chilo suppressalis* [54], *Periplaneta americana* [45] and *Apis mellifera* [3]. The objective of this study was to determine if essential oil terpenoids could interact with a tyramine receptor from the southern cattle tick.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted: (+) α-pinene 98%, isoeugenol 98%, eugenyl acetate (Berje Inc., Carteret, NJ), carvacrol 98%, vanillin 99%, piperonyl alcohol 98%, citronellic acid 98%, (±)-camphor 96%, ±-linalool 98%, piperonal 98%, limonene 97%, (±)-limonene oxide (97%), 1,4-cineole (85%) and (+)-pulegone (97%). Test compounds were diluted in certified DMSO (Fisher Scientific), and then serially diluted in Hank's Balanced Salt Solution (HBSS, Life Technologies, Carlsbad, CA). The final concentration of DMSO used in experiments (exposed to cells) was 0.1%. Compounds were prepared and frozen (−20 °C) prior to use, compounds were thawed only once for use in assays.

2.2. Cloning, CHO cell culture and transfection

The southern cattle tick's tyramine receptor, RmTAR1, was originally isolated from the Gonzalez strain (amitraz-susceptible strain). The Gonzalez strain originated in Zapata County, Texas in 1984, and has been maintained in culture by the USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL), Kerrville, TX at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL), Edinburg, TX [5]. RmTAR1 cDNA was synthesized by GeneScript USA Inc. (Piscataway, NJ), and cloned into a pCDNA3.1(-) expression vector (Life Technologies, Carlsbad, CA) for expression in Chinese hamster ovary (CHO or CHO-K1) cells (ATCC, Manassas, VA). Cell culture materials were obtained from Life Technologies, unless otherwise stated. CHO cells were maintained in a NuAire humidified water jacket incubator (Plymouth, MN); the incubator was maintained at normal cell growth conditions (37 °C, 90% humidity and 5% CO_2). Cells were maintained in 1X Ham's F12K (Kaighn's modification) medium supplemented with 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid). CHO cells were subcultured when cell confluence reached 80%–90%.

Stable transfection of CHO cells with RmTAR1 along with a $G\alpha_{qi(5)}$ G-protein chimera was performed in 96-well cell culture plates (Corning), as previously described [15]. Briefly, CHO cells were transfected using Lipofectamine 2000® (Life Technologies) with OPTI-MEM (Life Technologies) supplemented with normal cell growth medium, according to the manufacturer's protocols. Cells were transfected with 2.5 μg of RmTAR1 plasmid cDNA and 2.5 μg of $G\alpha_{qi5}$ plasmid cDNA. Transfected cells were maintained in normal growth medium containing 100 μg/mL of Geneticin® (G418 sulfate; Corning). Single-cell colonies were selected in 96-well plates over four weeks. Successfully stably transfected cells were determined with a calcium mobilization assay and by RT-PCR.

2.3. Calcium mobilization assay

CHO cells that were stably transfected with cDNA coding for the RmTAR1 and $G\alpha_{qi(5)}$ were counted using a Countess® Automatic Cell Counter (Life Technologies), according to manufacturer's protocols. CHO cells were placed into a black-wall clear-bottom 96-well plate (Corning) at a cell density of 27,500 cells per well, total volume of 100 μL. Cells were incubated, at normal cell culture conditions, for 24-hr prior to performing the calcium mobilization assay. The calcium mobilization assay was performed with a Fluo-4 NW Calcium Assay Kit (Life Technologies), according to the manufacturer's protocol. Briefly, the cells were incubated in HBSS (Hank's Balanced Salt Solution), 20 mM HEPES, a calcium-sensitive dye (Fluo-4), and 2.5 mM probenecid; cells were incubated in this medium for 90 min at 37 °C prior to the start of the assay. Fluorescence was monitored using a FlexStation 1 (Molecular Devices, Sunnyvale, CA). Basal fluorescence was monitored for 20 s prior to the addition of screening compounds. The fluorescence readings were measured every 1.5 s for 120 s. To monitor the effect of terpenoids against RmTAR1 a minimum of four wells per plate with a minimum of four plates (cells from four different passages) were utilized for each agonist or antagonist screen. Agonist screens were performed at 10 μM, and antagonist screening was performed at 10 and 100 μM. These concentrations of terpenoids were chosen based on preliminary range-finding experiments. Several controls for each plate were performed, which included blank wells (calcium fluorescent indicator, probenecid and HBSS with HEPES buffer (10 mM), and DMSO vehicle). Autofluorescence of each of the test compounds was also monitored and contained wells that had the test compound at each concentration, calcium fluorescent indicator, probenecid, and HBSS with HEPES buffer, but did not contain cells. Each plate also contained cells expressing the receptor and chimeric G-protein exposed to the vehicle (vehicle control). Non-receptor mediated effects were monitored for each treatment, and consisted of cells not expressing the receptor (regular CHO-K1 cells), but were transfected with the G-protein chimera and were exposed to the various terpenoids treatments (control for the verification of RmTAR1 response).

2.4. Data analysis

Concentration-response curves were analyzed in GraphPad Prism (La Jolla, CA). One-way ANOVA ($\alpha = 0.05$) and a paired *t*-test were used to test the data for statistically significant differences using SAS 9.3 (Cary, NC). Student-Newman-Keuls (SNK) post-hoc analysis was used to determine significant difference between

treatments with 95% confidence ($\alpha = 0.05$).

3. Results

RmTAR1 was successfully expressed and coupled to a chimeric $G\alpha_{qi(5)}$ protein; recently a pharmacological assessment of RmTAR1 has been described [15]. A tyramine concentration-dependent response of CHO cells stably expressing RmTAR1 along with the G-protein chimera ($G\alpha_{qi(5)}$) resulted in an EC_{50} of 15 nM [15]. The stably transfected cells expressing this tyramine receptor and $G\alpha_{qi(5)}$ chimera provides a system, amenable to high-throughput screening for functional analysis of compounds interacting with this tyramine receptor.

Monoterpenoids and related aromatic compounds (Fig. 1) were examined as potential ligands against CHO cells stably expressing RmTAR1 plus the G-protein chimera. Terpenoids with a variety of functional groups, including hydrocarbons, ketones, oxides, aldehydes, ethers, acids and alcohols were examined (Fig. 1). Screening of essential oil terpenoids was performed to determine if they could interact with and activate the receptor alone (agonist screen) or interact with/modulate the tyramine receptor in the presence of the receptor's endogenous ligand, tyramine. Agonist screening was performed at a single concentration of 10 μM, and calcium fluorescence was monitored in real-time (Fig. 2), whereas antagonist/modulation screening was performed at 10 μM and 100 μM, and the compounds were preincubated with cells for 30 min prior to the addition of 150 nM tyramine (Fig. 3).

Pulegone, a cyclic monoterpenoid, was the only screened terpenoid identified to agonize (activate) the receptor at 10 μM (Fig. 2). Pulegone increased the relative fluorescence, presumably by interacting with the expressed tyramine receptor (RmTAR1), which interacted with the expressed $G\alpha_{qi(5)}$ chimera, resulting in the increase of intracellular calcium. α-Pinene numerically decreased the intracellular calcium, compared to the vehicle, but this was not statistically significant. Agonist screening was not performed at 100 μM because of the high fluorescence of compounds in cells not expressing RmTAR1; however, these off-target effects were not observed when compounds were pre-incubated.

Terpenoids were also screened as antagonists or modulators against the expressed tyramine receptor (RmTAR1) at 10 μM and 100 μM (Fig. 3). For the antagonistic or modulator screening, compounds were incubated with CHO cells expressing the RmTAR1 and G-protein chimera ($G\alpha_{qi(5)}$, for 30 min, and calcium fluorescence was monitored upon the addition of tyramine at 150 nM; data were normalized to the 150-nM tyramine response (100%). At 10 μM, two compounds (carvacrol and isoeugenol) were able to significantly increase the tyramine response. Carvacrol and isoeugenol increased calcium fluorescence elicited by 150 nM tyramine by 320% and 337%, respectively (Fig. 3). Antagonistic or modulatory effects of essential oil terpenoids were also examined

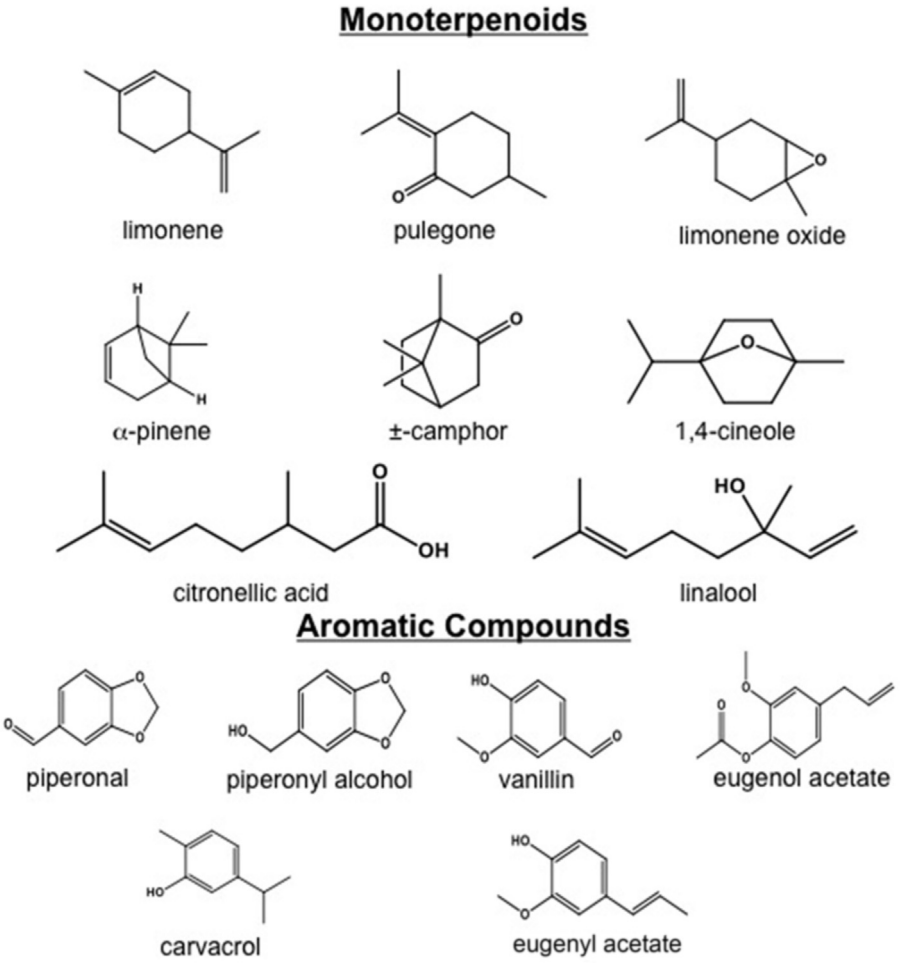

Fig. 1. Terpenoid chemical structures.
Monoterpenoids and related aromatic compounds containing various functional groups, were investigated against the recombinant RmTAR1 expressed in CHO cells with a $G\alpha_{qi(5)}$ chimera.

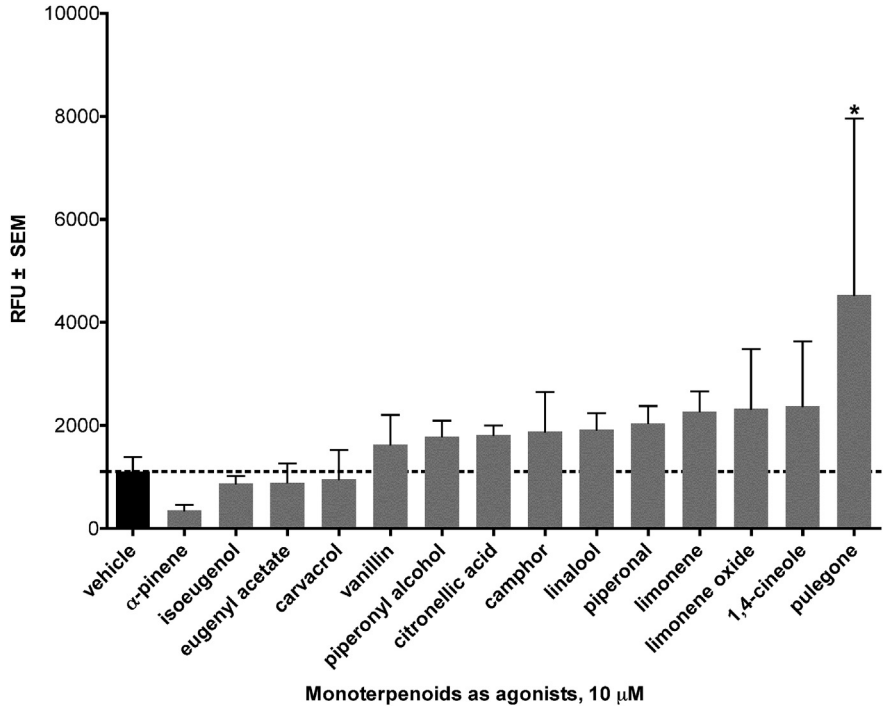

Fig. 2. Terpenoids as agonists against RmTAR1 and a G-protein chimera. Several monoterpenoids and related aromatic compounds were screened at 10 μM to determine if they could activate or agonize the heterologously expressed RmTAR1 plus $G\alpha_{qi(5)}$. Each terpenoid or related aromatic compound assay was replicated four times from different passages of cells (each replicated consisted of a treatment, blank, autofluorescent control, vehicle control, non-receptor mediated control in a minimum of four wells). An asterisk indicates a statistically significant difference, compared to the vehicle (0.1% DMSO). Analysis was performed with one-way ANOVA ($\alpha = 0.05$).

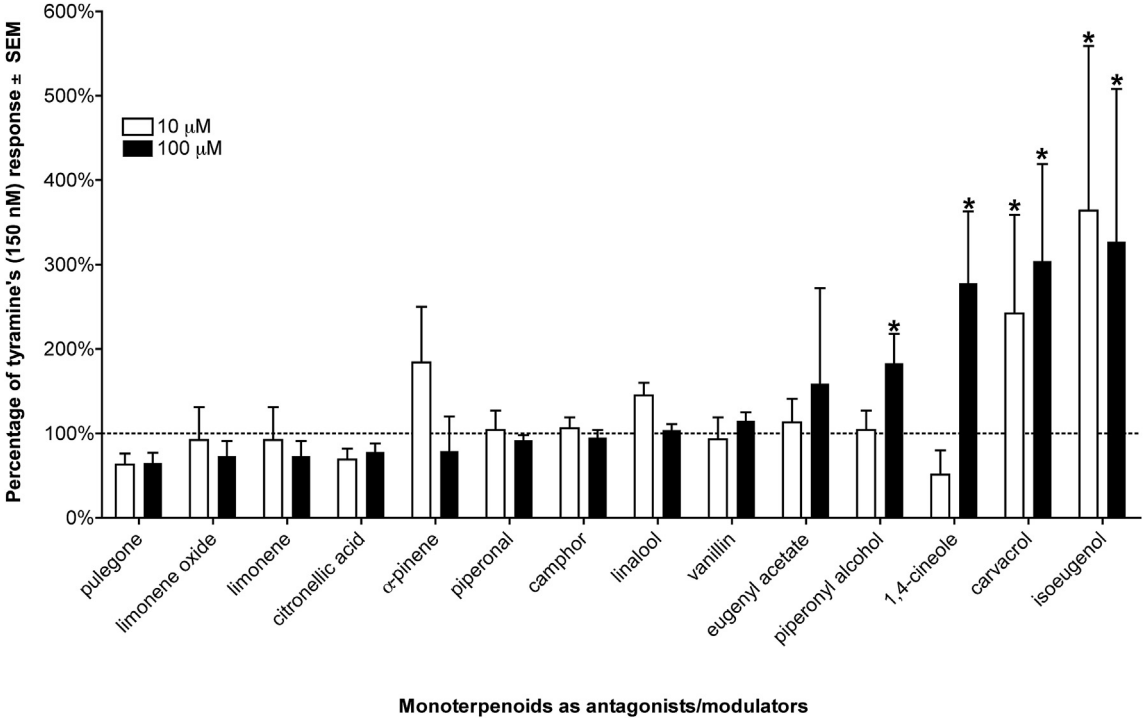

Fig. 3. Terpenoids screened as antagonists or negative modulators at 10 μM and 100 μM. Several monoterpenoids and related aromatic compounds were incubated at two concentrations (10 μM and 100 μM) for 30 min prior to the addition of 150 nM tyramine. This was performed to determine if the terpenoid would act as an antagonist or modulator of the tyramine receptor RmTAR1 plus $G\alpha_{qi(5)}$, expressed in CHO cells. The terpenoid response was normalized to 150 nM tyramine, which is represented as a dashed line at 100%. Each terpenoid or related aromatic compound assay was replicated four times from different passages of cells (each replicated consisted of a treatment, blank, autofluorescent control, vehicle control, non-receptor mediated control in a minimum of four wells). An asterisk indicates a statistically significant difference when compared to tyramine (150 nM). Analysis was performed with one-way ANOVA ($\alpha = 0.05$).

at a terpenoid concentration of 100 μM; tyramine concentration remained at 150 nM (Fig. 3). Four terpenoids: piperonyl alcohol, 1,4-cineole, carvacrol, and isoeugenol increased the 150 nM tyramine response significantly by 183%, 189%, 278%, 304% and 327%, respectively (Fig. 3).

4. Discussion

The development of acaricide resistance in the southern cattle tick [13], along with increased public concern about the safety of conventional pesticides, has resulted in the need to identify new and safer alternatives to control pests in the food supply, including the southern cattle tick. These alternatives may include new chemistry and/or the identification of novel or underutilized biochemical targets. Several essential oils have been shown to be toxic to the cattle fever tick (*R. annulatus*) and to the southern cattle tick (*R. microplus*) [8,22,26,36,37,39,40,46]. Here, we report on the development of an expression system for the southern cattle tick's tyramine receptor (RmTAR1), and present evidence that this receptor may be a potential target of biopesticides. We show that two monoterpenoids, and three related aromatic compounds interact with the southern cattle tick's tyramine receptor. It has been previously suggested that oxygenated terpenoids enhance receptor interaction for a tyramine receptor from *D. melanogaster* [10]. We found similar results, that is, certain oxygenated terpenoids were the only compounds to act as an agonist (pulegone; Figs. 1 and 2) or a modulator (piperonyl alcohol, 1,4-cineole, carvacrol, and isoeugenol; Figs. 1 and 3). Pulegone was the only compound that had a statistically significant effect as an agonist. However, it is structurally dissimilar to tyramine, the receptor's endogenous ligand. Pulegone does not have an aromatic ring or hydroxyl group. Instead pulegone has a saturated 6-membered ring with a carbonyl, which functions as hydrogen bond acceptor compared to the hydroxyl group of tyramine that functions as a hydrogen bond donor. Three out of the four monoterpenoid-related aromatic compounds are somewhat structurally similar to tyramine; that is they have an aromatic ring and hydroxyl group. We have demonstrated that carvacrol, along with piperonyl alcohol, isoeugenol and 1,4-cineole increase the activity of the endogenous ligand, tyramine, but do not have a significant effect on their own. These results indicate that once tyramine activates its receptor (RmTAR1), it results in a conformation change of the receptor protein that allows these terpenoids to stabilize and enhance the signaling activity of RmTAR1. We have previously described this effect with an American cockroach octopamine receptor when this receptor is constitutively active and expressed in yeast cells [17]. Additionally, a similar modulatory response was also reported with this tyramine receptor (RmTAR1) and the amitraz metabolite (BTS 27271) [15]. Carvacrol was previously shown to displace the binding of ^{3}H-tyramine, decrease the basal level of cAMP, and increased the intracellular concentration of calcium in cells heterologously expressing a type-1 tyramine receptor from *D. melanogaster* [10]. However, functional studies were not performed with the aromatic monoterpenoid in the presence of tyramine.

Preliminary post-transcriptional gene silencing (RNAi) studies have shown that this tyramine receptor (RmTAR1) in the southern cattle tick may be a viable target for tick control. The injection of dsRNA complementary to specific coding regions of RmTAR1 resulted in an increase in southern cattle tick mortality, and caused gross anatomical differences in the southern cattle tick's digestive system. These preliminary results indicate that the tyramine receptor is of physiological importance to the southern cattle tick, and may result in the opportunity to disrupt normal tick physiology using chemical compounds that interact with the southern cattle tick's tyramine receptor [16,49].

GPCRs, including tyramine receptors, are an underutilized and novel target for agrochemical discovery. The widespread insecticide resistance that has been reported with the southern cattle tick has led to an urgent need to identify under-utilized targets for tick control. Additionally, there is a growing interest in looking for natural compounds to control arthropod pests, including the use of essential oils and their terpenoids. We suggest that the tyramine receptor from the southern cattle tick may provide an underutilized target for tick control. Additionally, we have found some naturally occurring chemistry that interacts with a tyramine receptor from the southern cattle tick (RmTAR1).

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