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Can NO-indomethacin counteract the topical gastric toxicity induced by indomethacin interactions with phospholipid bilayers?

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ABSTRACT

Nitric oxide (NO)-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) have been developed to overcome the gastrointestinal and cardiovascular toxicity of NSAIDs, by chemically associating a NO-releasing moiety with commercial NSAIDs. Since increasing evidence supports that NSAIDs toxicity is related to their topical actions in membrane lipids, this work aims to evaluate the impact of adding a NO-releasing moiety to parent NSAIDs regarding their effect on lipid bilayers. Thus, the interactions of NO-indomethacin and indomethacin (parent drug) with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers were described herein at pH 3.0 and 7.4. Diverse experimental techniques were combined to characterize the partitioning and location of drugs in DMPC bilayers, and to analyze their effect on the lipid phase transition and the bilayer structure and dynamics. The partitioning of NO-indomethacin into DMPC bilayers was similar to that of charged indomethacin and smaller than that of neutral indomethacin. Both drugs were found to insert the DMPC bilayer and the membrane location of indomethacin was pH-dependent. NO-indomethacin and indomethacin induced a decrease of the main phase transition temperature of DMPC. The effect of these drugs on the bilayer structure and dynamics was dependent on diverse factors, namely drug ionization state, drug:lipid molar ratio, temperature and lipid phase. It is noteworthy that NO-indomethacin induced more pronounced alterations in the biophysical properties of DMPC bilayers than indomethacin, considering equivalent membrane concentrations. Such modifications may have *in vivo* implications, particularly in the gastric mucosa, where NO-NSAIDs-induced changes in the protective properties of phospholipid layers may contribute to the occurrence of adverse effects.

1. Introduction

Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs), called NO-NSAIDs, have been developed to circumvent the well-described gastrointestinal (GI) and cardiovascular (CV) toxicity associated with NSAIDs long-term therapy [1,2]. The rational design of these drugs was based on evidences that NO is a protective mediator that contributes to the integrity maintenance of the GI mucosa and vasculature [3,4]. As NO-NSAIDs consist of a parent NSAID chemically linked to a NO-releasing moiety, these drugs act as prodrugs as their *in vivo* cleavage generates the parent NSAID and the protective mediator [2,4]. Thus, these drugs may combine the remarkable efficacy of NSAIDs to treat painful and inflammatory conditions and the beneficial actions of NO release. Diverse *in vitro* and animal studies have been supporting the development of NO-NSAIDs by demonstrating their anti-inflammatory efficacy, GI and CV tolerability, and even cardioprotective properties [2]. Naproxcinod, a NO-hybrid of naproxen, completed phase III clinical studies for the treatment of knee and hip osteoarthritis [2]. However, naproxcinod application was refused by FDA in 2010 due to concerns about GI and CV tolerability in long-term therapy [5], highlighting the necessity to comprehensively describe the pharmacological actions of novel drugs before commercialization.

Biological membranes have been gaining attention in drug development, as membrane lipids, beyond membrane proteins, are nowadays

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considered potential therapeutic targets. Membrane-lipid therapy has emerged since alterations in membrane lipid composition and structure are related to diverse pathophysiological processes [6,7]. Therefore, understanding drug effects on membrane lipids may give insights into their therapeutic actions and toxic properties. Concerning NSAIDs, studying drug-membrane interactions seems to be particularly relevant, as their therapeutic properties involves the inhibition of cyclooxygenase (COX), a membrane-bound enzyme [8], and their toxicity mechanisms seem to comprise alterations in membrane lipids [9]. NSAIDs topical actions seem to disrupt the protective properties of phospholipids of the GI mucosal barrier [10–15], contributing to the occurrence of NSAIDs-induced GI toxicity. Concerning the CV system, NSAIDs have been reported to induce morphological changes in human erythrocytes and to disorder the isolated unsealed human erythrocyte membrane [16–20]. As hypertension has been associated with alterations in membrane lipid composition and fluidity of human erythrocytes [21–23], it is conceivable that the NSAIDs-induced modifications in erythrocytes may be related to their ability to cause CV adverse effects. Therefore, studying lipid-drug interactions may provide significant information related to the therapeutic and toxic properties of pharmaceuticals.

This work provides a comparative study of the interactions of NO-indomethacin, a NO-NSAID, and its parent NSAID, indomethacin, with phospholipid bilayers to assess their impact in membrane lipids. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was the chosen phospholipid to obtain liposomes, used as membrane model, once phosphatidylcholines are the main glycerophospholipid of mammalian cell membranes and human gastric mucosa [24,25]. DMPC was also the lipid choice as it is in the fluid phase at physiological temperature (37 °C), as most of glycerophospholipids found in biological membranes, and it allows to mimic ordered lipid phases by simply controlling temperature [24,26]. The chemical structure of NO-indomethacin, indomethacin and DMPC are displayed in Fig. 1. The drug-phospholipid interactions were evaluated at pH 7.4 and pH 3.0 to account for the pH gradient found in the gastric mucosa (pH ~ 2 in the gastric lumen to pH ~ 7 in the mucosal epithelium) [27]. Diverse experimental approaches were used to characterize the drug-DMPC interactions, concerning the partition coefficient and the membrane location of drugs, as well as the drugs effect on the phase transition behavior and on the bilayer structure and dynamics. This study intends to evaluate the impact of adding NO-releasing moieties to parent NSAIDs concerning their actions on membrane lipids, which may ultimately be related to the pharmacological effects of NO-NSAIDs.

2. Materials and methods

2.1. Materials

Indomethacin, methyl 5-DOXYL-stearate (5-MeSL), Trizma® base, perchloric acid, ascorbic acid, monopotassium phosphate, dimethyl sulfoxide (DMSO), Ludox® AM-30 colloidal silica, polidocanol [Sigma-Aldrich Co. (St. Louis, MO)]; 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) [Avanti Polar Lipids, Inc. (Alabaster, AL)]; NO-indomethacin [Cayman Chemical (Ann Arbor, MI)]; 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) and 1,6-diphenyl-1,3,5-hexatriene (DPH) [Molecular Probes (Invitrogen Corporation, Carlsbad, CA)]; formic acid [Merck & Co. (Kenilworth, NJ)]; ammonium molybdate [J.T. Baker (Mexico City, Mexico)]; *n*-propanol [Mallinckrodt Inc. (Paris, KY)]; and methanol [VWR International S.A.S. (Fontenais-sous-Bois, France)] were at least analytical grade and used with no further purification. Formate (10 mM, pH 3.0) and Tris HCl (10 mM, pH 7.4) buffers were prepared by dissolving formic acid or Trizma® base (respectively) in Milli-Q water and adjusting pH with NaOH or HCl solutions.

2.2. Preparation of liposomes

Large unilamellar vesicles (LUVs) were produced by the lipid film hydration method followed by extrusion [28]. A lipid film was prepared by placing a solution of DMPC in chloroform/methanol (3:2, v/v) in a round-bottom flask, evaporating the solvents under a N_2 stream and leaving under vacuum to remove organic solvent traces. The lipid film was hydrated with a buffer solution at 40 °C for 30 min and vortexed, yielding multilamellar vesicles (MLVs). DMPC LUVs were obtained by extruding the MLVs suspension through a polycarbonate filter (100 nm pore diameter) at 40 °C.

Labeled DMPC LUVs were prepared by adding the probe to the chloroform/methanol solution prior to the evaporation, hydration and extrusion procedures. The probe:lipid molar ratio was 1:300 and 1.5:100 for fluorescent and spin probes, respectively.

The incubation and incorporation procedures were used for adding drugs to DMPC LUVs. In the incubation procedure, a certain amount of a drug stock solution was added to preformed DMPC LUVs, followed by an incubation period. Stock solutions of indomethacin in Tris HCl buffer were prepared for studies at pH 7.4. For indomethacin studies at pH 3.0 and for all studies with NO-indomethacin, the drug stock solutions were prepared in DMSO to ensure drug dissolution. The total amount of DMSO was 1% (v/v) and 5% (v/v) for studies with indomethacin and

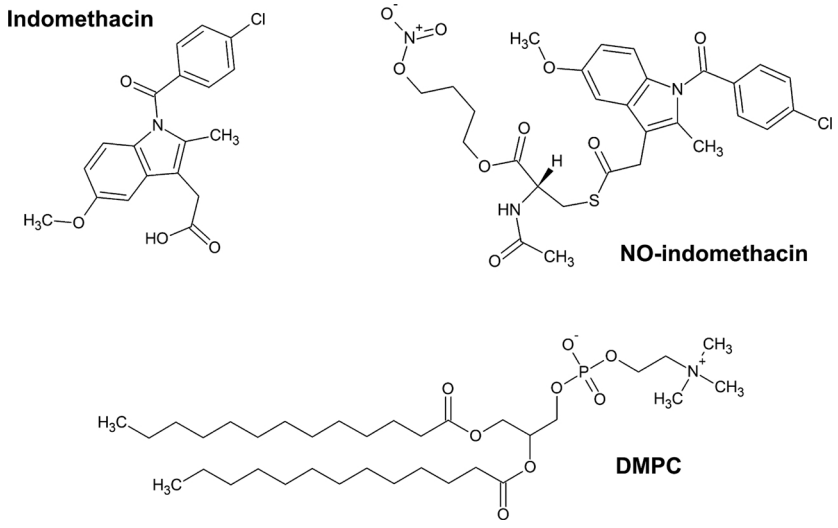

Fig. 1. Chemical structure of indomethacin, NO-indomethacin and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC).