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Novel Antagonists of Alcohol Inhibition of L1-Mediated Cell Adhesion: Multiple Mechanisms of Action

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ABSTRACT

1-Octanol antagonizes ethanol inhibition of L1-mediated cell adhesion and prevents ethanol teratogenesis in mouse whole embryo culture. Herein, we identify a new series of alcohol antagonists and study their mechanism of action. Cell aggregation assays were carried out in ethanol-sensitive, human L1-transfected NIH/3T3 cells in the absence and presence of 100 mM ethanol or 2 mM 1-butanol and candidate antagonists. Antagonist potency for 1-alcohols increased progressively over 5 log orders from 1-pentanol (C5) to 1-dodecanol (C12). Antagonist potency declined from 1-dodecanol (C12) to 1-tridecanol (C13), and 1-tetradecanol (C14) and 1-pentadecanol (C15) were inactive. The presence and position of a double bond in the 1-butanol molecule determined whether a compound was a full agonist (1-butanol), a mixed agonist-antagonist (2-buten-1-ol), or an antagonist (3-buten-1-ol). Increasing the concentration of agonist (1-butanol or ethanol) overcame the antagonism of 3-buten-1-ol, benzyl alcohol, cyclopentanol, and 3-pentanol, but not that of 4-methyl-1-pentanol, 2-methyl-2-pentanol, 1-pentanol, 2-pentanol, 1-octanol, and 2,6-di-isopropylphenol (propofol), suggesting that the mechanisms of antagonism may differ between these groups of compounds. These findings suggest that selective straight, branched, and cyclic alcohols may act at multiple, discrete sites to antagonize the actions of ethanol and 1-butanol on L1-mediated cell-cell adhesion.

Ethanol is a pleiotropic, weak central nervous system (CNS) drug (Charness et al., 1989). Ethanol potency is orders of magnitude less than that of other psychoactive drugs. This low potency indicates that the brain does not express a high-affinity ethanol receptor; rather, ethanol is believed to produce its CNS effects by interacting at millimolar concentrations with components of diverse neurotransmitter systems (Diamond and Gordon, 1997). For many years, the prevailing view was that ethanol modified synaptic activity by altering the biophysical properties of neuronal membranes, thereby disrupting indirectly the function of various membrane proteins (Goldstein, 1983). Recent research suggests that ethanol interacts directly with small regions of selective neuronal proteins (Slater et al., 1993; Franks and Lieb, 1994; Harris, 1999).

The immunoglobulin neural cell adhesion molecule L1 is a multifunctional, transmembrane protein that binds to L1 molecules on adjacent cells and to selective proteins in the extracellular matrix, cell membrane, and cytoskeleton (Crossin and Krushel, 2000). L1 interactions control cell-cell and cell-matrix events that are essential for growth cone mobility, axon pathfinding, axon fasciculation, and neuronal migration. L1 binding also triggers a series of transmembrane signaling events, resulting in neurite outgrowth and changes in growth cone morphology. L1 is expressed in the developing nervous system, where it plays a critical role in CNS development (Fransen et al., 1995, 1998; Demyanenko et al., 1999), and in the mature CNS, where it may be involved in learning and memory (Lüthi et al., 1994; Rose, 1995).

Mutations in the gene for L1 are associated with hydrocephalus, agenesis of the corpus callosum, cerebellar dysplasia, and a variety of other brain malformations (Fransen et al., 1995). Because children with fetal alcohol syndrome have neuropathology similar to that of children with L1 mutations, we studied the effects of ethanol on L1-mediated cell-cell adhesion (Charness et al., 1994; Ramanathan et al., 1996). Clinically relevant concentrations of ethanol inhibited L1-mediated cell adhesion in NG108-15 neuroblastoma × glioma hybrid cells, cerebellar granule cells, and selected human L1-transfected murine fibroblasts (Charness et al., 1994; Ramanathan et al., 1996; Wilkemeyer and Charness, 1998; Wilkemeyer et al., 1999). Similar concentrations of ethanol also inhibited L1-mediated neurite outgrowth in cerebellar granule cells (Bearer et al., 1999).

ABBREVIATIONS: CNS, central nervous system; DMSO, dimethyl sulfoxide; EC_{EQ}, aqueous concentration that produces molar membrane concentration equivalent to 100 mM ethanol.

Structure-activity analysis of various straight-chain, branched-chain, and cyclic alcohols revealed surprisingly strict structural requirements for alcohol inhibition of L1-mediated cell-cell adhesion (Wilkemeyer et al., 2000). The potency of methanol, ethanol, 1-propanol, and 1-butanol increased as a function of carbon chain length and membrane-buffer partition coefficient (Charness et al., 1994; Ramanathan et al., 1996). In contrast, 1-pentanol and higher 1-alcohols had no effect on L1-mediated cell-cell adhesion. The activity of 1-butanol, a four-carbon 1-alcohol, was abolished by the presence of a double bond between the carbons 3 and 4; however, the presence of methyl groups at carbon 2 or 3 was associated with an increase in potency (Wilkemeyer et al., 2000). These findings imply that ethanol and other small alcohols inhibit L1-mediated cell-cell adhesion by binding within a well defined, hydrophobic pocket of a target protein, possibly L1.

The existence of a specific binding pocket for ethanol predicts the discovery of drugs that can block ethanol's effects. Strikingly, very low concentrations of both the five-carbon alcohol 1-pentanol and the eight-carbon alcohol 1-octanol abolished the effects of ethanol on L1-mediated cell-cell adhesion (Wilkemeyer et al., 2000). 1-Octanol also blocked the effects of ethanol on the morphology of dividing neural cells (Wilkemeyer et al., 2000) and prevented apoptosis and dysmorphology in cultured mouse embryos (Chen et al., 2001). 1-Octanol is a toxic compound that could not be used clinically. However, the identification of a single compound that blocks ethanol teratogenesis suggests the possibility of safer alcohol antagonists. Herein, we identify a new series of alcohol antagonists and examine their structure-activity relation and mechanism of action.

Materials and Methods

Reagents. Alcohols were purchased from Sigma-Aldrich (St. Louis, MO); all other chemicals were purchased from Sigma-Aldrich or as indicated. Most alcohols were diluted in phosphate-buffered saline (0.13 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, and 0.002 M KH_2PO_4) or dimethyl sulfoxide (DMSO), as indicated.

Cell Culture. NIH/3T3 cells were cultured at 37°C in Dulbecco's minimum Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% normal calf serum (Intergen, Purchase, NY) and 400 μg/ml G418 (Invitrogen), in an atmosphere of 90% air and 10% CO_2. Three subclones were used in these studies: 2B2-L1, 2A2-L1, and Vec-1A5. The 2B2-L1 and 2A2-L1 cell lines are ethanol-sensitive subclones derived from a stable transfection of NIH/3T3 cells with human L1 cDNA; Vec-1A5 is a subclone from a transfection with the empty expression vector (Wilkemeyer and Charness, 1998).

Cell Adhesion Assay. Cell-cell adhesion was measured using a modified short-term aggregation assay of subconfluent cells (Wilkemeyer and Charness, 1998; Wilkemeyer et al., 2000). Cells were detached by gentle agitation in calcium-free and magnesium-free phosphate-buffered saline supplemented with 0.1 mg/ml DNase, mechanically dissociated to obtain a single-cell suspension, and diluted to 350,000 cells/ml. One milliliter of the cell suspension was added per well (4.5 cm²) to a 12-well plate. Agonists and test antagonists were added simultaneously, plates were sealed with parafilm to prevent evaporation, and the cells were gently shaken for 30 min at room temperature or on ice. Cells were viewed at a final magnification of 200×, and each well was scored for single and adherent cells in five to six microscopic fields of view. Approximately 100 cells/field of view and 600 cells/well were counted. The percentage of adherent cells was calculated for each microscopic field of view and then averaged for each well.

Agonists and antagonists were initially tested at aqueous concen-

trations that were calculated to produce molar membrane concentrations (~10 mM) equivalent to those produced by an aqueous concentration of 100 mM ethanol (EC_{EQ}). Calculations were based on membrane-buffer partition coefficients, which were obtained from a published source (McCreery and Hunt, 1978) or were derived by dividing the octanol/water partition coefficient by 5.

L1-mediated cell-cell adhesion was defined as the difference in percentage of adherent cells between an L1-transfected cell line (2B2-L1 or 2A2-L1) and a vector-transfected cell line (Vec-1A5). This component of cell adhesion is fully inhibited by Fab fragments of an anti-L1 polyclonal antibody (Wilkemeyer and Charness, 1998). Agonist inhibition of cell adhesion was calculated as 100 × (1 − the ratio of L1-mediated cell-cell adhesion in the presence and absence of agonist).

Agonists are defined as compounds that inhibit L1-mediated cell-cell adhesion. Antagonists are defined as compounds that have no effect on L1-mediated cell-cell adhesion and block the action of agonists. Molecules that are neither agonists nor antagonists are referred to as inactive. Antagonist activity was calculated as 100 × (1 − [(% inhibition cell adhesion by agonist plus antagonist)/(%inhibition cell adhesion by agonist)]).

Results

Structure-Activity Analysis of Alcohol Antagonists. The antagonists 1-pentanol and 1-octanol structurally resemble the agonist 1-butanol (Fig. 1) but do not inhibit L1-mediated cell-cell adhesion (Wilkemeyer et al., 2000). We hypothesized that other molecules that resemble agonists, but do not inhibit L1-mediated cell-cell adhesion, would also be antagonists. Each candidate antagonist was tested at a single aqueous concentration that was calculated to produce a molar membrane concentration equivalent to that produced by a buffer solution of 100 mM ethanol (EC_{EQ}). This concentration of ethanol maximally inhibited (62 ± 7%) L1-mediated cell-cell adhesion. Most of the candidate molecules blocked more than 60% of the activity of 100 mM ethanol or 2 mM 1-butanol (Table 1). In contrast, at the EC_{EQ} (Table 1) and at 10 times the EC_{EQ} (data not shown), DMSO, cyclopropylethanol, 1-tetradecanol, and 1-pentadecanol were neither agonists nor antagonists. To confirm that alcohols did not have an effect on the non-L1 component of cell adhesion, we performed aggregation assays on NIH/3T3 cells transfected with an empty vector. The mean percentage ± S.E.M. of cell adhesion for four independent assays was as follows: control, 15 ± 1; 6 mM 1-butanol, 13 ± 2; 50 μM 1-octanol, 12 ± 1; 10 mM 3-buten-1-ol, 14 ± 0; 0.7 mM 1-pentanol, 16 ± 2; 0.7 mM 4-methyl-1-pentanol, 15 ± 3; and 1% DMSO, 13 ± 0. Figure 1 shows the structure of the agonist and antagonist molecules discussed in this report.

Compounds Related to 1-Butanol. 3-Buten-1-ol differs from 1-butanol only by the presence of a double bond between carbon atoms 3 and 4. Alone, 3-buten-1-ol had no effect on L1-mediated cell-cell adhesion (Wilkemeyer et al., 2000); however, 9.6 mM 3-buten-1-ol (EC_{EQ}) blocked 64 ± 16% of the agonist activity of 100 mM ethanol (Table 1). The presence of a double bond between carbons 2 and 3 in 1-butanol had a different effect. At a concentration of 15 mM (EC_{EQ}), 2-buten-1-ol not only partially inhibited L1-mediated cell-cell adhesion (Fig. 2) but also partially antagonized the actions of 1-butanol (Fig. 2) and ethanol (data not shown). At higher concentrations (150 mM), 2-buten-1-ol maximally inhibited L1-mediated cell-cell adhesion and neither antagonized nor augmented the actions of 1-butanol or ethanol. Of note, 11 mM (EC_{EQ}) 2-butanol, which resembles 1-butanol and 1-pro-
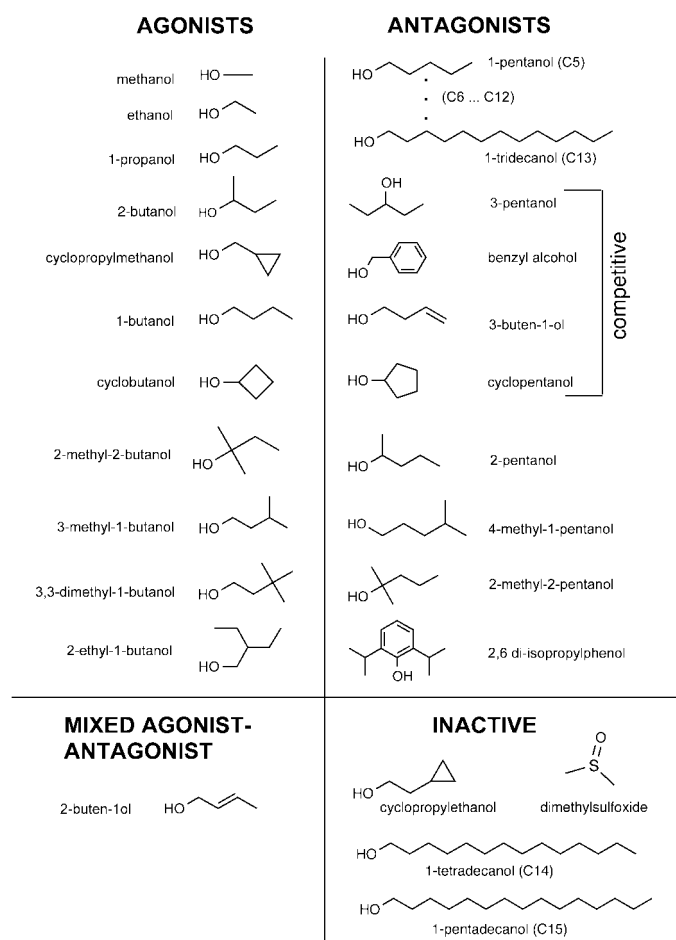
Fig. 1. Structure-activity relation of alcohols. Alcohols are categorized as agonists (inhibit L1-mediated cell-cell adhesion), antagonists (block the actions of agonists), mixed agonist-antagonist (inhibit L1-mediated cell-cell adhesion at lower potency than predicted with weak antagonist activity at low concentrations), or inactive (having neither agonist nor antagonist activity).

panol, was a full agonist ($63 \pm 9\%$ inhibition, $n = 6$) and had no antagonist activity (data not shown).

Cutoff Effect for Antagonist 1-Alcohols. 1-Octanol is a 200-fold more potent antagonist than 1-pentanol (Fig. 3) (Wilkemeyer et al., 2000). 1-Octanol is also 37-fold more lipid-soluble than 1-pentanol (Table 1). These data suggest that 1-octanol and 1-pentanol interact with a hydrophobic moiety within a putative antagonist binding site. We therefore asked whether the antagonist potency for 1-alcohols increases as a function of chain length and membrane-buffer partition coefficient and whether there is a cutoff above which longer 1-alcohols are less potent or inactive.

An artificial cutoff can be observed if a series of increasingly hydrophobic alcohols are incompletely soluble in water. To avoid this problem, we evaluated DMSO as a solvent for the 1-alcohols; 120 mM (1%) DMSO did not inhibit L1-mediated cell adhesion, nor did it antagonize ethanol inhibition of L1-mediated cell-cell adhesion (Table 1). At the concentrations used in our experiments, all 1-alcohols were soluble in DMSO.

Concentration-response curves were determined for antagonism of ethanol (100 mM) inhibition of L1-mediated cell-cell adhesion by a series of 1-alcohols dissolved in 1% DMSO (Fig.

3). Antagonist potency increased progressively over 5 log orders from 1-pentanol (C5) to 1-undecanol (C11). The potency of 1-dodecanol (C12) was only slightly greater than that of C11, whereas 1-tridecanol (C13) was approximately 25-fold less potent than C12. 1-Tetradecanol (C14) showed only minimal antagonist activity and 1-pentadecanol (C15) was completely inactive (Table 1). Because the test concentrations of C14 and C15 were very low (80 and 2.7 nM, respectively), we also evaluated higher concentrations (2–10 μM) to ensure that we did not miss an antagonist effect. Even at these higher concentrations, C14 and C15 were inactive (data not shown).

4-Methyl-1-pentanol, a Bivalent Antagonist. Molecules that contain multiple representations of 1-butanol were more potent agonists than 1-butanol (Wilkemeyer et al., 2000), consistent with the hypothesis that agonists interact with a selective recognition site. If the antagonist site is similarly selective then molecules that contain multiple representations of an antagonist should be more potent than the antagonist itself. 4-Methyl-1-pentanol can present a 1-pentanol molecule to a putative antagonist site from two different orientations (Fig. 4, inset). Concentration-response curves were determined for 1-pentanol and 4-methyl-1-pentanol antagonism of 2 mM butanol inhibition of L1-mediated cell-cell adhesion. 1-Hexanol was used as a control for the number of carbons in 4-methyl-1-pentanol. Antagonist potency was estimated from a linear regression analysis of the antagonist concentration-response curves. The aqueous concentration of 4-methyl-1-pentanol that produced half-maximal effect (32 μM) was approximately 7-fold greater than that of 1-pentanol (223 μM) and approximately 2.5 times that of 1-hexanol (80 μM) (Fig. 4). These ratios were then adjusted, based on the membrane buffer partition coefficient, to take into account the difference in membrane concentration of each antagonist. With this correction, 4-methyl-1-pentanol was approximately 3.7-fold more potent than 1-pentanol, whereas 1-hexanol was only 0.7-fold as potent as 1-pentanol.

Multiple Mechanisms of Antagonism. We showed previously that when tested in NIH/3T3 cells against a submaximally effective concentration of 1-octanol, increasing concentrations of 1-butanol reduced, but did not eliminate, the antagonist effect of 1-octanol (Wilkemeyer et al., 2000). This finding suggested that 1-octanol was a noncompetitive antagonist. We next used L1-expressing NIH/3T3 cells to investigate the mechanism of inhibition for two structurally dissimilar antagonists, 3-buten-1-ol and 1-octanol. Concentration-response curves for 1-butanol inhibition of L1-mediated cell-cell adhesion revealed that 2 mM 1-butanol produced approximately 90% of the maximal agonist response (Fig. 5A). Concentrations of 1-butanol above 75 mM altered membrane morphology and therefore were not used. Increasing concentrations of 1-butanol progressively reduced and then eliminated the antagonist effect of 3 mM 3-buten-1-ol. In contrast, increasing concentrations of 1-butanol did not eliminate the antagonist effect of 5 μM 1-octanol. These experiments suggest that alcohol inhibition of L1-mediated cell-cell adhesion can be antagonized through different mechanisms.

We next studied the mechanisms of inhibition for 10 different antagonists. Each antagonist was tested at a concentration that blocked 50 to 80% of the actions of 2 mM 1-butanol (Fig. 5B). Concentration-response curves for 1-butanol (0–75 mM) were determined in the absence and presence of a

TABLE 1

Antagonist activity and membrane/buffer partition coefficient for a series of alcohols and a nonvolatile anesthetic. Cell adhesion assays were performed in the absence and presence of the indicated compounds, as described in the text. Antagonist activity was measured against 100 mM ethanol for all of the compounds or against 2 mM 1-butanol, where indicated. The aqueous concentration of each antagonist was calculated to produce membrane concentrations equivalent to 100 mM ethanol. Membrane/buffer partition coefficients ($P_{m/b}$) were obtained or calculated from published octanol/water partition coefficients. The compounds are sorted by decreasing membrane/buffer partition coefficients. The $P_{m/b}$ for ethanol is 0.096 and for 1-butanol is 1.52.
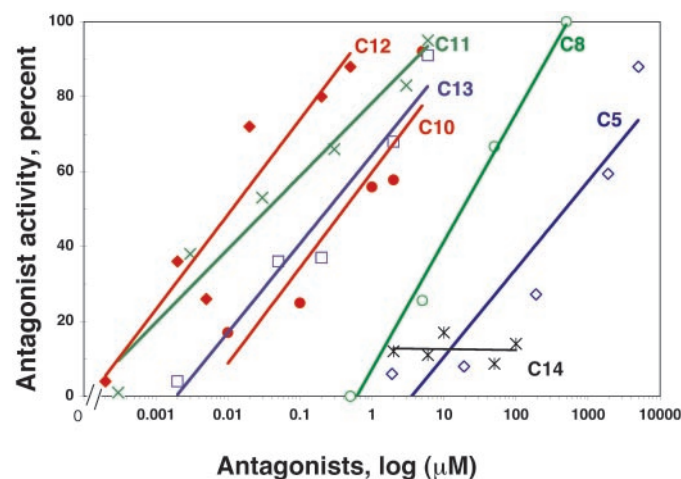
| Concentration | Alcohols | Antagonist Activity | | $P_{m/b}$ |
		Ethanol	Butanol	
mM		*% ± S.E.M.*		
2[a]	1-Pentadecanol	6 ± 7		347,560
2[a]	1-Tetradecanol	12 ± 6		112,468
0.26	1-Tridecanol	37 ± 10		36,394
0.35	1-Dodecanol	82 ± 11		26,979
3	1-Undecanol	89 ± 15		3,810
5	1-Decanol	93 ± 4		1,910
2[a]	2,6-Di-isopropylphenol	87 ± 7	68 ± 3	1,233
16	1-Nonanol	95 ± 4		604
50	1-Octanol	67 ± 9	82 ± 13	189
190	1-Heptanol	104 ± 4		51.4
450	1-Hexanol	88 ± 5		21.4
1,100	4-Methyl-1-pentanol	91 ± 8	95 ± 20	9.36
1,800	2-Methyl-2-pentanol	82 ± 5	61 ± 4	5.2
700[a]	1-Pentanol	58 ± 12	55 ± 7	5.02
2,100	3-Pentanol	92 ± 6	59 ± 13	4.69
2,200	2-Pentanol	75 ± 8	74 ± 7	4.38
3,800	Benzyl alcohol	87 ± 8	62 ± 17	2.52
4,300	Cyclopentanol	99 ± 1	83 ± 5	2.24
9,600	3-Buten-1-ol	67 ± 12		1
11,600	Cyclopropylethanol	19 ± 8		0.83
120,000[a]	Dimethyl sulfoxide	15 ± 16		0.012

[a] Aqueous concentration higher than that required to produce the same membrane concentration as 100 mM ethanol.

single concentration of each antagonist (Fig. 5b). Increasing concentrations of 1-butanol eliminated the antagonist activity of 3-pentanol, cyclopentanol, benzyl alcohol, and 3-buten-1-ol. In contrast, concentrations of up to 75 mM butanol did not eliminate the antagonist activity of 2-methyl-2-pentanol, 1-octanol, 1-pentanol, 2-pentanol, 4-methyl-1-pentanol, and 2,6-di-isopropylphenol (propofol). The two groups of compounds did not differ in their antagonism of 2 mM 1-butanol.
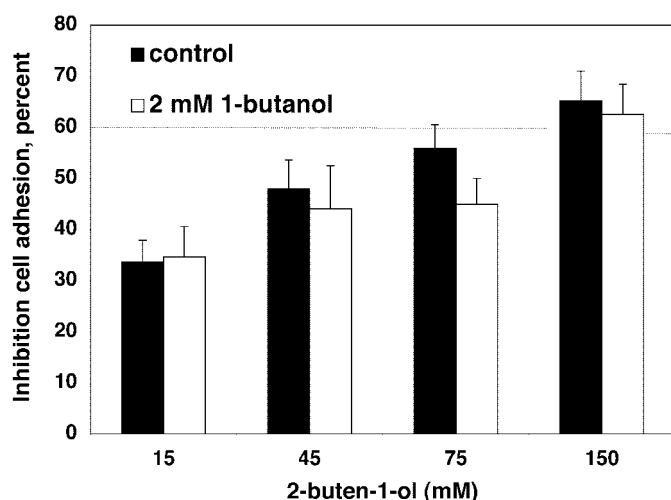
Fig. 2. Agonist and antagonist effects of 2-buten-1-ol. Cell adhesion assays were performed in human L1-expressing NIH/3T3 cells in the presence of the indicated aqueous concentrations of 2-buten-1-ol and in the absence (control, ■) or presence (□) of 2 mM 1-butanol. Columns represent the mean ± S.E.M. for the percentage of inhibition of cell adhesion from 4 to 11 independent experiments. The horizontal dashed line indicates the mean percentage of inhibition of cell adhesion by 2 mM 1-butanol alone (60 ± 5%, $n = 11$). The inhibition of cell adhesion produced by 15 mM 2-buten-1-ol plus 1-butanol (35 ± 6%, $n = 11$) and by 15 mM 2-buten-1-ol alone (34 ± 4%, $n = 10$) was significantly less than that produced by 2 mM 1-butanol alone (paired t test, $t = 5.21, 5.31$, respectively; $p < 0.001$).

Fig. 3. Antagonist effects of long-chain alcohols. Cell adhesion assays were performed in human L1-expressing NIH/3T3 cells in the presence of ethanol (100 mM) and the indicated aqueous concentrations of long-chain alcohols (antagonists). Shown are the means for the antagonist activity of the indicated alcohols ($n = 4$–16). Lines are fit by log-linear regression analysis. Shown below in parentheses are EC_{50} values (micromolar) based on the aqueous concentrations and EC_{50} values (micromolar) based on calculated membrane concentrations and expressed relative to 1-pentanol, as determined from the membrane-buffer partition coefficient (EC_{50} aqueous, EC_{50} membrane). Open blue diamond: C5, 1-pentanol (540, 540); open green circle: C8, 1-octanol (3, 98); filled red circle: C10, 1-dodecanol (0.4, 140); green cross: C11, 1-undecanol (0.03, 20); filled red diamond: C12, 1-dodecanol (0.01, 28); open blue square: C13, 1-tridecanol (0.25, 1625); and black asterisk: C14, 1-tetradecanol. 1-Pentadecanol (C15) exhibited no antagonist activity (Table 1).
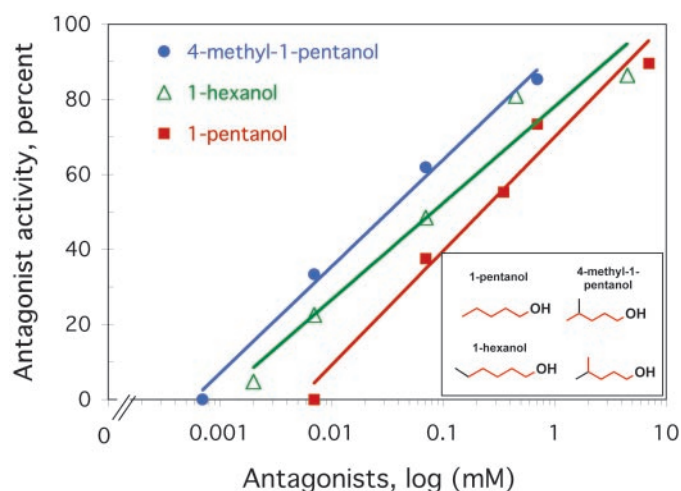
Fig. 4. Antagonism of 1-butanol effects by 1-pentanol, 1-hexanol, and 4-methyl-1-pentanol. Cell adhesion assays were performed in human L1-expressing NIH/3T3 cells in the presence of 1-butanol (2 mM) and the indicated concentrations of 1-pentanol, 4-methyl-1-pentanol, or 1-hexanol. Shown are the mean $\pm$ S.E.M. antagonist activity for the indicated aqueous concentrations of 4-methyl-1-pentanol (blue circle), 1-hexanol (green triangle), and 1-pentanol (red square). Inset, location of 1-pentanol molecules within the parent structures is outlined in red. Note that 4-methyl-1-pentanol, which can present a 1-pentanol moiety from two possible orientations, is approximately 4-fold more potent than 1-pentanol, after adjusting for differences in calculated membrane concentration (see *4-Methyl-1-pentanol, a Bivalent Antagonist* under *Results*).

Discussion

Our search for antagonists was guided by observations on the properties of agonists (Wilkemeyer et al., 2000). The potency of 1-alcohols increased as a function of chain length with an abrupt cutoff between 1-butanol and 1-pentanol. 1-Butanol was both the most potent 1-alcohol and also the most constrained, its activity being readily altered by minor chemical modifications. Restriction of movement between carbons 3 and 4 of 1-butanol (e.g., 3-buten-1-ol or cyclopropylethanol) or the placement of methyl groups adjacent to the hydroxyl group of 1-butanol (e.g., 2-pentanol and 2-methyl-2-pentanol) abolished agonist activity. In contrast, the presence of methyl or ethyl groups at carbon 2 or 3 increased agonist potency (e.g., 3-methyl-1-butanol, 2-ethyl-1-butanol, or 3,3-dimethyl-1-butanol). These data imply that interactions near carbons 1 and 4 of 1-butanol are critical for agonist activity.

The marked sensitivity of 1-butanol activity to chemical modification was most apparent in experiments with 2-buten-1-ol and 3-buten-1-ol. The presence of a double bond between carbons 2 and 3 of 1-butanol converted a full agonist (1-butanol) to a mixed agonist-antagonist (2-buten-1-ol). 2-Buten-1-ol inhibited L1-mediated cell-cell adhesion, albeit with lower potency than expected based on predicted membrane concentration. At low concentrations, 2-buten-1-ol partially inhibited L1-mediated cell-cell adhesion and partially antagonized the agonist activity of 1-butanol. At higher concentrations, 2-buten-1-ol acted as a full agonist and did not antagonize the actions of 1-butanol. Moving the double bond of 2-buten-1-ol from carbons 2 and 3 to carbons 3 and 4 transformed the molecule from a mixed agonist-antagonist to a competitive antagonist, 3-buten-1-ol. Hence, the presence and position of a double bond determined whether the resulting molecule is a potent agonist (1-butanol), a mixed agonist-

antagonist (2-buten-1-ol), or a competitive antagonist (3-buten-1-ol).

1-Propanol seems to be a less constrained agonist than 1-butanol. The presence of a methyl group adjacent to the hydroxyl group of 1-propanol (2-butanol) or the restriction of movement between carbons 2 and 3 (cyclopropylmethanol) did not affect agonist activity. However, placement of an ethyl group adjacent to the hydroxyl group of 1-propanol converted this molecule from an agonist to an antagonist (3-pentanol). Thus, even for the smaller 1-propanol molecule, sufficiently large substitutions in the vicinity of the hydroxyl group may produce steric hindrance and retard agonist interactions.

Our data suggest a model for agonist and antagonist effects on L1-mediated cell-cell adhesion (Wilkemeyer et al., 2000) (Fig. 6). We postulate the existence of an agonist target comprising a discrete hydrophobic binding site adjacent to a hydrophilic allosteric site. Agonist effects occur only when a molecule binds to the hydrophobic site and orients its hydroxyl group toward the allosteric site. The agonist target can be envisioned as a 1-butanol receptor that narrowly accommodates a flexed 1-butanol molecule. Restricting movement between carbons 3 and 4 of 1-butanol may abolish agonist activity by preventing bound 1-butanol from presenting its hydroxyl group to the allosteric site. An extended conformation of 1-butanol (3-buten-1-ol) or longer 1-alcohols (e.g., 1-pentanol) may be inactive as agonists because they are unable to bind coordinately to closely spaced hydrophobic and hydrophilic allosteric sites. Methanol, ethanol, and 1-propanol may be less potent than 1-butanol, because they are less hydrophobic and bind with lower affinity to the hydrophobic site. However, because they are small enough to maintain the correct orientation between their hydrophobic methylene groups and hydrophilic hydroxyl groups, they are still agonists.

A requirement for coordinate binding to two sites may also account for the observation that substituents adjacent to the hydroxyl group reduce agonist activity. Because the 1-butanol molecule is already a "tight fit", even minor degrees of steric hindrance might disrupt hydrogen acceptor activity at the allosteric site; in contrast, the less constrained 1-propanol molecule tolerates a methyl group but not an ethyl group adjacent to the hydroxyl group. The existence of a 1-butanol receptor would also explain why molecules that present a butanol moiety from multiple orientations are more potent agonists than 1-butanol (Wilkemeyer et al., 2000). For these divalent (3-methyl-1-butanol, 2-ethyl-1-butanol) and trivalent (3,3-dimethyl-1-butanol) 1-butanol molecules, the probability of presenting a 1-butanol moiety in the correct orientation to a 1-butanol receptor is greater than for 1-butanol.

A somewhat different picture of an antagonist binding site emerges from the structure-activity analysis of various antagonist alcohols. Most of the alcohols tested for antagonist activity were selected because they resembled agonists but did not inhibit L1-mediated cell-cell adhesion. The majority of these alcohols proved to be antagonists, although there were a few exceptions. 2-Buten-1-ol, which resembles 1-butanol, was a mixed agonist-antagonist. Cyclopropylethanol, which resembles the agonist cyclopropylmethanol, was inactive (neither an agonist nor an antagonist). One compound, propofol, does not clearly resemble any of the agonists but was an antagonist as well.

A.
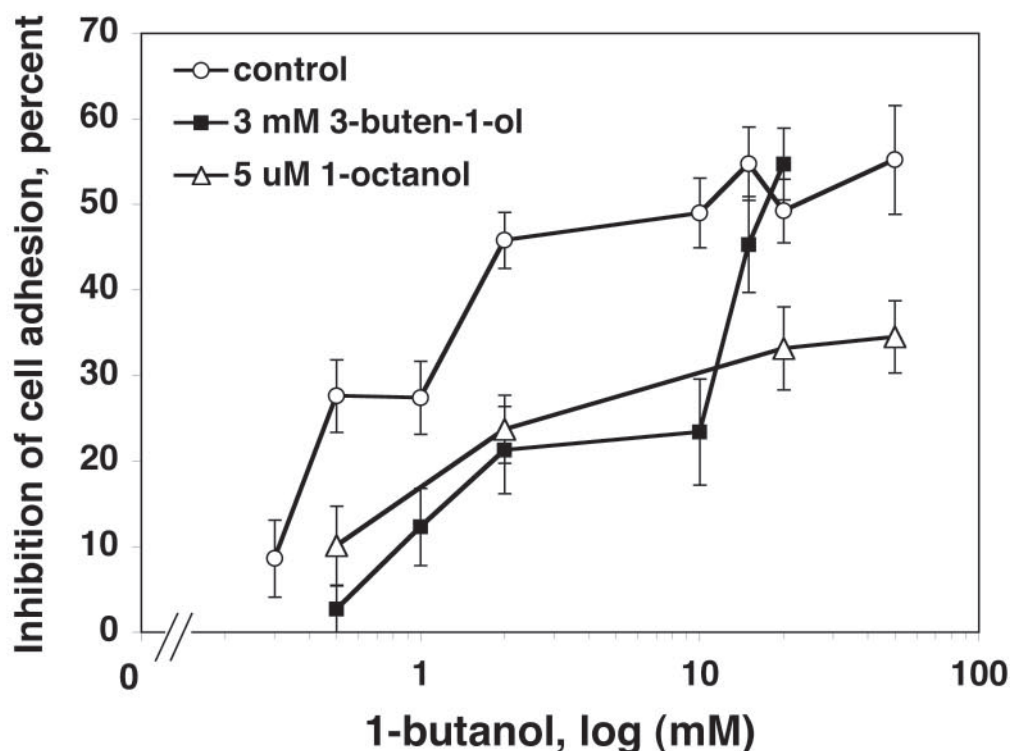
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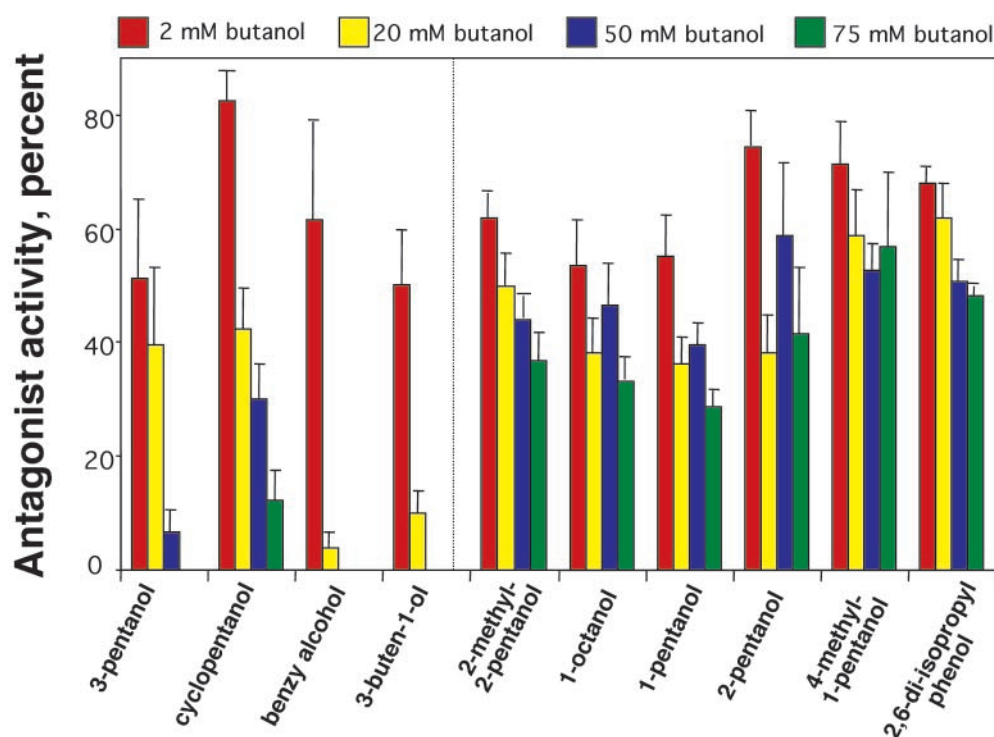
Fig. 5. Different mechanisms of antagonism of 1-butanol by alcohols. Cell adhesion assays were performed with NIH/3T3 cells expressing human L1 in the presence of increasing concentrations of 1-butanol and the indicated antagonists. A, concentration-response curves for 1-butanol inhibition of cell-cell adhesion (control, ○) were determined in the presence of IC_{50} concentrations for 3-buten-1-ol (3 mM) (■) and 1-octanol (5 μM) (△). Shown is the mean ± S.E.M. percentage of inhibition of cell adhesion for 5 to 12 experiments. Note that increasing concentrations of 1-butanol eliminate antagonism by 3-buten-1-ol, but not by 1-octanol. B, shown are the mean ± S.E.M. percentage of antagonist activity for the indicated compounds against 2 mM (red), 20 mM (yellow), 50 mM (blue), and 75 mM (green) 1-butanol ($n = 3$–14). The dashed vertical line separates antagonists whose actions were overcome by increasing concentrations of 1-butanol (left) from those that were not (right). Most of the antagonists were tested at aqueous concentrations that produce the same molar membrane concentration as an aqueous solution of 50 to 100 mM ethanol. For 3-buten-1-ol (3000 μM), 1-octanol (5 μM), 1-pentanol (700 μM), and 4-methyl-1-pentanol (40 μM), the approximate EC_{50} (against 2 mM 1-butanol) was used. The remaining concentrations were as follows: 3-pentanol (2000 μM), cyclopentanol (4300 μM), benzyl alcohol (3800 μM), 2-methyl-2-pentanol (1800 μM), 2-pentanol (2200 μM), and 2,6 di-isopropylphenol (2 μM).
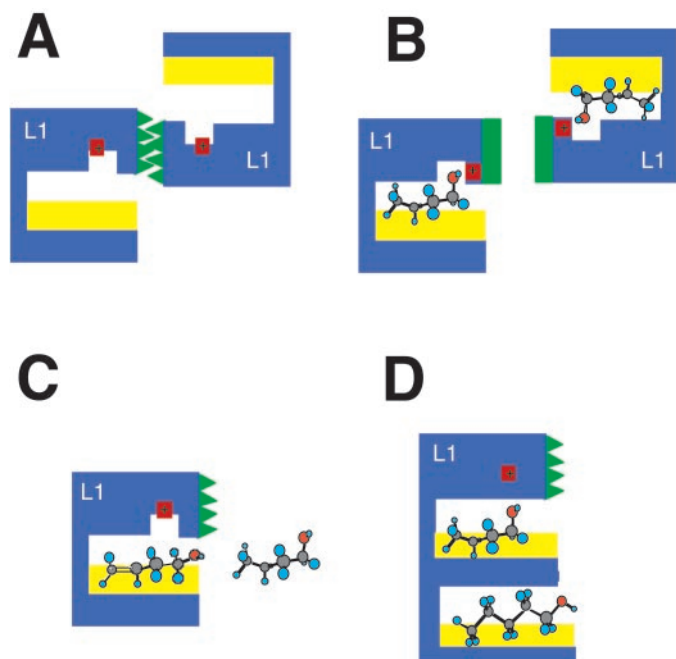
Fig. 6. Models of agonist and antagonist actions on L1-mediated cell-cell adhesion. A, homophilic binding of L1 molecules. A putative homophilic binding site is depicted in green. B, agonists, illustrated with 1-butanol, must bind to a hydrophobic recognition site (yellow) and orient their hydroxyl groups toward a hydrophilic allosteric site (red), producing a conformational change, perhaps in L1, that leads to reduced L1-mediated cell-cell adhesion. C, competitive antagonists, illustrated with 3-buten-1-ol, compete for agonist binding at the hydrophobic agonist recognition site, but are unable to orient their hydroxyl groups toward the allosteric site. D, noncompetitive antagonists, illustrated with 1-pentanol, may bind to a second hydrophobic site, at which they produce a conformational change that alters the relation of the agonist recognition site to the allosteric site. The model depicts L1 as the target protein, but the agonist and antagonist sites may reside on other proteins that interact with L1. Molecular models: red, oxygen; blue, hydrogen; and gray, carbon.

The membrane-buffer partition coefficients of the 1-alcohols increase with carbon chain length, and the series of 1-alcohols showed a striking increase in antagonist potency between C5 and C12. These data suggest that the antagonist 1-alcohols interact with a hydrophobic target site. Antagonist potency decreased between C12 and C13, and C14 and C15 showed little or no antagonism. The 1-alcohols from C13 to C15 are significantly more hydrophobic than the 1-alcohols from C5 to C12; therefore, the loss of activity with increasing carbon chain length indicates that the antagonist effect of C5 to C12 is not solely the result of their interaction with membrane lipids. More likely, these 1-alcohol antagonists target a cellular protein and bind within a hydrophobic pocket of restricted size. In contrast to the agonist site, which shows an abrupt cutoff between 1-butanol and 1-pentanol, the antagonist site shows a more gradual loss of recognition or binding of higher 1-alcohols. Because all of these alcohols were soluble in DMSO at the concentrations used, it is unlikely that this cutoff is an artifact of the increasing insolubility of the longer 1-alcohols.

If antagonists bind to a structurally selective site, then ligands that present an antagonist configuration from more than one orientation have a higher probability of correctly engaging the binding pocket. This may explain why the antagonist potency of 4-methyl-1-pentanol, which can present a 1-pentanol molecule from two different orientations, was greater than that of 1-pentanol. The two compounds differ only slightly in mem-

brane-buffer partition coefficient (Table 1); therefore, differential access to a hydrophobic site would probably not account for the difference in potency. Even after adjusting for the difference in membrane concentration predicted by partition coefficient, 4-methyl-1-pentanol was almost 4-fold more potent than 1-pentanol. A comparable discrepancy in agonist potency was observed between trivalent (3–3-dimethyl-1-butanol) and monovalent 1-butanol (Wilkemeyer et al., 2000).

The antagonist activity of four alcohols, 3-buten-1-ol, cyclopentanol, 3-pentanol, and benzyl alcohol, could be overcome by increasing concentrations of agonist. Each bears a close structural resemblance to a different alcohol agonist (Fig. 1). There are obvious similarities between 3-buten-1-ol and 1-butanol and between cyclopentanol and cyclobutanol. 3-Pentanol resembles the agonist 1-propanol, except for the presence of an ethyl group adjacent to the hydroxyl group. Benzyl alcohol, when aligned with 1-butanol, has features in common with 3-buten-1-ol, except that the ring structure ensures that the double bond is shared between carbons 2 and 3 and carbons 3 and 4. The structural similarity of these antagonists with alcohol agonists might enable them to compete for agonist binding at a common hydrophobic binding site (Fig. 6C).

The antagonist activity of a second group of alcohols could not be surmounted by increasing concentrations of agonist. All six molecules, 1-pentanol, 4-methyl-1-pentanol, 1-octanol, 2,6,-di-isopropylphenol, 2-pentanol, and 2-methyl-2-pentanol, present a linear array of at least five carbons and may be too large to fit within a delimited agonist binding pocket. These antagonists may interact with a second target site to alter the spatial relation between the hydrophobic agonist binding site and the allosteric site (Fig. 6D). In this scenario, the interaction of the agonist with the allosteric site would be impaired at all agonist concentrations. Noncompetitive antagonism can also result from covalent or high-affinity binding of an antagonist to a receptor; however, this mechanism is not consistent with the rapid reversibility of 1-octanol's antagonist activity (Wilkemeyer et al., 2000). The inactivity of 1-tetradecanol, 1-pentadecanol, and cyclopropylethanol suggests that the antagonist binding sites discriminate both molecular size and molecular shape.

Our hypothetical models depict alcohol modulation of cell-cell adhesion through direct interactions of agonists and antagonists with the L1 molecule (Fig. 6). Although this is one putative mechanism for alcohol effects on cell adhesion, it is not the only one. L1 also engages in heterophilic binding with a number of extracellular, transmembrane, and intracellular proteins (Crossin and Krushel, 2000), each of which might present alcohol binding sites. L1 is also phosphorylated at multiple sites, with important effects on L1 internalization and cell adhesion (Zisch et al., 1997; Kamiguchi and Lemmon, 1998). Conceivably, alcohols target the kinases or phosphatases that regulate the state of L1 phosphorylation or the sites at which proteins interact with L1 to alter its function. There is ample precedent for the regulation of ethanol sensitivity by phosphorylation. Phosphorylation modulates the response to ethanol for the glycine receptor (Mascia et al., 1998), *N*-methyl-D-aspartate receptor (Miyakawa et al., 1997; Anders et al., 1999), GABA$_A$ receptor (Hodge et al., 1999), mGluR5 metabotropic glutamate receptors (Minami et al., 1998), N-type and P/Q-type calcium channels (Solem et al., 1997), nerve growth factor (Hundle et al., 1997), nucleoside transporters (Coe et al., 1996), and serotonin 5-hydroxytryptamine$_{1c}$ receptors (Sanna et al., 1994). Further

research is required to learn the precise molecular targets of alcohol agonists and antagonists that modulate L1-mediated cell-cell adhesion.

The specificity of alcohol interactions with targets that regulate cell adhesion is unique among all defined neuronal targets of ethanol. Alcohols interact with discrete regions of the GABA$_A$ (Mihic et al., 1997) and ρ1 GABA$_C$ receptors (Wick et al., 1998), strychnine-sensitive glycine receptor (Mascia et al., 1996, 2000; Mihic et al., 1997; Wick et al., 1998; Ye et al., 1998), neuronal (McKenzie et al., 1995) and peripheral (Zhou et al., 2000) nicotinic acetylcholine receptors, G protein-linked inwardly rectifying potassium channels (Lewohl et al., 1999), Shaw2 potassium channel (Harris et al., 2000), and serotonin 5-hydroxytryptamine$_3$ receptors (Lovinger, 1999). Each of these targets exhibits different cutoffs for 1-alcohols. Mutagenesis studies of the glycine receptor indicate that the size of the alcohol cutoff is a function of the molecular volume of single amino acids at a key location within the ethanol binding site (Wick et al., 1998). Strikingly, ethanol itself is an antagonist for anesthetic actions at a mutated glycine receptor that is insensitive to ethanol but retains sensitivity to other anesthetics, consistent with competition of ethanol and anesthetics for a single binding site (Beckstead et al., 2001). At least for the G protein-coupled inwardly rectifying potassium channels, 1-octanol is not an ethanol antagonist (Lewohl et al., 1999). Taken together, these data suggest that a variety of neuronal proteins present alcohol binding pockets, but each one differs in its specificity for straight chain, branched chain, and cyclic alcohols.

Acknowledgments

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