Effects of Insecticidal Ketones Present in Mint Plants on GABA$_A$ Receptor from Mammalian Neurons

Mariela Eugenia Sánchez-Borzone, Leticia Delgado Marin, Daniel Asmed García

Instituto de Investigaciones Biológicas y Tecnológicas (IBYBT), CONICET-Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, Córdoba 5016, Argentina

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ABSTRACT

Background: The genus Mentha, an important member of the Lamiaceae family, is represented by many species commonly known as mint. The insecticidal activity of Mentha oil and its main components has been tested and established against various insects/pests. Among these, the ketone monoterpenes that are most common in different Mentha species demonstrated insect toxicity, with pulegone being the most active, followed by carvone and menthone. Considering that the GABA$_A$ receptor (GABA$_A$-R) is one of the main insecticide targets on neurons, and that pulegone would modulate the insect GABA system, it may be expected that the insecticidal properties of Mentha ketones are mediated by their interaction with this receptor. Objective: In order to discern the pharmacological actions of these products when used as insecticides on mammalian organisms, we evaluated the pharmacologic activity of ketones, commonly present in Mentha plants, on native GABA$_A$-R from rats. Materials and Methods: Determination of ketones effects on allosterically enhanced benzodiazepine binding, using primary cultures of cortical neurons, which express functional receptors and MTT assay to evaluate their cell toxicity. Results: Our results seem to indicate that ketone components of Mentha, with proven repellent or insecticidal activity, were able to behave as GABA$_A$-R negative allosteric modulators in murine cells and consequently could exhibit convulsant activity in mammalians. Only pulegone at the highest assayed concentration (2 mM) showed a significant reduction in cell viability after exposure for 24 hr. Conclusion: The present results strongly suggest that the ketone components of Mentha are able to exhibit convulsant activity in mammalian organisms, but functional assays and in vivo experiments would be necessary to corroborate this proposed action.

Key words: Cell culture, GABA$_A$ receptor, insecticide, ketones, Mentha, toxicity

SUMMARY

• The pharmacological activity of insecticide ketones, commonly present in Mentha plants, was evaluated on native GABA$_A$ receptor from mammalian neurons.
• All studied compounds: pulegone, menthone and dihydrocarvone, were able to behave as negative allosteric modulators and could exhibit convulsant activity in mammalian organisms.
• Cytotoxicity assays demonstrated that only pulegone affected the cell viability.

INTRODUCTION

The genus Mentha, one of the important members of the Lamiaceae family, is represented by many species commonly identified as mint, which has been known for its medicinal and aromatherapy properties. The insecticidal activity of Mentha oil and its main components has been tested and established against various insects/pests.\textsuperscript{[1]} Mentha’s repellent properties against agricultural pests were investigated in a series of experiments by Odeyemi et al.\textsuperscript{[2]} and Kumar et al.\textsuperscript{[3]} Its repellent activity was also demonstrated in mosquito control, and thus for diseases of public health concern such as malaria, yellow fever, dengue, and viral encephalitis.\textsuperscript{[4–6]} Many assays have reported insect mortality caused by Mentha toxicity.\textsuperscript{[7,8]} and some have evaluated its antifeedant activity.\textsuperscript{[3,9]} Species of the genus Mentha have been reported to contain a range of constituents.\textsuperscript{[10]} The monocyclic ketones most commonly found in Mentha species are pulegone, menthone, carvone, and, to a lesser extent, dihydrocarvone.\textsuperscript{[11,12]} The GABA$_A$ receptor (GABA$_A$-R) is a major insecticide target along with the voltage-dependent sodium channel, the nicotinic receptor, and acetylcholinesterase.\textsuperscript{[13,14]} Important insecticides acting at the GABA$_A$-R (e.g., lindane, α-endosulfan, dieldrin, and fipronil) recognize the picrotoxinin site, a noncompetitive antagonist site, to block GABA-induced chloride flux.\textsuperscript{[15]} GABA$_A$-R in mammalian, and even in various insect species, differs a lot in their subunit combinations and sensitivities to different ligands.\textsuperscript{[16–18]} The structure and nature of binding sites in housefly GABA receptors have been shown to be different from those in rat GABA receptors, and the differences may be related to the selectivity of antagonists for housefly versus rat receptor.\textsuperscript{[19]}

Abbreviations used: GABA: gamma aminobutyric acid, GABA$_A$R: GABA$_A$ receptor, MTT: 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, DMEM: Dulbecco’s modified minimum essential medium, [3H]TBOB: [3H]-t-Butylbicycloorthobenzoate

Correspondence: Dr. Daniel Asmed García, Instituto de Investigaciones Biológicas y Tecnológicas (IBYBT), CONICET-Universidad Nacional de Córdoba, Cátedra de Química Biológica, FCEFYN, Av. Vélez Sarsfield 1611, Córdoba 5016, Argentina. E-mail: dagarcia@efn.uncor.edu

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We described very recently the effects of carvone isomers on the mammalian GABA_\_R, demonstrating their inhibitory activity on this receptor. In this work, we evaluated the pharmacologic activity of the other monoterpene ketones commonly present in Mentha (pulegone, menthone, and dihydrocarvone; see structures in Figure 1) on native GABA_\_R from rats by determining their effects on allosterically enhanced benzodiazepine binding using primary cultures of cortical neurons, which express functional receptors,[20,21] in order to discern the pharmacologic activity of these products on mammalian organisms when used as insecticides. We also investigated the possible neurotoxic effects of Mentha components in the same cell culture system at concentrations relevant to their neuroactive ranges.

MATERIAL AND METHODS

Materials

(R)-(+)-Pulegone (purity 99%) (IUPAC name: (5R)-5-methyl-2-propan-2-ylidenecyclohexan-1-one), (–)-menthone (purity 99%) (IUPAC name: (2S,5R)-5-methyl-2-propan-2-ylcyclohexan-1-one), (+)-dihydrocarvone (mixture of isomers: ~77% n-(+)-dihydrocarvone and ~20% iso-(+)-dihydrocarvone) (IUPAC name: 2-methyl-5-prop-1-en-2-ylcyclohexan-1-one), γ-aminobutyric acid (GABA), picrotoxin, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyIormazam (MTT), Dulbecco’s modified minimum essential medium (DMEM), trypsin, soybean trypsin inhibitor, DNase, amino acids, and poly-l-lysine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal calf serum was obtained as insecticides. We also investigated the possible neurotoxic effects of Mentha components in the same cell culture system at concentrations relevant to their neuroactive ranges.

Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 17-day-old rat fetuses, as previously described.[20] The cell suspension (1.6 × 10^6 cells/ml) was seeded in 24× or 96× multiwell plates, according to the experiment, precoated with poly-l-lysine and finally incubated for 6–7 days in a humidified 5% CO\_2/95% air atmosphere at 37°C. Twenty millimolar cytosine arabinoside was added after 48 h in culture to prevent glial proliferation.

[3H]Flunitrazepam binding

The benzodiazepine binding to intact cultured cortical neurons was determined as previously described,[20] using nearly 2.0 nM [3H] flunitrazepam. Seven hundred fifty micromolar of ketones and variable concentrations of GABA, between 0 and 200 μM, were added to the incubation media for 30 min of incubation at 25 °C. Nonspecific binding was determined in the presence of 20 μM diazepam.

Cell viability

After 6–7 days in vitro, the cells were exposed to different concentrations of each compound for 30 min or 24 h. Ketones were added after solubilization in 0.2 ml of culture medium previously extracted from each well. Cell viability was determined by measuring the reduction of MTT to a colored formazan salt by mitochondrial reducing activity, as described previously.[20]

Data analysis

Data shown represent the mean ± standard error of mean (SEM). Sigmoid curves were fitted to concentration response data and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The two-tailed Student’s t-test and one-way ANOVA were used to compare data. A P value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of Mentha ketones on the benzodiazepine binding enhanced by GABA

To evaluate the activity of Mentha ketones on native GABA_\_R from mammals, their effects were observed on [3H]flunitrazepam binding stimulated by the agonist GABA in primary cultures of rat cortical neurons. The results demonstrated that GABA was able to enhance radioligand binding in a dose-dependent manner as expected, showing an EC_{50} value of 4.4 μM [Figure 2 and Table 1]. This result is consistent with that reported previously.[20,21] All Mentha ketones studied in the present work were able to right shift the concentration–response curve of the effect of GABA on [3H]flunitrazepam binding. At the beginning of each curve, pulegone and dihydrocarvone showed a negative effect in the absence of GABA (control samples), while menthone induced an increase; later, all ketones slowly enhanced the binding as the GABA concentration increased. Fitting the data to sigmoid curves revealed a rise in the EC_{50} value for the GABA-induced increase in [3H] flunitrazepam binding to 123.7, 64.8, and 66.7 μM in the presence of pulegone, menthone, and dihydrocarvone, respectively. At the same time, the maximum response induced by GABA 200 μM (174% with respect to basal) was significantly reduced by all ketones [Figure 2 and Table 1] (P < 0.05, one-way ANOVA). Taking into account these effects, we can clearly consider all Mentha compounds as negative allosteric modulators, at least on mammalian neurons. It should be noted that the allosteric behavior of the receptor was tested by determining the improvement of [3H]flunitrazepam binding exerted by GABA and its reduction by a noncompetitive GABA antagonist.

Pulegone is used as a flavoring agent, in perfumery and aromatherapy,[1] and an enantiomeric form ((R)-(++)-pulegone) was described as a psychoactive compound with the profile of an analgesic drug.[24] Tong and Coats[25] suggested that pulegone and other monoterpenoids act as positive allosteric modulators of the GABA_\_R in insects. However, this result supported by the 36Cl uptake enhancement did not correlate very well with the increase found in [3H]TBOB binding in the same work, since a reducing effect should be expected according to their suggested function as GABA allosteric agonist. The inhibitory effect of pulegone on GABA-stimulated [3H]flunitrazepam binding described in the present work clearly indicates its activity as a negative allosteric modulator in murine cortical neurons. In another report, Bessette[26] described opposite effects of pulegone on [3H]TBOB binding in house...
Concentration–response curves for the \[
3^5\text{Hf}2
\] binding to GABA \(_A\)-R would be necessary to identify the exact binding site(s) of the ketones. Thus, the present results strongly suggest that the ketone components of Mentha are able to exhibit convulsant activity in mammalian organisms, but functional assays and \textit{in vivo} experiments would be necessary to corroborate this proposed action.

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**Conflicts of interest**

There are no conflicts of interest.

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MARIELA EUGENIA SÁNCHEZ-BORZONE, et al.: Effects of insecticidal ketones on GABA\(_A\) receptor

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