Inhibitory Effects of Carvone Isomers on the $GABA_A$ Receptor in Primary Cultures of Rat Cortical Neurons

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ABSTRACT Carvone is a natural terpene which can be purified as R-(−) or S-(+) enantiomers. There are many reports about its antibacterial, antifungal, and insecticide activities, and also of some effects on the nervous system, where both enantiomers showed different potencies. Considering that the $GABA_A$ receptor is a major insecticide target, we studied the pharmacological activity of both carvone enantiomers, and of thujone as a reference compound acting on the receptor, on native $GABA_A$ by determining their effects on benzodiazepine recognition sites using primary neuronal cultures. Both isomers were able to inhibit the GABA-induced stimulation of [^{3}H]flunitrazepam binding, suggesting their interaction with the $GABA_A$ receptor as negative allosteric modulators. Their activity was comparable to that described for thujone in the present article, with the R-(−)-carvone being the more similar and potent stereoisomer. The different configuration of the isopropenyl group in position 5 thus seems to be significant for receptor interaction and the bicycle structure not to be critical for receptor recognition. The concentrations necessary to induce negative modulation of the receptor were not cytotoxic in a murine neuron culture system. These results confirm that, at least partially, the reported insecticidal activity of carvones may be explained by their interaction with the $GABA_A$ receptor at its noncompetitive blocker site. *Chirality 26:368–372, 2014.*

KEY WORDS: carvone; thujone; stereoisomer; $GABA_A$ receptor; cytotoxicity; convulsant

INTRODUCTION

Carvone is a terpene natural product mainly found in spearmint (*Mentha spicata*) and caraway (*Carum carvi L.*). Spearmint is mostly used in cuisine and is mainly produced in the USA, China, and South America. Caraway is found naturally in Northern and Central Europe, Siberia, Turkey, Iran, India, and North Africa, and is one of oldest spices cultivated in Europe. Since carvone possesses a chiral center, it can be purified as R-(−) or S-(+) enantiomers from these plants, the former being the major enantiomer in spearmint, while the S-(+) isomer is found in caraway.[1]

There are many reports about the biological activities of carvone. S-(+)-carvone was found to be a good potato-sprouting inhibitor.[2] Carvone has both antibacterial and antifungal activity,[2,3] and can also be used as an insecticide against the fruit fly.[4] There are some studies that show the effects of carvone on the nervous system. The inhalation of R-(−) and S-(+)-carvone modifies the motor activities of mice.[5] S-(+)-carvone and R-(−)-carvone also have a depressant effect in the central nervous system (CNS),[6] and both were able to reduce peripheral nerve conduction in a dose–response manner, although they showed different potencies.[7]

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate CNS. $GABA_A$ receptors ($GABA_A$-Rs) are ligand-gated Cl^- ion channels activated by GABA and by its agonists, such as muscimol or isoguvacine, modulated by numerous therapeutically important drugs, including barbiturates, anesthetics, and benzodiazepines, and blocked by picrotoxinin-like convulsants. Known allosteric modulations include the enhanced binding of benzodiazepine agonists by GABA.[8,9]

The $GABA_A$-R is a major insecticide target along with the voltage-dependent sodium channel, the nicotinic receptor, and acetylcholinesterase.[10,11] Important insecticides acting at the $GABA_A$-R (i.e., lindane, α-endosulfan, dieldrin, and fipronil) recognize the picrotoxinin or noncompetitive antagonist site to block GABA-induced chloride flux.[12]

Thujone, in its α- and β-conformation, is the toxic agent in absinthe, a liqueur popular in the 19th and early 20th centuries that has adverse health effects. Habitual ingestion of absinthe is associated with hallucinations, sleeplessness, convulsions, and increased risk of psychiatric illnesses and suicides.[13] The United States National Toxicology Program has recommended thujone for genotoxicity, neurotoxicity, reproductive toxicity, chronic toxicity, and carcinogenicity testing in rats and mice.[14] Thujone is also the active ingredient of wormwood oil and some other herbal medicines and is reported to have antinociceptive, insecticidal, and anthelmintic activity. In the nervous system, it was described as a potent convulsant compound acting at the noncompetitive blocker site of the $GABA_A$-R, with α-thujone being the more potent isomer.[15,16]

Considering that carvone and thujone are monoterpenic ketones, both showing insecticidal activity, we studied the pharmacological activity of both carvone enantiomers and of thujone (mixture of α- and β-isomers) (see structures in Fig. 1) on native $GABA_A$-R by determining their effects on benzodiazepine recognition sites using primary cultures of cortical neurons which express functional receptors.[17,18] The inclusion of thujone in this work, as a reference compound acting on $GABA_A$-R, would permit explaining the mechanism of action involved in an eventual pharmacological

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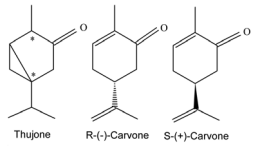

Fig. 1. Chemical structures of the compounds analyzed. Both isomers of carvone are included. *Chiral carbons in thujone structure.

activity of carvone isomers. Finally, we determined the neurotoxic effects at relevant concentrations according their neuroactive ranges.

MATERIALS AND METHODS

Materials

R-(−)- and S-(+)-carvone (5-isopropenyl-2-methyl-2-cyclohexen-1-one; optical purity >98%), thujone (1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-one; α≈70%, ≈β10%), γ-aminobutyric acid (GABA), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dulbecco's modified minimum essential medium (DMEM), trypsin, soybean trypsin inhibitor, DNase, amino acids, and poly-L-lysine were obtained from Sigma-Aldrich (St Louis, MO, USA). Fetal calf serum was obtained from PAA (Pasching, Austria) and [^{3}H] Flunitrazepam (84.9 Ci/mmol) from Perkin Elmer (Boston, MA, USA). All the other chemicals were of analytical grade. All ketones were prepared as 1 M stock solutions in pure DMSO, light protected, and stored at 4 °C. Stock solutions were diluted before each experiment in buffered solution, maintaining a 0.25% (v/v) DMSO final concentration.

Cell Cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 17–18-day-old rat fetuses, as previously described.[17] Pregnant animals were killed by cervical dislocation and fetuses extracted. Neocortices were dissected with forceps, mechanically minced, and cells then dissociated by mild trypsinization (0.02% w/v) at 37 °C for 10 min followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were resuspended in a DMEM (5 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with insulin, penicillin, and 10% fetal calf serum. The cell suspension (1.6 x 10^6 cells/ml) was seeded in 24x- or 96x 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. 20 μM cytosine arabinoside was added after 48 h in culture to prevent glial proliferation.

[^{3}H]Flunitrazepam Binding

The benzodiazepine binding to intact cultured cortical neurons was determined as previously described[17] using ≈2.0 nM [^{3}H]flunitrazepam. Prior to incubation with the radioligand, the plates were washed three times with 1 ml/well of HEPES buffer (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 9 mM glucose adjusted to pH 7.4), and the binding assay took place in the culture well (24x multiwell plate) in the presence of the HEPES buffer, [^{3}H]flunitrazepam, and GABA and/or ketone solutions according to the experiment. GABA was added to the incubation media at variable concentrations between 0 and 200 μM, while ketones were added to the incubation media at a constant final concentration of 750 μM. After 30 min of incubation at 25 °C without shaking, a cold buffer was added and rapidly removed by suction. The cells were rinsed three times with cold buffer, then disaggregated in 0.2 M NaOH overnight, and their radioactivity was determined by liquid scintillation counting (with scintillation liquid: 25% v/v Triton X-100 and 0.3% w/v diphenyloxazole in toluene). Basal binding was determined in HEPES solution containing 0.25% DMSO in the absence of drugs. Nonspecific binding was determined in the presence of 20 μM diazepam. All experiments were run simultaneously with a parallel experiment that determined the increase of [^{3}H] flunitrazepam binding induced by 100 μM GABA, used as a positive assay control, and its reduction by picrotoxin. Data are expressed as the percentage of basal specific binding.

Cell Viability

Following 7 d *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.[17,19] Briefly, the cultures were rinsed and incubated for 15 min with a solution of MTT (250 μg/ml) dissolved in a HEPES buffer solution at 37 °C. After washing off the excess MTT, the cells were disaggregated with 5% of sodium dodecyl sulfate (SDS), and the colored formazan salt was measured at 560 nm in a spectrophotometer plate reader. Data are expressed as a percentage of nontreated cultures.

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, San Diego, CA, USA). A two-tailed Student's *t*-test as well as one-way analysis of variance (ANOVA) were used.

RESULTS AND DISCUSSION

To assess the activity of ketones on $GABA_A$-R, their effects were observed on [^{3}H] flunitrazepam binding. It has been previously reported that an increase of [^{3}H]flunitrazepam binding corresponds to potentiation of the $GABA_A$-R.[17,20,21] In the present work, GABA was able to stimulate radioligand binding in a dose-dependent manner as expected, showing an EC_{50} value of 5.75 μM (Fig. 2). This result is in good agreement with those reported previously.[22,23] The concentration-response curve of the effect of GABA on [^{3}H]flunitrazepam binding was shifted to the right in the presence of all ketones studied. Fitting the data to sigmoid curves revealed a rise in the EC_{50} value for GABA-induced increase in [^{3}H]flunitrazepam binding to 74.44, 90.02, and 15.69 μM in the presence of thujone, R-(−)-carvone, and S-(+)-carvone, respectively. The maximum response induced by GABA (182% with respect to basal) was notably reduced by all ketones, to values not different from basal (Fig. 2, Table 1). Considering all the above results, there is a clear inhibitory effect of all the compounds studied on the GABA-induced increase in benzodiazepine

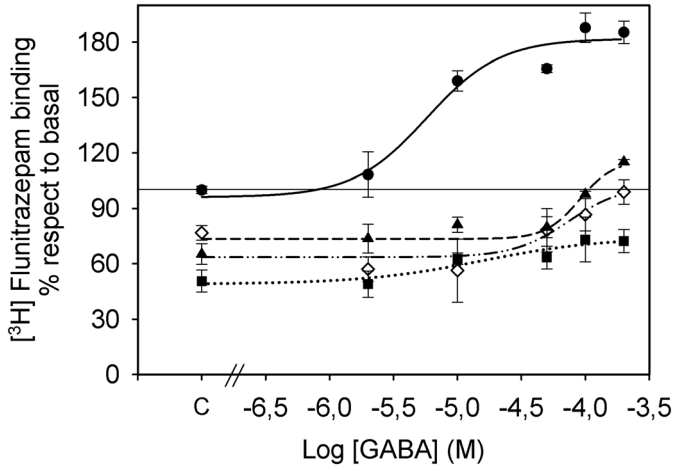

Fig. 2. Concentration–response curves for the increase in [^{3}H]flunitrazepam binding induced by GABA in primary cultures of cortical neurons. The points correspond to experiments made in the presence of GABA and in the absence (—●—) or in the presence of 750 μM thujone (·-·◊·-·), R-(−)-carvone (---▲---) or S-(+)-carvone (·····■·····). Lines represent sigmoid curves fitted to the data. The values are expressed as the percentage of basal binding (without GABA or any compound). C: control values in the absence of GABA. Results are means ± SEM of 3–6 independent experiments done in triplicate.

TABLE 1. Effect of ketones on GABA-induced increase of [^{3}H] flunitrazepam binding

Compound	Log EC_{50}	EC_{50} (μM)	E_{max} (%)
GABA	−5.24 ± 0.185	5.75	182 ± 5.9
Thujone	−4.13 ± 0.473	74.44	103 ± 32.6
R-(−)-carvone	−4.05 ± 0.107	90.02	116 ± 10.2
S-(+)-carvone	−4.80 ± 0.890	15.69	74 ± 16.7

EC_{50} values were calculated from data shown in Fig. 2, as explained in the Materials and Methods section. E_{max} corresponds to the percentage increase with respect to the basal binding (without GABA or any compound). A minimum of six concentrations were used for each curve. The values correspond to the mean ± SEM.

binding, indicating a negative allosteric effect on the $GABA_A$-R. It is important to note that, in all experiments, the allosteric behavior of the receptor was checked by determining the enhancement of [^{3}H]flunitrazepam binding exerted by GABA 100 μM in several samples (159.06 ± 5.4%, with respect to control) and its reduction by picrotoxin 200 μM (82.36 ± 1.64%, with respect to control), a noncompetitive GABA antagonist ($P < 0.01$, Student's *t*-test).

Figure 3 shows the effect of ketones on cell viability in primary neuronal cultures, determined by the cell capacity to reduce the MTT salt. The concentrations assayed for each compound were selected considering ranges that include concentrations effective on GABA-induced [^{3}H]flunitrazepam binding (between 100 and 2000 μM for all compounds). No ketone significantly reduced cell viability after exposure for 30 min (incubation time of the binding experiment) or for 24 h, indicating the absence of cytotoxic effects in this cell assay system (with respect to 100% viability, one-way ANOVA).

Even though primary cultures of cortical neurons are enriched in GABAergic neurons,[24] their exposure to different concentrations of GABA did not reduce the initial concentration of GABA in the incubation medium. Furthermore, the release of endogenous GABA by cells exposed to basal conditions of the binding assay did not reach a sufficient concentration to elicit $GABA_A$R-mediated responses.[17] Thus, the results obtained with this binding assay system are valid for describing possible positive or negative allosteric modulation on the receptor. The ability of all the ketones studied in this work to reduce GABA-induced enhancement of [^{3}H]flunitrazepam binding suggests that they behave as negative allosteric modulators of the $GABA_A$-R.

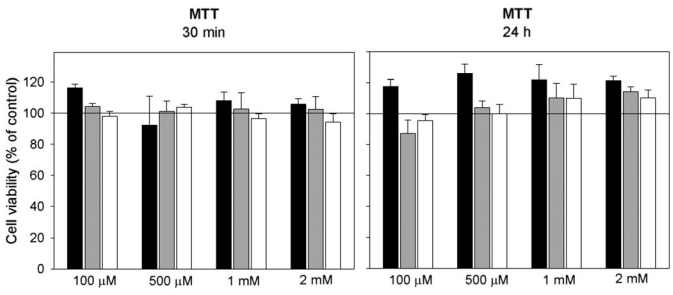

Fig. 3. Cell viability MTT assay in the presence of ketones. The bars represent the percentage of cell viability with respect to 100% control (without any compound) after 30 min or 24 h of exposure to each compound: thujone (black bars), R-(−)-carvone (gray bars) and S-(+)-carvone (white bars). The concentrations ranges used were 100–2000 μM for all compounds, which include the concentration used on [^{3}H]flunitrazepam binding experiments. All values are mean ± SEM of 3 independent experiments done in triplicate.

Thujone was shown to modulate the $GABA_A$-R by acting at the noncompetitive blocker site of the receptor, with α-thujone being 2.3 times more active than β-thujone in binding assays, which provided a reasonable explanation for some of the actions of absinthe as expressed in the Introduction, and for its only pharmacological action listed in the Merck index: "convulsant."[15,25] Absinthe contains not only α-thujone as the supposed active ingredient but also many other candidate toxicants, including ethanol. Even though this alcohol acts as an enhancer of neuronal $GABA_A$R function, its presence does not alter the inhibitory action of α-thujone on the receptor.[15] The present work is the first report in which this restraining effect of thujone is shown through the allosteric behavior of the GABA-induced increase of benzodiazepine binding, which is also inhibited by channel blockers such as picrotoxin.[8,26] In this context, the decrease in allosteric behavior reported here for R-(−)-carvone and S-(+)-carvone credibly suggests a possible participation of these molecules at the noncompetitive $GABA_A$-R blocker site, which may be corroborated by using radioligands that recognize the picrotoxinin site as [^{3}H] ethynylbicycloorthobenzoate ([^{3}H]EBOB) or t-[^{35}S] butylbicyclophosphorothionate ([^{35}S]TBPS).[15,18]

The EC_{50} values for the ketones studied clearly show a greater inhibitory effect for thujone and R-(−)-carvone than for S-(+)-carvone, despite the latter also demonstrating evident capacity to reduce the E_{max}.

There are not many reports about the effects of carvones on the nervous system. Kubota *et al.*[27] reported that R-(−)-carvone showed a sedative effect, while S-(+)-carvone elicited a stimulant effect based on contingent-negative-variation data in the EEG. Heuberger *et al.*[28] analyzed the effects of both enantiomers, administered by inhalation, on human autonomic nervous system parameters and found that, while S-(+)-carvone effected autonomic arousal, its isomer caused increases in both physiological and behavioral arousal. Another report showed that the effects of carvone on the locomotive activity of mice also depend on the chirality of this ketone, given that R-(−)-carvone showed more sedative and relaxing effects while S-(+)-carvone demonstrated both sedative and activating effects.[5,6] De Sousa *et al.*[6], analyzing a range of *in vivo* experiments, suggested that R-(−) and S-(+)-carvone have a depressant effect on the CNS, with S-(+)-carvone showing possible anticonvulsant-like activity. More recently, Gonçalves *et al.*[7] observed that both R-(−)- and S-(+)-carvone reduced peripheral nerve conduction in a dose–response manner but with different potencies, with R-(−)-carvone being less potent than its enantiomer; thus, it appears that both compounds interact with a receptor at the same site, but with different affinities. In another report, Gonçalves *et al.*[29] described the ability of R-(−)-carvone to induce increases in cytosolic calcium concentration through TRPV1 activation.

Our results, using an allosteric model on the $GABA_A$-R, demonstrate that both carvones are able to interact with the receptor, probably by recognizing the picrotoxinin site, as was proposed earlier by De Sousa *et al.*[6] Thus, although the interaction with this blocking site is described mainly for convulsant agents, as explained above, some compounds act at the same site in the chloride channel operating as negative allosteric modulators but have anticonvulsant activity. For example, the sesquiterpenoid lactone bilobalide has some structural similarities to picrotoxinin, including a lipophilic side chain and a hydrophilic cage, and is also a noncompetitive antagonist at $GABA_A$-R.[26,30,31] The lack of convulsant action in

an agent that reduces GABA action may be important for enhancement of cognition. For bilobalide and other compounds, the lack of convulsant action may result from subunit selectivity, but this has yet to be established.[26,32] On the other hand, the differences between both carvones in their inhibitory effect on GABA-induced benzodiazepine binding also sustain the idea proposed by Gonçalves *et al.*[7] that R-(−) and S-(+)-carvone may have different affinities with receptors in general, and with $GABA_A$-R in particular (present work).

Although thujone presents an asymmetric bicyclic ring structure, it does have some similarities with the molecular structure of carvones, such as: (i) the presence of a keto group, (ii) the presence of a three carbon group (isopropyl in thujone and isopropenyl in carvones), and (iii) the presence of a methyl group. In addition, the distances between the keto group and both the methyl and the isopropyl or isopropenyl groups are very similar in thujone and carvones. Considering that R-(−)-carvone and thujone show analogous activities on the receptor while S-(+)-carvone appears less active (see EC_{50} values), the different configuration of the carvone isopropenyl group in position 5 would be substantial for receptor interaction, and the bicycle structure not critical for receptor recognition. The importance of the chiral center at carbon 5 in the carvone molecule for interaction with the $GABA_A$-R was previously proposed by De Sousa *et al.*[6] based on *in vivo* experiments, as explained above.

Since many reports indicate that carvones and thujone could have cytotoxic effects, we analyzed their effects on cell viability. Carvones have been described as antibacterial and antifungal agents,[1 and references therein] and also as an inhibitor of the viability and proliferation of Hep-2 cells.[33] Thujone is considered toxic to brain and liver cells, as demonstrated in several articles.[34–36] In this study, no compound was found to produce cell damage in a murine neuron culture system, either after short (30 min) or long exposure times (up to 24 h), as demonstrated by the MTT assay at concentrations necessary for activity at the $GABA_A$-R. The results of this MTT assay are indicative of a lack of mitochondrial failure.

CONCLUSION

We have found that both carvone isomers are able to inhibit the GABA-induced stimulation of [^{3}H]flunitrazepam binding, suggesting their interaction with the $GABA_A$-R as negative allosteric modulators. Their activity is comparable to that described for thujone in the present article, with R-(−)-carvone being the more similar and potent stereoisomer. These results confirm that, at least partially, the reported insecticidal activity of carvones may be explained by their interaction with the $GABA_A$-R at its noncompetitive blocker site.

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