Multiple effects of short-chain alcohols on binding to rat heart muscarinic receptors

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Short-chain alcohols inhibited the equilibrium binding of agonists and antagonists to rat heart muscarinic receptors. Methanol, ethanol, propan-2-ol and propan-1-ol, when used at low concentrations, behaved as pseudo-competitive antagonists. Their rank order of potency paralleled their relative partition coefficients, suggesting that this inhibition was simply due to the interaction of the alcohols with a hydrophobic part of the receptor or with membrane lipids. The four alcohols increased the dissociation rate constant of [³H]oxotremorine M from the high-affinity agonist receptors and decreased the stability of this receptor state. These effects might reflect increased membrane fluidity and/or decreased hydrophobic interactions (see below). By contrast, the effects of alcohols on the association and dissociation rates of *N*-[³H]-methscopolamine (an antagonist) were not correlated to their relative octanol/water partition coefficient (a measure of their affinity for biophases). Alcohols, at the relatively high concentrations necessary for increased membrane 'fluidity', are known to affect the relative stability of various protein conformations. We believe that the effects of alcohols on antagonist binding to rat heart muscarinic receptors reflected changes in the activation energy of association and dissociation reactions, the inhibition of equilibrium binding being mainly due to decreased 'hydrophobic interactions'.

Molecular explanations of local anaesthesia generally ascribe the loss of neurotransmission to the anaesthetic's capacity to perturb lipid and/or protein components of nerve membranes. Alcohols and other local anaesthetics may indeed change membrane fluidity (Paterson *et al.*, 1972; Seeman, 1972; Chin & Goldstein, 1977, 1981, and references cited therein), their relative potencies being well correlated with their octan-1-ol/water partition coefficients.

In some cases, the reaction catalysed by membrane enzymes involves large conformational changes, and a re-arrangement of the lipid 'belt' surrounding the enzymes may become necessary. This reaction is greatly facilitated by lipid 'melting', so that a large decrease in enzyme activation energy may be observed at the lipid transition temperature. Adenylate cyclase activation by low alcohol concentration (e.g. see Stock & Schmidt, 1978; Uhlemann *et al.*, 1979) might be explained by a closely related mechanism, i.e. a facilitation

by increased membrane fluidity of the coupling between the catalytic unit and the activating GTP-binding protein.

Interpretation of alcohol effects in terms of membrane fluidity alone is, however, an oversimplification even when there is a good correlation between the alcohol potencies and their octan-1-ol/water partition coefficients. Indeed, this octan-1-ol/water partition coefficient is a very good representation of drug partitioning in all biophases, including not only membranes but also proteins, nucleic acids etc. (Hansch & Dunn, 1972). For example, leucine aminopeptidase (a soluble enzyme) is inhibited by alcohols, with a 'hydrophobicity'-related specificity, by competitive interactions with the active site of the enzyme (Hill & Smith, 1957).

In fact alcohols and other anaesthetics also induce or facilitate denaturation or conformational changes of soluble (Seeman, 1972; Ueda & Kamaya, 1973) as well as integral membrane proteins (Davio & Low, 1982). A modification of the relative stability of various enzyme conformations appears to be responsible for the alcohol inhibition of Na⁺ + K⁺-stimulated ATPase (Sun,

Abbreviations used: [³H]NMS, [*N*-methyl-³H]-methscopolamine chloride; [³H]Oxo-M, [*methyl*-³H]oxotremorine M acetate.

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1976; Lin, 1980), these effects being correlated with the alcohol hydrophobicity (Mitjavila *et al.*, 1976).

There are few studies on the effects of alcohols on receptors. The dose- and chain-length-related inhibition of antagonist binding to rat brain muscarinic and α_1 receptors has been interpreted as reflecting merely an increase in membrane 'fluidity' (Fairhurst & Liston, 1979). A good correlation between the hydrophobicity of alcohols and their inhibitory effect on the binding of [D-Ala², D-Leu⁵]enkephalin to δ opiate receptors is also documented (Hiller *et al.*, 1981). By contrast, the inhibition of dihydromorphine binding, which occurs only at much higher alcohol concentration (Hiller *et al.*, 1981; Tabakoff & Hoffman, 1983), is not linearly related to the membrane/water partition coefficient (Tabakoff & Hoffman, 1983). A detailed study of alcohol effects on the nicotinic acetylcholine receptor demonstrates the existence of an alcohol-preferring hydrophobic site, able to modulate the receptor conformation (El-Fakahany *et al.*, 1983). Another 'ethanol-binding site' might also affect benzodiazepine binding: the enhanced binding of 1 nM-[³H]diazepam by a low (0.1 M) ethanol concentration (Burch & Ticku, 1980) is probably mediated, indirectly, via the picrotoxinin-sensitive protein (Ticku & Davis, 1981).

In the present work, we studied the effects of alcohols on equilibrium binding, and on the association and dissociation kinetics of a muscarinic antagonist ([³H]NMS) and a muscarinic agonist ([³H]Oxo-M) to rat heart receptors, in an attempt to characterize better the effects of alcohols on these receptors.

Materials and methods

Chemicals

[³H]NMS (specific radioactivity 54 Ci/mmol) and [³H]Oxo-M (specific radioactivity 84 Ci/mmol) were obtained from New England Nuclear Corp. (Dreieich, Germany). Alcohols (puriss) were purchased from Fluka (Buchs, Switzerland).

Methods

Preparation of rat cardiac membranes. Rat cardiac membranes were prepared by the method of Snyder & Drummond (1978) with a few modifications, as indicated in Waelbroeck *et al.* (1982). Proteins were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Scatchard analysis. Cardiac membranes (0.1 mg of protein/ml) were incubated in 1.2 ml of 50 mM-sodium phosphate buffer, pH 7.4, containing 1% (w/v) bovine serum albumin and 2 mM-MgCl₂, and various concentrations of [³H]NMS (0.2–4.0 nM)

in the presence or in the absence of 1 μ M-atropine. Alcohol was added as indicated. Incubation for 10 min at 25°C or for 2 h at 5°C allowed for a complete equilibration in the absence or in the presence of alcohols. The samples were then filtered on glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) and rinsed four times with 2 ml of ice-cold 20 mM-Tris/HCl buffer, pH 7.5, containing 0.25 M-sucrose, 2 mM-dithiothreitol and 1% (w/v) bovine serum albumin. The dissociation constant and receptor concentration were calculated by the Scatchard (1949) procedure by linear-regression analysis (correlation coefficient $r \geq 0.95$).

Competition curves. Cardiac membranes (0.1 mg of protein/ml) were incubated as indicated above, in the presence of a fixed tracer concentration (1.0 nM-[³H]NMS or 1.5 nM-[³H]Oxo-M, unless otherwise indicated) and of increasing concentrations of unlabelled drug. Non-specific binding was determined in the presence of 1 μ M-atropine. The samples were filtered after a 10 min incubation at 25°C as indicated above, and specific binding was determined as the difference between total and non-specific binding.

Dissociation rates. Cardiac membranes (0.1 mg of protein/ml) were preincubated for 10 min at 25°C as indicated above, in the presence of the tracer (1.0 nM-[³H]NMS or 1.5 nM-[³H]Oxo-M) and in the presence or in the absence of 1 μ M-atropine in a total volume of 1080 μ l. Dissociation was then induced by the addition of atropine (final concentration 1 μ M) in the absence or in the presence of alcohols as follows. A 120 μ l volume of water (control) or unlabelled drug (final concentration as indicated in the legends of Figs. 6, 7 and 8) was added to each tube at time zero. Specific binding was measured at appropriate time intervals as explained above. 'Control' binding (after addition of water alone) was constant. The dissociation rate constant and initial binding were calculated by linear-regression analysis of $\ln B$ as a function of time (B representing specific binding) (correlation coefficients $r > 0.9$). The 'initial binding' calculated by this method was within 5% of 'control' binding observed after addition of 120 μ l of water.

Association rates. Cardiac membranes (0.1 mg of protein/ml) were incubated for the indicated time intervals in the presence of 1.0 nM-[³H]NMS or 1.5 nM-[³H]Oxo-M and in the presence or in the absence of 1 μ M-atropine or of the indicated concentrations of alcohols. The association kinetics were analysed according to eqn. (1):

$$\ln \left(\frac{B_{eq.}}{B_{eq.} - B_t} \right) = k_{obs.} \cdot t \quad (1)$$

assuming that variation of the tracer concentration (less than 10% of the total tracer concentration)

was negligible (Bennett, 1978), where B_t is the specific binding at a given time t , $B_{eq.}$ is the specific binding at equilibrium and $k_{obs.} = k_{on} L + k_{off}$, where k_{on} is the association rate constant, L is the tracer concentration and k_{off} is the dissociation rate constant. The plot of $\ln[B_{eq.}/(B_{eq.} - B_t)]$ was linear as a function of time for both tracers (results not shown). A plot of ' $k_{obs.}$ ' values as a function of $[^3H]NMS$ concentration (0.1–4.0 nM) yielded a straight line (results not shown), with an apparent K_D value equal to the K_D value found by Scatchard-plot analysis of equilibrium binding ($K_D = 1.2$ nM) and the y -axis intercept corresponding to the k_{off} value found by dissociation-rate studies (Table 1).

Results and discussion

We have previously shown that $[^3H]NMS$, an antagonist, recognizes all binding sites with the same affinity, whereas $[^3H]Oxo-M$, a labelled

agonist, binds only to a high-affinity subclass of muscarinic receptors at the ligand concentrations used in these experiments (Waelbroeck *et al.*, 1982). As shown in Fig. 1(a), atropine (a muscarinic antagonist) displaced $[^3H]NMS$ and $[^3H]Oxo-M$ binding, as expected for a non-selective drug, i.e. a drug that recognizes both classes of receptors (high-affinity and low-affinity binding sites) with the same affinity. By contrast, alcohols inhibited $[^3H]Oxo-M$ binding at lower concentrations than those required for inhibiting $[^3H]NMS$ binding [Figs. 1(b)–1(e)].

The alcohols behaved (at least at low concentration) as 'competitive' antagonists for $[^3H]NMS$ binding, i.e. they decreased the affinity of all binding sites (with high affinity and with low affinity for agonists) to the same extent, without affecting total receptor concentration (Fig. 2). The corresponding experiment, with $[^3H]Oxo-M$ as tracer, could not be performed, owing to the high non-specific binding, which made Scatchard

Fig. 1. Atropine and alcohol competition curves for $[^3H]NMS$ and $[^3H]Oxo-M$ binding. Binding of 1.0 nM- $[^3H]NMS$ (●) and 1.5 nM- $[^3H]Oxo-M$ (○) was measured at 25°C in the absence or in the presence of the indicated concentrations of atropine (a), methanol (b), ethanol (c), propan-1-ol (d) or propan-2-ol (e). The results are expressed as percentage of specific binding in the absence of unlabelled drug. This experiment is representative of two others.

Fig. 2. Scatchard analysis of $[^3\text{H}]\text{NMS}$ binding in the absence or in the presence of alcohols

$[^3\text{H}]\text{NMS}$ (0.2–4.0 nM) binding was measured in the absence (●) or in the presence of 2.0 M-methanol (○), 2.0 M-ethanol (△) or 0.4 M-propan-1-ol (■). The results were analysed by the Scatchard (1949) procedure. This experiment is representative of two to four others.

analysis of data in the presence of alcohols very difficult to interpret. 'Competition curves' by alcohols were shifted to the right at increasing $[^3\text{H}]\text{Oxo-M}$ concentrations, implying that the inhibition of $[^3\text{H}]\text{Oxo-M}$ binding was also at least partially competitive (results not shown).

The increase of the $[^3\text{H}]\text{NMS}$ dissociation constant (K_D) in the presence of alcohols was much larger at 25°C (Fig. 3) than at 5°C (Fig. 4). This result, and the fact that propan-2-ol was less active than propan-1-ol, suggested that the degree of inhibition might be related to the partition coefficient of alcohols between water and an organic phase. The excellent correlation between the logarithm of the membrane/water partition coefficient of the alcohols (Seeman, 1972) and the logarithm of the alcohol concentration inhibiting 50% of $[^3\text{H}]\text{NMS}$ or $[^3\text{H}]\text{Oxo-M}$ binding (Fig. 5) (Hansch & Dunn, 1972) supported this hypothesis. As alcohol effects on soluble proteins (Hill & Smith, 1975), biological membranes (Seeman, 1972) and lipid bilayers (Paterson *et al.*, 1972) may all be correlated to their octan-1-ol/water partition coefficient, no distinction could be made, at this point, between true 'competitive' inhibition

Fig. 3. $[^3\text{H}]\text{NMS}$ dissociation constant and total-receptor concentration as a function of alcohol concentrations at 25°C

The equilibrium dissociation constant (K_D) of $[^3\text{H}]\text{NMS}$ and the total concentration of muscarinic receptors (percentage of control) were measured in the absence (●) or in the presence of the indicated concentrations of methanol (○), ethanol (△), propan-2-ol (■) or propan-1-ol (□). Averages of three to seven experiments are shown.

(interaction with part of the muscarinic receptor 'binding site') or a membrane-fluidity-related effect. We therefore measured the effects of alcohols on the association and dissociation kinetics of both tracers: we expected that interaction of the alcohols with the binding sites would lead to a decreased association rate (competitive inhibition), whereas an increased membrane fluidity might decrease tracer binding by facilitating the dissociation reaction (i.e. increasing the dissociation rate).

Fig. 4. $[^3\text{H}]\text{NMS}$ dissociation constant and total receptor concentration as a function of alcohol concentration at 5°C

The K_D of $[^3\text{H}]\text{NMS}$ and the total concentration of muscarinic receptors was measured at 5°C in the absence (●) or in the presence of the indicated concentrations of methanol (○), ethanol (△), propan-2-ol (■) or propan-1-ol (□). Averages of three to seven experiments are shown.

As shown on Fig. 6, $[^3\text{H}]\text{Oxo-M}$ (Fig. 6b) and $[^3\text{H}]\text{NMS}$ (Fig. 6a) showed first-order dissociation kinetics, suggesting that only one type of drug-receptor complex was present. Ethanol (1M) increased significantly the dissociation rate of $[^3\text{H}]\text{Oxo-M}$ and slightly the dissociation rate of $[^3\text{H}]\text{NMS}$. The dissociation kinetics of $[^3\text{H}]\text{Oxo-M}$ and $[^3\text{H}]\text{NMS}$ remained first-order in the presence of the four alcohols tested (methanol, 1.0 and 2.0M; ethanol, 1.0 and 2.0M; propan-1-ol, 0.2 and 0.4M; propan-2-ol, 0.4 and 1.0M; three experiments; Fig. 6 and Tables 1 and 2). 'Dose-effect' curves were therefore obtained by measuring the percentage of tracer dissociated after a 3 min isotopic dilution (with $1\text{ }\mu\text{M}$ -atropine), in the absence or in the presence of alcohols (Figs. 7 and 8). The dissociation rate constants measured by this method were in good agreement with those obtained from complete dissociation kinetic studies (Fig. 6 and Tables 1 and 2).

The four alcohols tested increased markedly the $[^3\text{H}]\text{Oxo-M}$ dissociation rate constant, their relative potency being similar to that expected for a

Fig. 5. Relationship between the alcohol concentration inhibiting tracer binding by 50% (I_{50}) and the membrane/water partition coefficient

The logarithm of the alcohol concentration leading to 50% inhibition of 1.0 nM - $[^3\text{H}]\text{NMS}$ (●) or 1.5 nM - $[^3\text{H}]\text{Oxo-M}$ (○) binding (I_{50}) was plotted as a function of the log of the membrane/water partition coefficients ($\log P$) (Seeman, 1972). Correlation coefficients for linear regression (r) were 1.00 for $[^3\text{H}]\text{Oxo-M}$ and 0.97 for $[^3\text{H}]\text{NMS}$. Averages of three experiments are shown.

'hydrophobicity'-related effect (compare Fig. 7 with Fig. 5). This was not the case for $[^3\text{H}]\text{NMS}$: methanol was as potent as ethanol, and propan-2-ol (up to 1.0M) did not affect the $[^3\text{H}]\text{NMS}$ dissociation rate, despite its high membrane/water partition coefficient (0.276 as compared with 0.14 for ethanol and 0.45 for propan-1-ol; Seeman, 1972) (Fig. 8).

The increase of the dissociation rate of both tracers, large as it may appear, did not fully account for the observed inhibition of binding. We first checked that equilibrium binding was reached after 10 min of incubation, both in the absence and in the presence of alcohols. This being established, we calculated the percentage of 'unexplained inhibition' of tracer binding (Fig. 9), assuming at first approximation that a doubling of the dissociation rate of the tracer should lead to a 50% decrease of tracer binding (at the same tracer concentration).

In the case of $[^3\text{H}]\text{NMS}$ binding, the receptor concentration was not significantly decreased (except in the presence of 1M-propan-1-ol; Fig. 3) so that the 'unexplained inhibition' could be ascribed entirely to a decrease of the association rate constant in the presence of alcohols. This was corroborated by analysing association kinetics in the absence and in the presence of alcohols. As shown in Table 1, propan-1-ol was not significantly

Fig. 6. Dissociation of the [^3H]NMS-receptor and [^3H]Oxo-M-receptor complexes in the presence of 1M-ethanol. After a 10min preincubation, [^3H]NMS (a) and [^3H]Oxo-M (b) dissociation was induced by isotopic dilution (by 1 μM -atropine) in the absence (●) or in the presence (○) of ethanol (final concentration 1 M). Specific binding was measured after the indicated time periods. This experiment is representative of two others.

Table 1. [^3H]NMS kinetic constants in the absence and in the presence of four alcohols

The tracer concentration used $L = 1.0\text{ nM} = 0.8 K_D$. k_{off} was calculated as indicated in the Materials and methods section, in two to four experiments (consisting in ten determinations performed in duplicate). The standard deviations (s.d.) were equal to $\pm 10\%$ of the average values. k_{obs} was calculated as indicated in the Materials and methods section, in two experiments consisting in three to five determinations performed in duplicate. The standard deviations (s.d.) were equal to $\pm 10\%$ of the average values. $k_{\text{on}}L$ was calculated as $k_{\text{obs}} - k_{\text{off}}$. The expected B_{eq} was calculated as $B_{\text{eq}} = 1/(k_{\text{off}}/k_{\text{on}}L + 1)$, and expressed as fraction of the control value (in the absence of alcohol).

Alcohol	Concn. (M)	k_{off} (min^{-1})	k_{obs} (min^{-1})	$k_{\text{on}}L$ (min^{-1})	B_{eq} (% of control)	
					Expected	Observed
None (control)	—	0.42	0.72	0.30	100	100
Methanol	1.0	0.46	0.74	0.28	91	92
	2.0	0.61	0.90	0.29	77	77
Ethanol	1.0	0.48	0.76	0.28	88	80
	2.0	0.52	0.67	0.15	54	51
Propan-2-ol	0.4	0.42	0.63	0.21	80	85
	1.0	0.42	0.52	0.10	46	65
Propan-1-ol	0.2	0.45	0.72	0.27	90	82
	0.4	0.52	0.75	0.23	74	65

more potent than propan-2-ol in decreasing the [^3H]NMS association rate constant [the small difference between these two alcohols on Fig. 9(a) could be explained by the slight denaturing effect

of propan-1-ol; see Fig. 3]. The relative potencies of the four alcohols studied and, in particular, the similarity of propan-1-ol and propan-2-ol effects (Fig. 9) suggested that the decrease of the associ-

Table 2. [^3H]Oxo-M kinetic constants in the absence and in the presence of four alcohols

The tracer concentration used $L = 1.5 \text{ nM} = 0.9 K_D$. Constants were calculated as explained in Table 1 legend. The highest k_{obs} values (≥ 0.8) corresponded to only two determinations and were subject to the largest error (S.D. $\pm 15\%$ of the average value as compared with $\leq 10\%$ for smaller k_{obs} values).

Alcohol	Concn. (M)	k_{off} (min^{-1})	k_{obs} (min^{-1})	$k_{\text{on}}L$ (min^{-1})	B_{eq} (% of control)	
					Expected	Observed
Control		0.25	0.47	0.22	100	100
Methanol	1.0	0.29	0.57	0.28	105	63
	2.0	0.40	1.04	0.64	121	43
Ethanol	1.0	0.48	0.83	0.35	83	50
	2.0	0.78	1.04	0.27	51	19
Propan-2-ol	0.4	0.42	0.59	0.17	58	63
	1.0	0.54	6.69	0.18	50	28
Propan-1-ol	0.2	0.26	0.76	0.50	132	68
	0.4	0.39	1.01	0.72	130	40

Fig. 7. Effect of alcohols on the [^3H]Oxo-M-receptor complex dissociation rate constant

The [^3H]Oxo-M dissociation rate constant was measured after a 3 min isotopic dilution (in the presence of $1 \mu\text{M}$ -atropine) in the absence or the presence of increasing concentrations of methanol ($\circ$), ethanol (Δ), propan-2-ol ($\blacksquare$) or propan-1-ol ($\square$). The results are expressed as percentages of the control values (in the absence of alcohol). Averages of two to four experiments are shown.

Fig. 8. Effects of alcohols on the [^3H]NMS-receptor complex dissociation rate constant

The same experimental procedure as that indicated in Fig. 7 legend was used, in the presence or in the absence of methanol ($\circ$), ethanol (Δ), propan-2-ol ($\blacksquare$) or propan-1-ol ($\square$). Averages of two to four experiments are shown.

ation rate constant of [^3H]NMS was not correlated with alcohol membrane/water partition coefficients (methanol, 0.05; ethanol, 0.14; propan-2-ol, 0.28; propan-1-ol, 0.45; Seeman, 1972).

[^3H]Oxo-M binding in the presence of alcohols decreased after the first 5 or 10 min, suggesting that the high-affinity binding sites were progressively inactivated. This was further confirmed (Table 2) by the large discrepancy observed between 'expected' (from the kinetic constants) and measured tracer binding; indeed, a slight underestimation of ' B_{eq} ' in eqn. (1) leads to a large overestimation of ' k_{obs} '.

Fig. 9. 'Unexplained' inhibition of tracer binding (a) The $[^3\text{H}]\text{NMS}$ inhibition curve not due to an increased dissociation rate was calculated from the data of Figs. 1 and 8, assuming (as a first approximation) that a 2-fold increase of the dissociation rate should lead to 50% inhibition of tracer binding, any greater inhibition being due to a decreased association rate constant, decreased receptor concentration, or both. $\circ$, Methanol; Δ , ethanol; $\blacksquare$, propan-2-ol; $\square$, propan-1-ol. (b) $[^3\text{H}]\text{Oxo-M}$ inhibition not due to an increased dissociation rate [same as in (a), from the data of Figs. 1 and 7].

values, and therefore of the ' $k_{on}L$ ' values and 'expected' tracer binding.

Our findings may therefore be summarized as follows. (1) Alcohols behaved as pseudo-competitive inhibitors of $[^3\text{H}]\text{NMS}$ binding (Fig. 2). We suggest that this inhibition was due to a decrease in the strength of hydrophobic interactions, i.e. in the relative 'solubility' of free and bound $[^3\text{H}]\text{NMS}$ and receptor. This is supported by the good corre-

lation between the potency and hydrophobicity of alcohols (Fig. 5). On the other hand, the effects of alcohols on the $[^3\text{H}]\text{NMS}$ association and dissociation rate constants were not correlated with their relative membrane concentrations. The interpretation of this phenomenon is difficult, for the following reason. As discussed above, alcohols may inhibit hydrophobic interactions (methanol < ethanol < propan-2-ol $\leq$ propan-1-ol). The same alcohols may, however, also increase electrostatic interactions by decreasing the medium dielectric constant (methanol < ethanol < propan-1-ol < propan-2-ol). Since the relative contributions of hydrophobic and ionic interactions to the association and dissociation 'activation energies' are not known, it is therefore difficult to predict the effects and relative potencies of a given alcohol (and, in particular, of propan-1-ol and propan-2-ol) on the two rate constants. (2) The inhibition of $[^3\text{H}]\text{Oxo-M}$ binding by alcohols was relieved at least partly by increasing the tracer concentration. This inhibition of equilibrium binding of $[^3\text{H}]\text{Oxo-M}$ was due both to an increased dissociation rate and a progressive decrease in receptor concentration, the effects of alcohols on the association rate constant being difficult to assess. All these effects could be related to an increased membrane fluidity.

To conclude, the present data suggest that alcohols inhibit antagonist binding to rat heart muscarinic receptors mostly by decreasing hydrophobic interactions between ligand and receptors, whereas they inhibit agonist binding through increased membrane fluidity and/or decreased hydrophobic interactions.

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