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The membrane-activity of Ibuprofen, Diclofenac, and Naproxen: A physico-chemical study with lecithin phospholipids

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent non-specific inhibitors of the cycloxygenase pathway of inflammation, and therefore an understanding of the interaction process of the drugs with membrane phospholipids is of high relevance. We have studied the interaction of the NSAIDs with phospholipid membranes made from dimyristoylphosphatidylcholine (DMPC) by applying Fourier-transform infrared spectroscopy (FTIR), Förster resonance energy transfer spectroscopy (FRET), differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). FTIR data obtained via attenuated total reflectance (ATR) show that the interaction between DMPC and NSAIDs is limited to a strong interaction of the drugs with the phosphate region of the lipid head group. The FTIR transmission data furthermore are indicative of a strong effect of the drugs on the hydrocarbon chains inducing a reduction of the chain–chain interactions, i.e., a fluidization effect. Parallel to this, from the DSC data beside the decrease of T_m a reduction of the peak height of the melting endotherm connected with its broadening is observed, but leaving the overall phase transition enthalpy constant. Additionally, phase separation is observed, inducing the formation of a NSAID-rich and a NSAID-poor phase. This is especially pronounced for Diclofenac. Despite the strong influence of the drugs on the acyl chain moiety, FRET data do not reveal any evidence for drug incorporation into the lipid matrix, and ITC measurements performed do not exhibit any heat production due to drug binding. This implies that the interaction process is governed by only entropic reactions at the lipid/water interface.

1. Introduction

The nonsteroidal anti-inflammatory drugs (NSAIDs) are a wide non-chemically related group of compounds that are also called "aspirin-like drugs" for sharing similar therapeutic actions. They have been used since the introduction of acetylsalicylic acid (Aspirin) in 1899 [1]. NSAIDs are a large family of compounds classified into several subgroups, based on their chemical structure, like salicylates (Aspirin, Diflunisal), phenylacetic acids (Diclofenac), indoles (Indomethacin), oxicams (Piroxicam, Meloxicam), pyrazoles (Phenylbutazone) and sulphonanilides (Nimesulide) [2]. Despite their structural diversity, NSAIDs share most of the therapeutic actions as well as side-effects. Within the most widely prescribed and recognized drugs, Ibuprofen, Naproxen and Diclofenac are well-established NSAIDs, exhibiting anti-inflammatory, analgesic, antithrombotic and antipyretic properties [3]. Since 1974, Diclofenac is widely used in the long-term treatment of degenerative diseases. Some of its associated side-effects are hepatoxicity and liver injury [4]. Naproxen is used for the treatment of mild to moderate pain and fever. The most common side effects from Naproxen are headaches, dizziness, abdominal pain, nausea, and shortness of breath. Naproxen is associated with an increase in the risk to suffer heart attack and malignant effect on mucosal hydrophobicity [5]. One severe challenge encountered with the use of NSAIDs is their little gastrointestinal tolerability [6–9].

In most countries, Ibuprofen is freely available which allows self-medication. It has a wide range of side-effects including cardiovascular [10], renal and hepatic damage associated with long-time treatments [11]. They are also extensively medicated for rheumatoid arthritis, osteoarthritis, and musculoskeletal disorders. Recently, some of the NSAIDs have emerged as part of a new cancer chemotherapeutic and chemopreventive therapy [12,13].

The common mechanism of action of all NSAIDs is the inhibition of the cyclooxygenase (COX) enzyme. The COX pathway converts arachidonic acid in prostaglandins and thromboxans, which contribute

Abbreviations: NSAIDs, Nonsteroidal anti-inflammatory drugs; DMPC, dimyristoylphosphatidylcholine; FTIR, Fourier-transform infrared spectroscopy; ATR, attenuated total reflection; ITC, isothermal titration calorimetry; FRET, Förster resonance energy transfer spectroscopy; DSC, differential scanning calorimetry; T_m, temperature of main phase transition; COX, cyclooxygenase; Pβ, ripple phase; Lα, liquid crystalline phase

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to several physiological functions [14,15]. It is well known that there are at least two COX isoenzymes. The isoforms are called cyclooxygenase 1 and 2, and possess distinct enzymatic activities [16]. COX-1 is expressed constitutively in most tissues types and it is involved in the synthesis of prostaglandins which protect the stomach lining, intestine and kidney from damage. COX-2 is only expressed in a limited number of cell types and contributes among other activities to the inflammation processes in cells like fibroblasts, macrophages and monocytes [17]. Because of the high degree of structural similarity, most of the NSAIDs are non-selective inhibitors of COX-2 [18]. Most serious and sometimes fatal side-effects are gastrointestinal damage including ulceration and haemorrhage, renal toxicity, inhibition of the platelet aggregation that can lead to coagulation disorders which are side effects of non-selective (non-selective) COX-1 inhibition [19]. Additionally, studies have shown alternative COX-independent mechanisms of action inducing local ulcerations in the gastrointestinal tract [20]. These findings suggest the possibility of a direct membrane activity.

Artificial membranes are commonly used as a model for natural membranes to study drug–membrane interactions. The major constituents of the membrane are phospholipids. They are responsible for several features of the bilayer like stability and semi-permeable properties. Major lipidic components of eucaryotic cell membranes are phosphatidylcholines. The interactions of the NSAIDs with phospholipids can change the physicochemical properties of the membranes, which may be essential to understand the dynamics of the mechanism of action. The aim of this study was to investigate the interactions between dimyristoyl-phosphatidyl choline (DMPC) liposomes and three NSAIDs (Ibuprofen, Naproxen and Diclofenac as sodium salts). The results describe the molecular details of these interactions and the consequences for the membranes properties, by using Fourier-transform infrared spectroscopy (FTIR) in transmission and with attenuated total reflection (ATR), differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), and Förster resonance energy transfer spectroscopy (FRET).

2. Materials and methods

2.1. Lipid sample preparation

The lipid samples were prepared as aqueous dispersions at varying buffer (20 mM HEPES pH 7.4) content, depending on the sensitivity of the technique: 0.1 mM for the FRET-, 0.4 mM for the DSC-, and 20–40 mM for the FTIR-experiments. In all cases, the lipids were suspended directly in buffer, sonicated, and temperature-cycled several times between 5 and 70 °C and then stored at 4 °C before measurement. The drugs were dissolved in buffer and added to the phospholipid liposomes at the given molar ratios, and the measurements were done with these freshly prepared dispersions.

2.2. Fourier-transform infrared spectroscopy

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). For phase transition measurements, the lipid samples were placed in a CaF_2 cuvette with a 12.5 μm Teflon spacer. Temperature-scans were performed automatically between a low initial temperature (−20 °C to +10 °C) and 70 °C with a heating rate of 0.6 °C min^{-1}. For measurement of hydrated lipid samples, these were spread on a ATR Ge plate, and free water was evaporated. Every 3 °C, 50 interferograms were accumulated, apodized, Fourier-transformed, and converted to absorbance spectra.

For the evaluation of the gel to liquid crystalline phase behaviour, the peak position of the symmetric stretching vibration of the methylene band $\nu_s(CH_2)$ around 2850 cm^{-1} was taken, which is a sensitive marker of lipid order [21,22]. Furthermore, vibrational bands from the interface region (ester carbonyl stretch around 1725 to 1740 cm^{-1}), and the head group (antisymmetric stretch at 1260 to 1220 cm^{-1}, the choline group around 1100 to 1000 cm^{-1}, and the N–H stretching band at 1000 to 950 cm^{-1}) were analyzed. The instrumental wavenumber resolution is better than 0.02 cm^{-1}, the wavenumber reproducibility in repeated scans is better than 0.1 cm^{-1}.

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a VP-DSC calorimeter (MicroCal, Inc., Northampton, MA, USA) at a heating and cooling rate of 1 °C min^{-1}. The DSC samples were prepared by dispersing a known amount (5.6 mM) in 10 mM PBS buffer at pH 7.4. The samples were hydrated in the liquid crystalline phase by vortexing. Prior to the measurements the DSC samples were stored for a defined time at 4 °C (see text). The measurements were performed in the temperature interval from 5 °C to 65 °C. In the figures only the temperature range is shown where phase transitions were observed. Five consecutive heating and cooling scans checked the reproducibility of the DSC experiments of each sample [23]. The accuracy of the DSC experiments was ±0.1 °C for the main phase transition temperatures and ±1 kJ mol^{-1} for the main phase transition enthalpy. The DSC data were analyzed using the Origin software. The phase transition enthalpy was obtained by integrating the area under the heat capacity curve [24].

2.4. Isothermal titration calorimetry

Microcalorimetric experiments of drug binding to DMPC were performed on a MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA, USA) at various temperatures. The phospholipid samples at a concentration of 0.05 to 0.15 mM – prepared as described above – were filled into the microcalorimetric cell (volume 1.3 ml) and the drugs in the concentration range 0.5 to 5 mM into the syringe compartment (volume 100 μl), each after thorough degassing of the suspensions. After thermal equilibration, aliquots of 3 μl of the NSAID solution were added every 5 min into the lipid-containing cell, which was stirred constantly, and the heat of interaction after each injection measured by the ITC instrument was plotted versus time. The total heat signal from each experiment was determined as the area under the individual peaks and plotted versus the [drug]:[lipid] molar ratio. Since the instrument works in temperature equilibrium at a constant 'current feedback' corresponding to a power of approximately 74 μW, the occurrence of an exothermic reaction leads to a lowering of this current and of an endothermic reaction to an increase. All titration measurements, performed at constant temperatures, were repeated at least 2 times. As control, the drugs were also titrated into pure buffer.

2.5. FRET (Förster resonance energy transfer) spectroscopy

Intercalation of the NSAIDs into phospholipid liposomes composed of DMPC was determined in 20 mM Hepes, 150 mM NaCl, pH 7.4 at 37 °C by FRET spectroscopy applied as a probe dilution assay [25,26]. The drugs were added to the lipid aggregates which were labelled with the donor dye NBD-phosphatidylethanolamine (NBD-PE) and acceptor dye Rhodamine-PE. Intercalation was monitored as the increase of the ratio of the donor fluorescence intensity I_D at 531 nm to that of the acceptor intensity I_A at 593 nm (FRET signal) in dependence on time.

3. Results

3.1. Fourier-transform infrared spectroscopy/attenuated total reflectance (FTIR/ATR)

FTIR spectroscopy is most suitable for studying the interactions at the water lipid interface because this technique provides information