Surface activity of thymol: implications for an eventual pharmacological activity

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Abstract

In the present work, we studied the ability of thymol to affect the organization of model membranes and the activity of an intrinsic membrane protein, the GABA A receptor (GABA A -R). In this last aspect, we tried to elucidate if the action mechanism of this terpene at the molecular level, involves its binding to the receptor protein, changes in the organization of the receptor molecular environment, or both. The self-aggregation of thymol in water with a critical micellar concentration (CMC) \( \sim 4 \mu M \) and its ability to penetrate in monomolecular layers of soybean phosphatidylcholine (sPC) at the air-water interface, even at surface pressures above the equilibrium, lateral pressure of natural bilayers were demonstrated. Thymol affected the self-aggregation of Triton X-100 and the topology of sPC vesicles. It also increased the polarity of the membrane environment sensed by the electrochromic dye merocyanine. A dipolar moment of 1.341 Debye was calculated from its energy-minimized structure. Its effect on the binding of \(^{3}H\)-flunitrazepam (\(^{3}H\)-FNZ) to chick brain synaptosomal membranes changed qualitatively from a tendency to the inhibition to a clear activatory regime, upon changing the phase state of the terpene (from a monomeric to a self-aggregated state). Above its CMC, thymol increased the affinity of the binding of \(^{3}H\)-FNZ \( (K_{d - control} = 2.9, K_{d - thymol} = 1.7 \text{nM}) \) without changing the receptor density \( (B_{max - control} = 910, B_{max - thymol} = 895 \text{fmol/mg protein}) \). The activatory effect of thymol on the binding of \(^{3}H\)-FNZ was observed even in the presence of the allosteric activator gamma-aminobutyric acid (GABA) at a concentration of maximal activity, and was blocked by the GABA antagonist bicuculline. Changes in the dipolar arrangement and in the molecular packing of GABA A -R environment are discussed as possible mediators of the action mechanism of thymol.

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1. Introduction

Thymol is a compound classified among the group of monoterpenes. The latter are substances derived from isoprene hydrocarbon (2-methyl-1,3-butadiene) and originated by the attachment of two or more isoprene molecules. They can be found as components of many essential oils extensively used as fragrances in cosmetics, as flavoring food additives, as scenting agents in a variety of household products, as active ingredients in some old drugs and as intermediates in the synthesis of perfume chemicals [1]. The interest in isolated monoterpenes has been growing during the last years due to their eventual pharmaceutical or pharmacological utility.

Thymol is a widely known anti-microbial agent. Due to its bactericide action against oral bacteria, it is commonly incorporated in mouthwashes. Its action mechanism seems to be mainly related with harmful effects on both the cellular cytoplasmic membrane (perforation) and the generation of...
ATP[2,3]. It was also demonstrated an important fungicide activity of this terpene [4] that could involve effects on the membrane.

From the analysis of the chemical structure of thymol, it could be inferred that, from a biophysical point of view, this compound would have an amphiphilic and/or hydrophobic behavior. This suggests an ability of thymol to partition in the membrane from an aqueous phase as well as a capacity to affect the membrane organization and the surface electrostatics. This assumption may explain the effects of thymol on the permeability of membranes and on the activity of membrane intrinsic proteins such as ATPases or membrane receptors.

GABA<sub>4</sub> receptor (GABA<sub>4</sub>-R), a membrane intrinsic protein, is a postsynaptic receptor for gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system of mammals. GABA<sub>4</sub>-R belongs to the super-family of ligand gated ion channels. It is a pentamer with subunits of 50 kD with binding sites for several drugs including barbiturates, neurosteroids and benzodiazepines (BZDs), which behave as allosteric modulators[5–7]. Fluorinated (FNZ), a typical BZD, exerts its main pharmacological actions by enhancing the GABA inhibitory effects through its binding to BZD-receptor sites located at GABA<sub>4</sub>-R[8]. It is known that the activity of this complex protein may be affected by surface-active compounds[9–11]. The essential oil of Tagetes minuta L., a complex mixture of mostly hydrophobic terpene derivatives, as well as thymol, its major component, were able to inhibit the specific (receptor mediated) as well as the non-specific binding of FNZ[10,11]. Furthermore, thymol not only improved the coupling between the binding sites of BZDs and GABA but also enhanced the GABA-induced chloride permeability, probably through a modulation of the membrane organization at the supramolecular level[11]. Thymol has been considered as an allosteric modulator of the GABA receptors from insects [12]. Moreover, it was recently reported that this terpene was able to induce chloride currents through GABA<sub>4</sub>-R in rat brain membranes[13]. Considering the apparent hydrophobic nature of thymol, we wondered whether its effects on GABA<sub>4</sub>-R activity were exerted through its binding to the receptor protein, through changes in the supramolecular organization of the receptor environment, or both.

In the first part of this work, we studied the ability of thymol to incorporate in model membranes and to affect their supramolecular organization. Then, we investigated the effects of this terpene on the binding of FNZ to GABA<sub>4</sub>-R from chick forebrain synaptosomal membranes. Taking into account our previous reports, which described that the quantity and localization of BZDs partitioned in the membrane depended on the state of order and mobility of the membrane[14,15], we pointed our attention not only to the specific interaction of FNZ with GABA<sub>4</sub>-R but also to its non-specific interaction with the membrane as a whole. In this sense, FNZ was used as a probe for membrane organization.

2. Materials and methods

2.1. Materials

The BZD diazepam (DZ) was kindly supplied by Produ- tos Roche (Cordoba, Argentina). [3H]FNZ was purchased from New England Nuclear Chemistry (E.I. DuPont de Nemours & Co. Inc., Boston, MA, USA). The sPC was from Avanti Polar Lipids (Alabaster, AL, USA). GABA, bicuculline [(−)-bicuculline methobromide] and bromthymol blue (3′,3′′-dibromthymolstilbene) were from Sigma (St. Louis, MA, USA). Thymol (5-methyl-2-isopropylphenol) was obtained from local pharmaceutical companies and its purity was checked by gas chromatography (GC) using a Shimadzu GC-R1A with a flame ionisation detector and a DB-5 capillary column (30 m × 0.5 mm i.d.) at a N<sub>2</sub> flow rate of 0.9 mL/min. Other drugs and solvents were of analytical grade.

2.2. Animals

Chicks (Cobb, both sexes) were obtained from a commercial hatchery, INDACOR (Argentina). Birds in groups of 20 were housed in brooders (50 cm × 90 cm) on the evening of the day of hatch. The brooders were placed in a room (3 m × 3 m) isolated from external noises, with constant temperature (32 °C) and humidity at a 12 h light:12 h dark cycle (lights on at 07:00 h) with food and water freely available and maintained in these conditions until they reached 15 days old.

2.3. Determination of CMC

The CMC of thymol was determined by dispersing thymol in water at different final concentrations (between 0 and 58 μM) in the presence of the hydrophobic dye bromthymol blue (80 μM final concentration). In another experiment the effect of thymol on the CMC of Triton X-100 in water was evaluated. In this case, the concentration of Triton X-100 varied between 0 and 1 mM; thymol and the hydrophobic dye were present at constant final concentrations (1 mM and 80 μM, respectively). The samples were incubated at room temperature during 10 min and then the absorbance was measured, depending on the experiment, at 437 or 485 nm (the latter was chosen to avoid Triton X-100 interference) with a Beckman DU 7500 spectrophotometer. The whole absorbance spectrum of bromthymol blue, which exhibited a maximum at 432 nm in water, suffered a blue shift in low polar media, leading to an increment in the absorbance values at λ < λ<sub>max</sub> (wavelength with maximal absorbance) and a decrement at λ > λ<sub>max</sub> with respect to the values obtained in water (not shown). Beyond the concentration of thymol or...
of this experiment was to determine the maximum value of the surface pressure measured by the Wihelmy plate method. Reproducibility was a Minitrough II from KSV Instruments Ltd. (Helsinki, bidistilled water) and about 5 min were allowed for the solution of phospholipid were spread on an aqueous surface essentially according to Perillo et al. [17]. The equipment used was PC at the air–water interface 2.5. Penetration of thymol in monomolecular layers of absorbance of thymol at this wavelength was zero. chromatogram plotted with a Pharmacia XY recorder. The were detected by using a UV detector set at 254 nm and the peaks of phospholipid vesicles were detected through GF/B filters with an automatic harvester apparatus. These suspensions were homogenized and centrifuged at 30,000 × g during 20 min; the pellets were resuspended in 100 mM NaCl–50 mM Tris–HCl buffer pH 7.4 and maintained at −20°C. Immediately before their use, these samples were defrosted and diluted by adding 10 volumes of bidistilled water, centrifuged again at 30,000 × g during 20 min. and the pellets resuspended in the buffer indicated above, at the protein concentration required for the experiment. 

The incubation system contained, in a final volume of 250 μl, the membrane suspension at a final protein concentration of 0.25 mg/ml approximately, [3 H]-FNZ (minimum specific activity 70 Ci/mmol), 100 mM NaCl–50 mM Tris–HCl buffer pH 7.4 containing (non-specific) or not (total) DZ 10 μM (final concentration). Thymol was added to the system at variable or constant concentrations depending on the experiment. [3 H]-FNZ was also used at a constant concentration of 3 nM or covering a concentration range from 0.5 to 15 nM (saturation curve). In some experiments, the system contained GABA or bicuculline at a constant final concentration of 100 μM. Samples were incubated at 4°C in the dark for 1 h and then they were immediately filtered through GF/B filters with an automatic harvester apparatus. The filters rinsed and dried in the air, were placed in vials containing 2.5 ml of scintillation liquid (25% (v/v) Triton X-100, 0.3% (pv) diphenoxyazo in toluene) and the retained radioactivity was determined with a scintillation counter Rackbeta 1214 (Pharmacia-LKB, Finland) with a efficiency of 60% for tritium. Protein concentration was determined by the method of Lowry [18]. 

2.8. Data analysis

The specific binding of [3 H]-FNZ was calculated as total binding (measured in the absence of DZ) minus non-specific binding (in the presence of DZ). Eq. (1) was fitted to the data obtained by the saturation curves (specifically bound versus free [3 H]-FNZ), by a non-linear regression analysis performed by a computer aided least squares method [19].

\[ B = \frac{B_{max} F}{K_b + F} \]
where $B$ and $F$ are the bound and free $[^3H]$-FNZ concentrations, respectively, $B_{\text{max}}$ the maximal binding and $K_d$ the equilibrium dissociation constant.

Linear regression analysis were done by the least squares method. The data were statistically analyzed using a two-tailed Student’s $t$-test for independent samples or a one-way analysis of variance (ANOVA), according to the experiment [20]. The values shown represent the mean and the standard error of the mean (S.E.M.).

3. Results

3.1. Interaction of thymol with model membranes

3.1.1. Thymol’s self-assembly and its effect on Triton X-100’s self-aggregating structures

The self-aggregation of an amphiphilic molecule involves the appearance of a compartment with a polarity markedly different from that of water, where a hydrophobic molecule can be concentrated. In order to investigate an eventual self-assembly of thymol and that of Triton X-100 in the presence of thymol, the changes in the absorbance of a hydrophobic dye (bromthymol blue) was evaluated as a function of the concentration of the amphiphile (thymol or Triton X-100). Ethanol (used as solvent for thymol) did not affect the absorbance of bromthymol blue (Fig. 1a). The CMC was determined as the concentration of amphiphile at which the plot of absorbance versus concentration suffered an abrupt change in its slope. The results shown in Fig. 1b allowed determining a CMC value of approximately 4 $\mu$M for thymol. The CMC of Triton X-100 was 0.13 mM (Fig. 1c) and increased up to 0.62 mM in the presence of 1 mM thymol (Fig. 1d).

3.1.2. Effect of thymol on the size of soybean phosphatidylcholine (sPC) vesicles

Fig. 2 shows the elution patterns of sPC vesicles in a molecular filtration gel chromatography through Sephadex G-200. The control sample (without thymol) shows only one peak at the exclusion volume, the asymmetry of which suggests the existence of a certain degree of dispersion in the size of sPC vesicles. Thymol induced the appearance of a second peak, indicating a change in the size distribution of the vesicles.

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Fig. 1. Thymol’s self-aggregation in aqueous medium and its effect on Triton X-100 CMC. Percentual variation of the absorbance at 437 nm bromthymol blue, with respect to the control in water, as a function of the concentration of (a) ethanol (used as thymol vehicle), (b) thymol, (c) Triton X-100 or (d) Triton X-100 in the presence of 1 mM thymol. The arrows indicate the corresponding CMC value in each case. Points represent the mean ± S.E.M. of triplicates.

Fig. 2. Effect of thymol on the size of sPC vesicles. The graph represents the elution pattern of a molecular filtration chromatography through Sephadex G-200 of a suspension of sPC vesicles previously incubated in the absence (control) or presence (thymol) of 0.03 mM thymol.
of a second shorter peak at a higher elution volume. It is important to note that the absorbance of both sPC and thymol at the emission wavelength of the UV recorder (254 nm) are negligible. Hence, both peaks are due to light scattering. The latter peak would represent a population of vesicles of smaller size with respect to those in the former.

3.1.3. Penetration of thymol in monomolecular layers of sPC at the air–water interface

The penetration of thymol in the monomolecular layers of sPC was evidenced by an increment in the surface lateral pressure at constant area. Thymol penetrated from the aqueous subphase to the sPC monolayer compressed at initial pressures up to 47.8 mN/m (πcut-off) (Fig. 3). This value was determined by extrapolating the plot of Δπ versus πinitial to Δπ = 0.

3.1.4. Effect of thymol on the absorbance spectra of merocyanine

Fig. 4a shows that merocyanine exhibits two absorbance peaks in water, one of them at 500 nm and the other one at 540 nm, representing the dimer (peak 1) and the monomer (peak 2), respectively. As demonstrated previously [22,23], the λmax2 suffered hypochromic and hypochromic shifts as the polarity of the medium increases (here represented by the decreasing dioxane concentrations) (Fig. 4a). The λmax2 values obtained in the presence of Triton X-100 (at the maximal dye/detergent molar ratio assayed) with or without thymol (Fig. 4c), were interpolated in the plot of λmax2 versus D in Fig. 4b, and the values of D of the environment sensed by merocyanine in the Triton X-100 micelles were obtained (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>λpeak 1 (nm)</th>
<th>λpeak 2 (nm)</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>540</td>
<td>565</td>
<td>0</td>
</tr>
<tr>
<td>Triton X-100 + thymol</td>
<td>540</td>
<td>562</td>
<td>12</td>
</tr>
</tbody>
</table>

Values were taken from the merocyanine spectra shown in Fig. 4c in aqueous media, at the maximal merocyanine/Triton X-100 molar ratio assayed in the absence or in the presence of thymol (λpeak 1 and λpeak 2 are the measures at the wavelengths of maximal absorbance corresponding to the dimer and the monomer (at the lowest and the highest λmax, respectively). D values were calculated by interpolating the λmax values of the monomer in the graph of Fig. 4b.
3.2. Effects of thymol on the binding of FNZ to synaptosomal membranes

3.2.1. Effect of thymol at different concentrations on the specific and the non-specific binding of [3H]-FNZ

The aim of these experiments was to determine a range of thymol concentrations capable of affecting FNZ–membrane interaction. Thymol was added to the system at variable final concentrations between 0.66 and 666 µM in the presence of 3 nM [3H]-FNZ. At the lowest concentrations, thymol showed a tendency to inhibit the specific binding of [3H]-FNZ to its receptor site in GABA A-R. This effect was reverted at higher concentrations. Thymol was able to increment the percentage of ligand binding (with respect to the control without thymol) only at the highest concentrations assayed (333 and 666 µM) where an increase of about 25%, significantly different from control (P < 0.05, Student’s t-test), was observed. The Eq. (2) was fitted to the experimental data [3H]-FNZ bound versus thymol concentration (Fig. 5a):

\[
Y = \frac{\text{Min} + (\text{Max} - \text{Min})}{1 + 10^{(X - \text{IP})/\text{Hill's coefficient}}}
\]  

where, \(X\) is the log of thymol concentration, \(Y\) the percentage of specific binding of [3H]-FNZ, Max and Min the maximal and minimal percentages of binding, respectively, and IP the inflexion point of the curve which resulted 56.4 µM. The value of IP would represent the turning point from the inhibitory towards the activatory regime of thymol’s action.

In another experiment, the effect of ethanol on the specific binding of [3H]-FNZ was evaluated at the same concentrations applied when used as thymol vehicle (between 0.5 and 15 nm) were used. That terpene concentration was able to induce a maximal effect on the binding of [3H]-FNZ (see Fig. 5a).

3.2.2. Effect of thymol on the affinity and the maximal specific binding of [3H]-FNZ to GABA A-R

In these experiments, a constant thymol concentration of 666 µM (000 g/ml) and variable [3H]-FNZ concentrations (between 0.5 and 15 nm) were used. That terpene concentration was able to induce a maximal effect on the binding of [3H]-FNZ (see Fig. 5a). Fig. 6a shows typical saturation plots obtained in the absence (control) and in the presence of thymol. Eq. (1) was fitted to the experimental data (see Section 2) and the kinetic parameters of the specific binding were determined. The results obtained from seven independent experiments showed that thymol induced a significant decrement in the value of \(K_d\) (\(K_d\)-control = 2.9 ± 0.2, \(K_d\)-thymol = 1.7 ± 0.1 nm; P < 0.01, Student’s t-test) without a significant change in \(B_{max}\).
they are expressed as a percentage of \([3\ H]-\text{FNZ}\) binding to synaptosomal membranes. These results are shown in Table 2 where to the control (zero) of the specified binding with respect to the control (without any of the drugs). The numbers in parentheses indicate the mean of independent experiments done by triplicate. The percentage reported previously [25].

\[
\text{Percentage of } [3\ H]-\text{FNZ} = \frac{\text{max} - \text{control}}{\text{max} - \text{thymol}} 
\]

The values represent the mean ± S.E.M. of the specific binding of \([3\ H]-\text{FNZ}\) in the presence of the indicated drug, with respect to the control (without any of the drugs). The numbers in parentheses indicate the number of independent experiments done by triplicate. The means labeled with the same number are significantly different between them (a–d: \(P < 0.05\); e: \(P < 0.01\); f: \(P < 0.001\); one-way ANOVA).

Amphipathic compounds are characterized by the coexistence of regions of contrasting polarity in its molecular structure. This kind of compounds, due to the hydrophobic effect, can self-aggregate spontaneously when its concentration in an aqueous media increases over a critical value named CMC. The type of the self-aggregating structure formed depends on thermodynamic and geometric restrictions associated to the hydrophilic–hydrophobic balance within the molecule. These concepts have been developed theoretically by Israelachvili [26] and then proved in many experimental systems [27], and references therein. Due to its 3.2.3. Effect of thymol, GABA and bicuculline on the specific binding of \([3\ H]-\text{FNZ}\)

The activatory effect of thymol at 666 \(\mu\text{M}\) on the specific binding of \([3\ H]-\text{FNZ}\) was quantitatively similar to the maximal effect \([24]\) and could be blocked by the antagonist bicuculline at a concentration that was also considered to be able to exert a maximal effect \([24]\). These results are shown in Table 2 where they are expressed as a percentage of \([3\ H]-\text{FNZ}\) binding with respect to the control, in the presence of the modulatory drugs. Together, thymol and GABA, induced a stimulant effect (±30%) higher than the one obtained with each drug added separately (17 and 24%, respectively) and could also be completely abolished by bicuculline. Moreover, bicuculline alone induced a decrement of about 8% with respect
the hydrophobic dye (bromothymol blue) stopped increasing as a function of thymol concentration (Fig. 1b).

Conversely, Triton X-100 is a cone-shaped molecule able to self-assemble in micelles having a hydrophobic core [29]. Thus, the partitioning of the hydrophobic dye inside that core started at the CMC and increased at higher concentrations of Triton X-100 due to the growing amount of micellar structures. This was evidenced by the continuous decrement in the dye absorbance at 485 nm, which started at the detergent’s CMC.

Thymol induced an increment in the CMC value of Triton X-100 (compare Fig. 1c and d). Because the CMC is related with the free energy of micellization by the expression \( \Delta G = RT \ln \text{CMC} \), where \( \Delta G = \gamma_{\text{micelle}} - \gamma_{\text{monomer}} \), in thermodynamic terms, the increment in CMC reflects either an increment in the stability of the monomer (\( \gamma_{\text{monomer}} \) decreases) or a decrement in that of the micelle (\( \gamma_{\text{micelle}} \) increases). Although none of these possibilities could be discarded, the latter was supported by other experiments (see below). Thus, thymol was able to penetrate in highly packed monomolecular layers up to a lateral surface pressure of 47.8 mN/m (Fig. 3). So, its penetration should be expected not only in bilayers like sPC vesicles or natural membranes whose lateral pressure is about 35 mN/m but also in micelles where the molecules are packed at even lower lateral pressures. Once in the membrane, thymol may be located at the surface at different depths within the polar head group region, in the hydrophobic core of the micelle or in the hydrocarbon chain region of the bilayers. Which ever was the localization, the presence of thymol could generate tensions and would affect the stability of the self-aggregating structure. This would be more probable if thymol were placed within the polar head group region of the amphipathic molecules (here represented by Triton X-100 and sPC). Drug partitioning towards a membrane generates asymmetries in membrane tensions, a condition that could lead to a curved equilibrium configuration of the membrane [30–33]. Our results indicated that, in the presence of thymol, bilayer vesicles were forced to acquire a higher spontaneous surface curvature and consequently a smaller size (Fig. 2) in order to attain a more relaxed state.

Merocyanine has already been used as an electrochromic dye [22,23]. The positions of the absorbance peaks of the membrane-associated merocyanine reflected the properties of the microenvironment surrounding the dye molecules, specially the polarity if the contribution of the refractive index is small [34]. Thymol induced a blue shift in the absorbance maximum corresponding to the monomeric (membrane bound) form of merocyanine. This result indicated that this terpene induced either an increase in the polarity (higher \( D \)) of the Triton X-100 water interface or a change in the dye position, within the Triton X-100 micelle, towards a more polar environment. In addition, the strong dipole moment of thymol, calculated from its energy minimized structure (1.341 Debye), might have exerted repulsive electrostatic interactions with the positively charged merocyanine, leading to the decrease in the partition coefficient of the dye as indicated by the increment in the \( A_{\text{peak}} / A_{\text{peak}2} \) ratio (Table 1).

4.2. Effects of thymol on the binding of FNZ to synaptosomal membranes

The tendency of thymol to inhibit the specific binding of \([\text{H}]\)FNZ at low concentration and then to show a stimulant effect as its concentration increases, suggests a possible bimodal effect of this terpene. The effect of thymol changes from slightly inhibitory to considerably stimulatory at approximately the concentration corresponding to the IP shown in Fig. 5a. The possible inhibitory effect at low concentrations would involve either a competition with the ligand for the same binding site or a negative allosteric modulation of this site. On the other hand, the increase in the specific binding would indicate a possible positive allosteric modulation.

Thymol, at an activatory concentration, was able to diminish the \( K_0 \) of \([\text{H}]\)FNZ binding to GABA\(_A\)-R from chick forebrain, indicating an increment in the ligand affinity, without affecting \( B_{\text{max}} \). These results, expected for a GABA agonist with a positive allosteric modulation of the specific binding of \([\text{H}]\)FNZ [11,35], suggested an action mechanism of thymol exerted through the GABA receptor site. The experiments of \([\text{H}]\)FNZ binding performed in the presence of GABA or its antagonist bicuculline, were pointed to test this hypothesis. Bicuculline, a competitive antagonist of GABA [24,25], not only abolished the stimulation of \([\text{H}]\)FNZ binding induced by GABA, as it was expected, but also eliminated the stimulation induced by thymol. This result supported the idea of considering this terpene as a GABA agonist previously suggested by Mohammadi et al. [13] who reported that thymol was able to induce chloride currents via GABA\(_A\)-R in the absence of GABA. Cysteinyl oxidation at the peptidic sequence of receptor subunits was reported to induce an abnormal conformation state of GABA\(_A\)-R, leading to an increased binding of \([\text{H}]\)FNZ, which could be prevented by GABA\(_A\)-R antagonists [36]. A similar mechanism cannot be ascribed to thymol positive allosteric modulation of GABA\(_A\)-R because an eventual redox effect of this well known anti-oxidant terpene [37] would be exerted in the opposite direction.

Finally and taking into consideration that the actual EC50 value of thymol for the activatory effect on the binding of \([\text{H}]\)FNZ would be between the IP calculated in Fig. 5a (56.4 \( \mu \)M) and the maximal stimulant effect (666 \( \mu \)M), it can be suggested that thymol would have an affinity for the GABA\(_A\)-R considerably lower than that of GABA (IC50\(_{\text{GABA}} \approx 3 \mu \)M) [11,35].

Interestingly a tonic BZD-sensitive GABAergic inhibition has been reported in cultures of cerebellar granule cells [38]. Bicuculline could block this tonic action of GABA and caused a reduction of about 20% in the binding of FNZ in the absence of GABA [25]. A similar tendency was also observed in the present work since bicuculline produced a
in the aqueous media leading to the increment in drophobic FNZ within thymol self-aggregating structures. However, an eventual partitioning of the hy-CMC value might lead to suspect that they had an activity of GABA were observed at concentrations above its observed.

in the partitioning of FNZ, in a membrane whose curvature poration of the drug. Consequently, the expected increase to compensate the tensions initially generated by the incor-
plex systems, posses many mechanisms (like lipid flip-flop) reason that follow: (a) the ability of thymol to incorpo-
rate to bilayers, even at the packing conditions of a natural membrane, and to affect their surface curvature were proved (Figs. 2–4); (b) because higher curvatures were associated with lower molecular packings, it would be expected that these conditions correlate with a higher FNZ partition. Al-
though changes in the curvature of natural membranes are able to be induced by small molecules [30], they would have a transient kinetics because cellular membranes, being com-
plicated systems, posses many mechanisms (like lipid flip-flop) to compensate the tensions initially generated by the incor-
poration of the drug. Consequently, the expected increase in the partitioning of FNZ, in a membrane whose curvature could have been affected by thymol, might eventually not be observed.

The fact that the stimulating effects of thymol on the ac-
tivity of GABA were observed at concentrations above its CMC value might lead to suspect that they had an arte-
factual origin. However, an eventual partitioning of the hy-
drophobic FNZ within thymol self-aggregating structures would have diminished the actual free FNZ concentration in the aqueous media leading to the increment in Kd. On the other hand, an increase in non-specific binding of FNZ might have arose from the entrapment of FNZ molecules inside thymol structures that might remain adsorbed to the membraneous phase after the filtration procedure used to sep-
arate the bound and free radioligand fractions in a satura-
tion experiment. As none of these effects was observed, the possibility of an artefactual result derived from a sequestra-
tion of FNZ inside thymol’s self-aggregating structures was discarded.

4.4. The frontier between specific and non-specific drug-membrane interactions

Several membrane properties, like microviscosity, curva-
ture and dipolar arrangement, emerge at the supramolecular level of organization from the self-aggregation of mem-
brane constituent molecules and can be modulated by, or can be the modulating factors of drug–membrane specific (receptor-mediated) interactions. Among the non-specific mechanisms by which membrane components can influ-
ence embedded-proteins such as membrane receptors, can be cited: (i) the coupling between hydrophobic mismatch and curvature stress [41]; (ii) changes in the lateral stresses profile (the depth-dependent distribution of lateral stresses within the membrane) which affect the conformation equi-
librium and the activity of intrinsic proteins the function of which involves a structural change accompanied by a depth-dependent variation in its cross-sectional area in the transmembrane domain [42]; and (iii) the dipolar arrange-
ment of the membrane which was shown to affect signifi-
cantly the insertion, folding, conformation and activity of membrane proteins [43–45].

Our studies on model membranes support the hypothesis that the allosteric modulation of thymol on GABA\textsubscript{A}-R in-
volves an effect on the supramolecular organization of the receptor environment through the mechanisms described above. The observed tendency towards a bimodal behavior of thymol (Fig. 5), may reflect a dissection of its effects on both the binding of FNZ and the coupling between the binding sites of FNZ and GABA, exerted in opposite directions, being the former more sensitive than the lat-
ter. A similar behavior had already been reported for the terpene tagetone [11]. From the correlation between the hydrophobicity and the binding affinity of over 30 differ-
ent BZDs, Borea and Bonora [46] obtained a parabolic shaped plot which demonstrated that those BZDs with mean hydrophobicities (e.g. FNZ) exhibited the highest efficiency of BZD-GABA\textsubscript{A}-R interaction, reflecting the optimal membrane-partitioning/receptor-binding ratio. The inhibitory modulation of the binding of FNZ induced by thymol, might be associated with the effects of this terpene on the increment in the polarity of the membrane environ-
ment above the value required for maintaining FNZ at the top of the plot of binding affinity versus hydrophobicity.

Concluding, (i) thymol can incorporate to membranes at the packing densities of natural membranes increasing the surface curvature and polarity, (ii) at concentrations above the CMC, this terpene behaves as an activator of the binding of FNZ to GABA\textsubscript{A}-R, and (iii) the latter is an allosteric effect of thymol on the binding site of GABA, which may be interpreted as an improvement in the coupling between the binding sites for FNZ and GABA.
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