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Analgesic Agents Share the Membrane Interactivity Possibly Associated with the Diversity of Their Pharmacological Properties

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Authors' contributions

This work was carried out in collaboration between both authors. Author HT designed and conducted the study, managed the experimental process and wrote the first draft of the manuscript. Author MM performed the experiments and the statistical analysis of the data and revised the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: Various drugs used for pain relief show the diversity of pharmacological properties besides their intrinsic analgesic activity. In order to verify a common mechanism, we studied the effects of selected analgesic agents on lipid bilayer membranes by paying attention to their induced physicochemical membrane modification and stereostructure specificity.
Methodology: Biomimetic membranes were prepared with different phospholipids and cholesterol to be unilamellar vesicle suspensions. The membrane preparations were treated with local anesthetics (lidocaine, bupivacaine and ropivacaine), phenolic sedatives/anesthetics (thymol, eugenol, guaiacol and propofol), non-steroidal anti-inflammatory drugs (ibuprofen and indomethacin), *N*-methyl-D-aspartate receptor antagonist (ketamine), and their stereoisomers at clinically-relevant concentrations, followed by measuring fluorescence polarization to determine the

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changes in membrane fluidity.
Results: All the tested drugs interacted with lipid bilayer membranes to modify their fluidity. Lidocaine, bupivacaine, ropivacaine, thymol, eugenol, guaiacol, propofol and ketamine increased the fluidity of neuronal mimetic membranes at 0.1-200 μM, whereas ibuprofen and indomethacin decreased the membrane fluidity at 100-200 μM. In neuronal and myocardial mimetic membranes consisting of 35-40 mol% chiral cholesterol, stereoisomers (25-200 μM) showed the enantiomer-specific membrane effects with the relative potencies being *R*(+)-bupivacaine > racemic bupivacaine > *S*(–)-bupivacaine, *S*(+)-ketamine > racemic ketamine, and *S*(+)-ibuprofen > racemic ibuprofen > *R*(–)-ibuprofen, which were correlated with those of their analgesic, anesthetic or cardiotoxic effects.
Conclusion: Analgesic agents share the ability to interact with lipid bilayers, directly influencing the properties and functions of biomembranes at a lipid level and indirectly modulating the activities of membrane-associated ion channels, receptors and enzymes through the conformational changes of proteins. The membrane interactivity possibly accounts for their pharmacological diversity.

Keywords: Analgesic agents; membrane interaction; lipid bilayer; pharmacological diversity; mechanism.

ABBREVIATIONS

NSAID, non-steroidal anti-inflammatory drug; NMDA, N-methyl-D-aspartate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]; POPI, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-myo-inositol), CL, cardiolipin; SM, sphingomyelin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMSO, dimethyl sulfoxide.

1. INTRODUCTION

Pain relief is achieved by medications with structurally- and mechanistically-different agents, including local anesthetics, sedatives/anesthetics, non-steroidal anti-inflammatory drugs (NSAIDs), *N*-methyl-D-aspartate (NMDA) receptor antagonists and anesthesia adjuncts (structures of representative agents shown in Fig. 1). In addition to the intrinsic analgesic activity, these agents show seemingly unrelated pharmacological effects. Local anesthetic lidocaine, bupivacaine and ropivacaine [1]; sedative/anesthetic thymol, eugenol [2], and propofol [3]; non-steroidal anti-inflammatory indomethacin [4] and ibuprofen [5]; and NMDA receptor antagonistic ketamine [6] have the properties to inhibit the growth of various bacterial and fungal species. Bupivacaine [7], propofol [8], indomethacin [9], ibuprofen [10] and ketamine [11] are able to inhibit the platelet aggregation induced by different agents. Lidocaine [12], thymol [13], propofol [14] and ibuprofen [15] scavenge free radicals or reactive oxygen species and inhibit the lipid peroxidation caused by them. Lidocaine, bupivacaine [16], propofol [17] and indomethacin [18] inhibit the proliferation, viability and invasion of different tumor cells and also induce their apoptosis. These analgesic agents show not only antimicrobial, antiplatelet, antioxidant and antitumor effects depending on molecular structures but also significant pharmacological differences even between stereoisomers as reported for antibacterial bupivacaine (racemic and *S*(–)-bupivacaine) [19] and antiplatelet ibuprofen (*S*(+)-, racemic and *R*(–)-ibuprofen) [10,20]. While their primary mode of action is referred to as the blockade of voltage-gated ion channels, the allosteric modulation of receptors, the inhibition of pathogenetically-responsible enzymes or the antagonism against relevant receptors, the pharmacological mechanism underlying such diverse effects with the structure-specificity remains poorly understood.

The diversity of pharmacological properties is not interpreted by the direct interaction with a specific functional protein alone, suggesting multiple molecular targets or a common target for analgesic agents. The physicochemical modification of biomembrane-constituting lipid bilayers is presumable as one of mechanisms for inhibiting microbial growth, platelet aggregation, lipid peroxidation and tumor cell proliferation. Although they are not essentially analgesics, antimicrobial peptides [21], antiplatelet benzodiazepines [22], antioxidant

phytochemicals [23] and antitumor drugs [24] commonly alter fluidity, order, elasticity or curvature of artificial and biological lipid membranes. Representative analgesic agents (Fig. 1) are structurally composed of an aromatic ring to confer hydrophobicity or lipophilicity on the molecule, an ionizable group to confer hydrophilicity and different substituents to provide chemical characteristics. Amphiphilic molecules interact hydrophobically and electrostatically with lipid bilayers, while highly hydrophobic molecules preferentially act on the deeper regions of lipid bilayers. Analgesic agents meeting such structural requirements would interact with lipid bilayer membranes [25] and their membrane interactions should at least partly underlie antimicrobial, antiplatelet, antioxidant and antitumor effects as well as analgesic effects [14,23-25]. Although the partition and distribution of membrane-interactive drugs into lipid bilayers are generally governed by their hydrophobicity, their potencies to modify the membrane physicochemical property are not necessarily determined by the order of hydrophobicity.

Besides membrane-embedded or membrane-bound ion channels, receptors and enzymes, local anesthetics, sedatives/anesthetics, NSAIDs, NMDA receptor antagonists and analgesic/ antinociceptive anesthesia adjuncts are presumed to act on membrane lipid components [11,14,23-26]. Therefore, we studied the effects of selected local anesthetics, phenolic sedatives/anesthetics, NSAIDs, NMDA receptor antagonists and their stereoisomers (Fig. 1) on lipid bilayer membranes in order to verify whether these analgesic agents mechanistically share the ability to interact with biomimetic membranes and show the structure-specificity. The drug and membrane interactions have been investigated by a variety of methodology including differential scanning calorimetry, magnetic resonance, electron spin resonance, fluorometry, etc. Because fluorescence polarization measurement has been most frequently used of spectroscopic methods for studying the membrane effects of drugs [26], we employed this method. The results are expected to provide not only an insight into the diversity of their pharmacological properties but also a novel experimental tool for discovering drugs and lead compounds.

2. MATERIALS AND METHODS

2.1 Chemicals

Local anesthetic lidocaine and bupivacaine; phenolic sedative/anesthetic thymol, eugenol, guaiacol and propofol; non-steroidal anti-inflammatory indomethacin, ibuprofen (racemate), *S*(+)-ibuprofen and *R*(–)-ibuprofen; and NMDA receptor antagonistic ketamine (racemate) and *S*(+)-ketamine were purchased from Aldrich

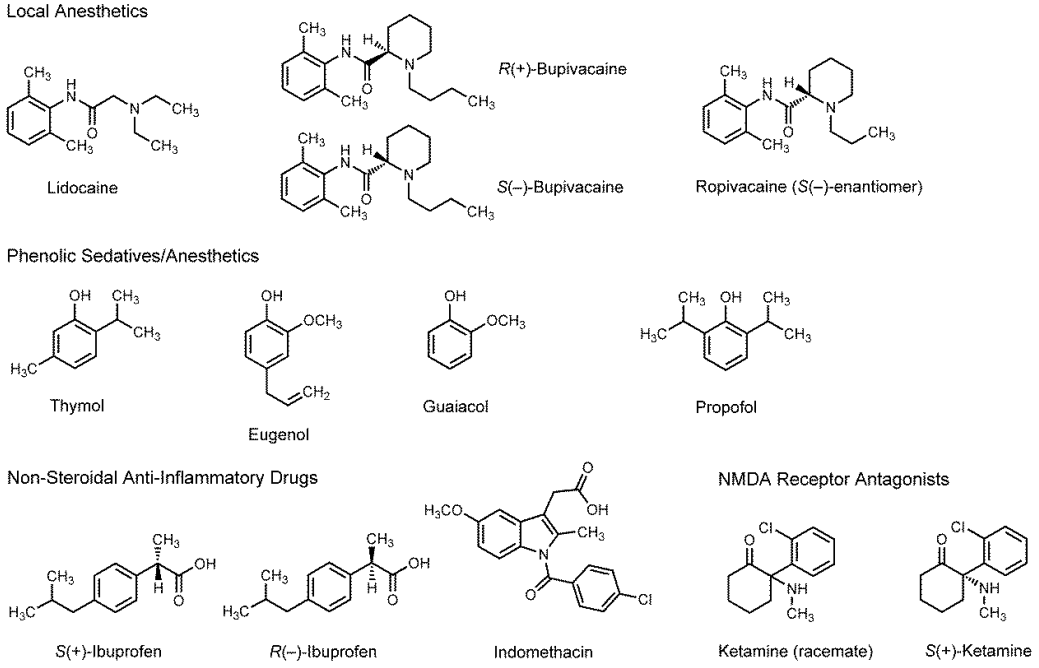

Fig. 1. Analgesic agents examined in this study

(Milwaukee, WI), Sigma (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan) or Tokyo Chemical Industrials (Tokyo, Japan). *S*(–)-Bupivacaine, racemic bupivacaine and *R*(+)-bupivacaine, and ropivacaine were supplied by Maruishi Pharmaceuticals (Osaka, Japan) and AstraZeneca (Södertälje, Sweden), respectively. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (POPI), bovine heart cardiolipin (CL) and porcine brain sphingomyelin (SM) were obtained from Avanti Polar Lipids (Alabaster, AL), cholesterol from Wako Pure Chemicals, and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Molecular Probes (Eugene, OR). Dimethyl sulfoxide (DMSO) of spectroscopic grade (Kishida; Osaka, Japan) and water of liquid chromatographic grade (Kishida) were used for preparing reagent solutions. All other chemicals were of the highest analytical grade available commercially.

2.2 Preparation of Biomimetic Membranes

DPH-labeled biomimetic membranes were prepared by the injection method for unilamellar vesicle preparation of Okimoto et al. [27] with some modifications as follows. The ethanol solutions (250 µL x 4) of phospholipids and cholesterol (total lipids of 10 mM) and DPH (50 µM) were repeatedly injected into 199 mL of 20 mM sodium phosphate buffer (pH 7.4, containing 100 mM KCl) under stirring at 50ºC. The molar ratio of DPH to membrane lipids was adjusted to be 1: 200 and the membrane structure after preparation and drug treatment was confirmed according to previous studies [28,29]. The lipid compositions of membranes were (1) 36 mol% POPC, 22 mol% POPE, 3.5 mol% POPS, 3.5 mol% SM and 35 mol% cholesterol to mimic neuronal membranes and (2) 25 mol% POPC, 16 mol% POPE, 10 mol% CL, 3 mol% POPS, 3 mol% POPI, 3 mol% SM and 40 mol% cholesterol to mimic myocardial membranes [26].

2.3 Determination of Membrane Interactions

The interactions of analgesic agents with biomimetic membranes to changes their fluidity were determined as reported previously [28,29]. In brief, the DMSO solutions of local anesthetics, phenolic sedatives/anesthetics, NSAIDs and ketamine were added to the suspensions of biomimetic membranes so that the final concentrations of tested drugs were analgesia-, sedation-, anesthesia- or cardiotoxicity-relevant 1-200 µM [26,30-33]. The concentration of DMSO vehicle was adjusted to be 0.5% (v/v) of the total volume so as not to affect the fluidity of intact membranes. DMSO of the corresponding volume was added to controls. After the reaction at 37ºC for 15 min, DPH fluorescence polarization was measured at 37ºC by an RF-540 spectrofluorometer (Shimadzu; Kyoto, Japan) equipped with a polarizer at 360 nm for excitation wavelength and 430 nm for emission wavelength. Polarization values were calculated according to the formula of Ushijima et al. [34]. The changes in fluorescence polarization were obtained by subtracting the polarization values of controls from those of drug treatments. Polarization decrease and increase mean an increase (membrane fluidization) and a decrease of membrane fluidity (membrane rigidification), respectively.

2.4 Statistical Analysis

All results were expressed as means ± S.E.M (n = 6-8). Data were statistically analyzed by a one-way analysis of variance (ANOVA), followed by a *post hoc* Fisher's protected least significant difference (PLSD) test using Stat View 5.0 (SAS Software; Cary, NC). *P* values less than 0.01 were regarded as statistically significant.

3. RESULTS

3.1 Membrane Interactions to Increase Fluidity

Local anesthetics and phenolic sedatives/ anesthetics concentration-dependently interacted with neuronal mimetic membranes to increase the membrane fluidity as shown by polarization decreases (Fig. 2). NMDA receptor antagonistic ketamine also fluidized the membranes. While all of them were membrane-interactive at a micromolar level, alkylphenols were more effective in fluidizing the membranes than local anesthetics and ketamine. Especially, propofol increased the membrane fluidity even at sub-micromolar concentrations (~0.1 µM). In alkylphenols, propofol was the most potent, followed by thymol, guaiacol and eugenol. In local anesthetics, the relative membrane-fluidizing potency was bupivacaine > ropivacaine > lidocaine.

3.2 Membrane Interactions to Decrease Fluidity

NSAIDs decreased the membrane fluidity at 25-200 μM by interacting with neuronal mimetic membranes as shown by polarization increases (Fig. 2, comparative effects shown for NSAIDs). Indomethacin was greater in membrane rigidification than ibuprofen.

3.3 Stereospecific Membrane Interactions

Local anesthetics stereospecifically interacted with myocardial mimetic membranes to show different potencies between bupivacaine stereoisomers at a cardiotoxically-relevant concentration [30] (Fig. 3). When comparing the polarization decreases at an equimolar concentration, the relative potency to fluidize the membranes was *R*(+)-bupivacaine > racemic bupivacaine > *S*(–)-bupivacaine. NMDA receptor antagonists similarly showed the stereospecificity to be *S*(+)-ketamine > racemic ketamine in interactivity with neuronal mimetic membranes at an anesthesia-relevant concentration [33] (Fig. 3).

Non-steroidal anti-inflammatory ibuprofen also stereospecifically interacted with neuronal mimetic membranes at a pharmacokinetics-relevant concentration [32], although the membrane fluidity was differently decreased depending on its stereostructures (Fig. 4). The relative potency of membrane rigidification was *S*(+)-enantiomer > racemate > *R*(–)-enantiomer.

4. DISCUSSION

Our main findings are as follows: (1) all the tested analgesic agents interact with biomimetic membranes to modify the fluidity but their interaction potencies vary in a structure-dependent manner, (2) their membrane interactions induce either fluidity increases or decreases depending on drug class, and (3) their membrane interactivities are discriminated between stereoisomers.

The rank order of membrane interactivity (local anesthetics: bupivacaine > ropivacaine > lidocaine, alkylphenols: propofol > thymol > guaiacol > eugenol, and NSAIDs: indomethacin > ibuprofen) almost agrees with that of analgesic activity of local anesthetics (bupivacaine > ropivacaine > lidocaine) [35,36], of sedative/anesthetic activity of alkylphenols (propofol > thymol) [37], and of analgesic activity of NSAIDs (indomethacin > ibuprofen) [38]. The relative potencies of membrane interactions also correlate to those of antimicrobial effects of local anesthetics (bupivacaine > ropivacaine > lidocaine) [1], of antiplatelet effects of NSAIDs (indomethacin > ibuprofen) [39], of antioxidant effects of alkylphenols (propofol > thymol > eugenol) [40], and of antitumor effects

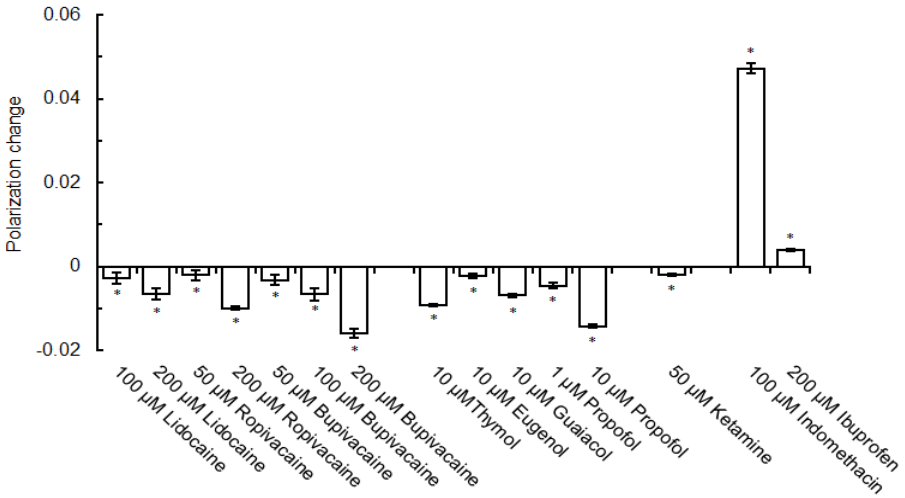

Fig. 2. Effects of local anesthetics, phenolic sedatives/anesthetics, ketamine and NSAIDs on biomimetic membranes. Neuronal mimetic membranes were subjected to the reactions with drugs, followed by measuring DPH fluorescence polarization. The polarization changes from controls are shown as means ± S.E.M (n = 6)

**P < 0.01 compared with control*

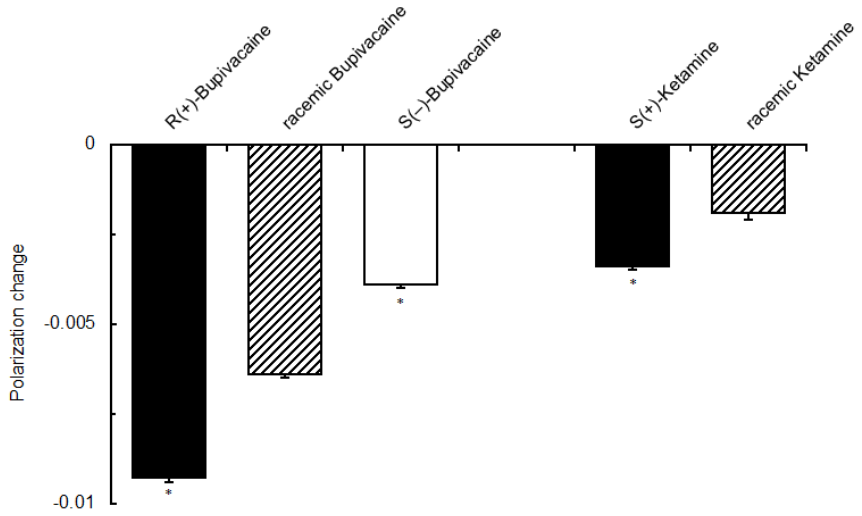

Fig. 3. Stereospecific fluidizing effects of bupivacaine and ketamine on biomimetic membranes. Myocardial and neuronal mimetic membranes were subjected to the reactions with 25 μM bupivacaine stereoisomers and 50 μM ketamine stereoisomers, respectively, followed by measuring DPH fluorescence polarization. The polarization changes from controls are shown as means ± S.E.M (n = 8)

**P < 0.01 compared with each racemate*

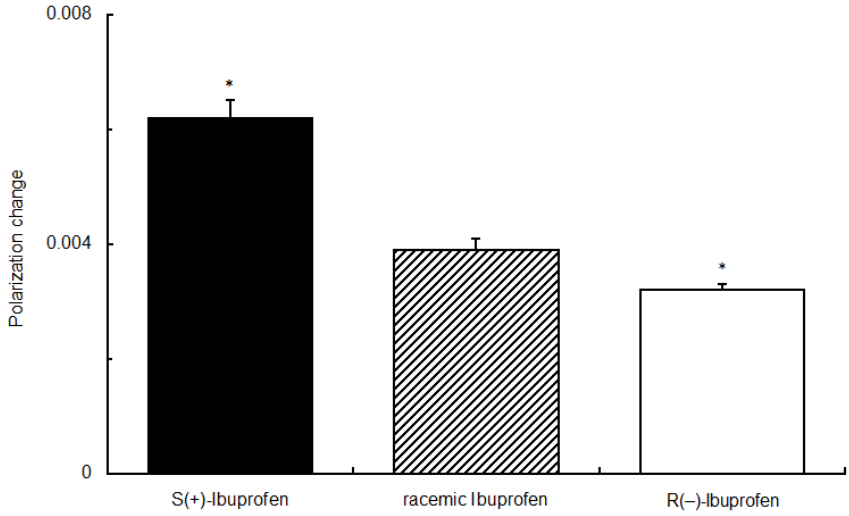

Fig. 4. Stereospecific rigidifying effects of ibuprofen on biomimetic membranes. Neuronal mimetic membranes were subjected to the reactions with 200 μM ibuprofen stereoisomers, followed by measuring DPH fluorescence polarization. The polarization changes from controls are shown as means ± S.E.M (n = 8)

**P < 0.01 compared with racemate*

(bupivacaine > lidocaine) [16]. While pipecoloxylide local anesthetics, NMDA receptor antagonists and propionate NSAIDs show stereostructure-dependent effects which are discriminable between enantiomers, the comparative potencies of membrane fluidity modification are correlated not only with those of pharmacological or toxicological effects of bupivacaine [41,42], ketamine [43] and ibuprofen stereoisomers [44], but also with those of their antibacterial [19] and antiplatelet effects [20].

Drug molecules interact hydrophobically with the aliphatic chains of membrane-constituting phospholipids and electrostatically with the phospholipid polar head groups. They penetrate into lipid bilayers with the preference of more hydrophobic molecules to deeper regions of the membranes. Since a fluorescent probe DPH aligns with phospholipid acyl chains and it is subject to the rotational restriction imparted by membrane fluid or rigid conditions, the membrane interactions determined in this study primarily reflect the hydrophobic membrane interactions which are greater in drugs with higher hydrophobicity. The rank order of membrane interactivity is almost the same as that of hydrophobicity, lipophilicity or partition coefficient of local anesthetics (bupivacaine > ropivacaine > lidocaine) [45], alkylphenols (propofol > thymol > eugenol) [46] and NSAIDs (indomethacin > ibuprofen) [47].

The preferential incorporations in lipid bilayers and the interactions with membranes vary by a slight structural difference of local anesthetics [45] and alkylphenols [46,48]. And furthermore, the effects to modify membrane fluidity were discriminated even between stereoisomers of bupivacaine, ketamine and ibuprofen. The opposite absolute configurations allow enantiomers to be discriminated by the interaction with another chiral molecule in membranes. Cholesterol with several chiral centers is contributable to the enantiomer-specific membrane interaction. From their induced DPH polarization changes, bupivacaine, ketamine and ibuprofen are assumed to penetrate into membrane lipid bilayers and align between phospholipid acyl chains, although the spaces to be occupied by drug enantiomers vary by their configurational differences. Cholesterol is oriented in membranes with a 3β-hydroxyl moiety anchoring to phospholipid polar head groups, a steroid ring adjoining fatty acyl chains and a flexible alkyl chain extending into hydrophobic membrane cores [49]. The adjacently aligning chiral cholesterol could provide lipid bilayers with the chirality which is responsible for the stereospecific membrane interactions of bupivacaine and ibuprofen enantiomers to modify the membrane fluidity depending on *R* (+)- or *S*(–)-configuration and *S*(+)- or *R*(–)-configuration, respectively.

Antimicrobial compounds and antibiotics directly act on phospholipids of cell membranes to alter membrane organization, fluidity, permeability and dynamics [50,51]. The resulting disturbance of membrane structures and functions produces antimicrobial effects. Antiplatelet drugs inhibit the platelet aggregation induced by mechanistically-different agonists, suggesting the common site of their actions, not confined to a receptor specific to each individual inducer. They change platelet membrane fluidity, with a resultant influence on the activity of phospholipase C, and subsequent inhibition of phosphoinositide breakdown, inhibiting intracellular Ca^{2+} mobilization and thereby resulting in platelet aggregation inhibition [22]. Antioxidant agents to scavenge free radicals either decrease the fluidity of lipid bilayers to reduce the radical mobility in rigid membranes [52] or increase the fluidity of lipid bilayers to make the interaction between antioxidant molecules and radicals more efficient in fluid membranes [53], causing the suppression of lipid peroxidation, because the fluidity governs the propagation of oxidant and antioxidant molecules in lipid bilayer membranes. Since the activation and suppression of cell proliferation occur in the lipid membrane environments, cell membranes and membranous organelles are considered as one of targets for antitumor agents [54]. Doxorubicin used for the treatment of a wide range of cancers has the property to alter the lipid bilayer fluidity and the membrane protein conformation of erythrocytes from leukemia patients [55]. The membrane interactions independent of cyclooxygenase inhibition underlie both beneficial anti-inflammatory effects and adverse gastrointestinal injury actions of NSAIDs [56]. Membrane fluidity modification linked to the inhibition of cyclooxygenase also plays a crucial role in tumorigenesis [57]. While antitumor drugs with different mechanisms modify the fluidity of membrane hydrophobic regions, the alteration of membrane fluidity affects the functions of cells and the induction of apoptotic pathways, leading to the death of tumor cells [58]. The membrane interactivity shared by analgesic agents is possibly associated with the diversity of their pharmacological properties.

Local anesthetics, thymol, eugenol and NSAIDs exert antibacterial and antifungal effects by damaging cell membranes, inhibiting dehydrogenase and increasing cell wall permeability [1], affecting the biosynthesis of a specific membrane component and the membrane integrity [59], disrupting cytoplasmic membranes [60] and inhibiting DNA synthesis [5], respectively. Although the medicinal product of propofol was suggested to support the growth of many microorganisms, such properties are attributed to soya bean oil and egg lecithin, not

propofol itself, contained in the propofol emulsion formulation like Diprivan® [61]. Propofol show the antibacterial activity based on membrane lysis and permeability increase. Bupivacaine [7], propofol [8] and NSAIDs [10] prevent platelet aggregation through multiple platelet signaling pathways, stimulated NO production and cyclooxygenase inhibition. Local anesthetics [12], alkylphenols [13] and NSAIDs [62] scavenge reactive oxygen species and inhibit their induced lipid peroxidation, contributing to the antioxidant effects. Local anesthetics [16], alkylphenols [17, 63,64] and NSAIDs [18] not only promote the apoptosis of various tumor cells but also inhibit tubulin and enzymes associated with tumor proliferation and progression. In addition to these mechanisms, the mode of membrane interaction is considered to be at least in part responsible for the diverse effects of analgesic agents.

Membrane lipid bilayers regulate or determine the functions of membrane-embedded or membrane-bound proteins such as voltage-dependent sodium and calcium channels, $GABA_A$ and NMDA receptors, and cyclooxygenase [65,66]. The intrinsic effects of analgesic agents would be the combined results of the interaction with these proteins to modulate their activities and the interaction with membrane lipids to alter the lipid environments surrounding functional proteins. The latter mechanistic interaction is also likely to produce the pharmacological effects independent of ion channels, receptors and enzymes.

Although the clinical implications of the membrane interactivity shared by analgesic agents may be beyond the scope of this study, the discussion on their effects besides the intrinsic analgesic activity would be valuable for speculating the practical applications of membrane-interactive drugs, possibly suggesting their supplemental roles. Local anesthetics and NSAIDs are used to prevent the incidence of pain on drug injection. Ketamine is frequently applied together with general anesthetics during clinical use. Membrane-acting drugs (lidocaine, ketamine, indomethacin, etc.) with the antimicrobial activity may be effective in reducing the risk of postoperative infections and sepsis. In dental applications such as impacted tooth extraction, membrane-acting antimicrobial NSAIDs and alkylphenols may decrease postoperative complications and exhibit the synergism with antibiotics. Surgery and the accompanying anesthesia induce the oxidative stress which is implicated in ischemia-reperfusion, inflammatory and cardiovascular injuries and cerebral damages as one of factors to increase postoperative morbidity and mortality. Protection against the pathological states relating to peri-, intra- and postoperative oxidative stress is expected for membrane-acting drugs (lidocaine, propofol, thymol, ketamine, etc.) with the antioxidant activity. In addition to the pain relief, membrane-acting antiplatelet and antiproliferative drugs potentially contribute to the prevention of thrombus formation and cancer. Among them, anesthetics with the antitumor activity might be ideal agents for cancer surgery.

5. CONCLUSION

Local anesthetics, phenolic sedatives/anesthetics, NSAIDs and NMDA receptor antagonists share the structure-dependent ability to interact with lipid bilayer membranes, directly influencing the properties and functions of biomembranes at a lipid level and indirectly modulating the activities of ion channels, receptors and enzymes by changing the conformation of membrane-associated proteins. The membrane interaction possibly accounts for the pharmacological diversity of analgesic agents and also may be an experimental clue to discover drugs or lead compounds with the analgesic potential and other activities from the point of view of a novel mechanism.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

The authors declare there are no conflicts of interest.

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