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Article

Molecular Interactions of Mefenamic Acid with Lipid Bilayers and Red Blood Cells

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O ácido mefenâmico é um anti-inflamatório não esteroidal (NSAID), também prescrito no tratamento da dor. No presente trabalho, os efeitos estruturais na membrana de eritrócitos humanos e de modelos moleculares foram investigados. Esta última foi composta em bicamadas construídas de dimiristoilfosfatidilcolina (DMPC) e dimiristoilfosfatidiletanolamina (DMPE), classes de lipídios encontrados nas porções interna e externa do eritrócito e da maioria das membranas celulares, respectivamente. Este artigo apresenta evidências de que o ácido mefenâmico interage com as membranas das células vermelhas, como segue: (*i*) em estudos de microscopia eletrônica de varredura (SEM) em eritrócitos humanos, foi observado que a droga induziu mudanças na forma, produzindo estomatócitos; (*ii*) estudos de difração de raios X mostraram que a droga interagiu com bicamadas de DMPC e efeitos de perturbação um pouco menores, em DMPE, foram detectados; (*iii*) medições de FT-IR mostraram que NSAID induziu a fluidização das cadeias acílicas de ambos, DMPC e DMPE; (*iv*) a espectroscopia de transferência de energia por ressonância Förster (FRET) indicou uma rápida intercalação do ácido mefenâmico nas cadeias hidrofóbicas do lipossoma DMPC.

Mefenamic acid is a nonsteroidal anti-inflammatory drug (NSAID), also prescribed to treat pain. In the present work, the structural effects on the human erythrocyte membrane and molecular models have been investigated. The latter consisted in bilayers built-up of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), classes of lipids found in the outer and inner moieties of the erythrocyte and most cell membranes, respectively. This report presents evidence that mefenamic acid interacts with red cell membranes as follows: (*i*) in scanning electron microscopy (SEM) studies on human erythrocytes it has been observed that the drug induced shape changes, forming stomatocytes; (*ii*) X-ray diffraction showed that the drug interacted with DMPC bilayers; somewhat lower perturbing effects on DMPE were detected; (*iii*) FT-IR measurements showed that NSAID induced fluidization of both DMPC and DMPE acyl chains; (*iv*) Förster resonance energy transfer spectroscopy (FRET) indicated a rapid intercalation of the mefenamic acid into DMPC liposome hydrophobic chains.

Keywords: nonsteroidal anti-inflammatory drugs, mefenamic acid, cell membrane, drug-membrane interactions, erythrocytes

Introduction

The use of experimental model systems that mimic the complexity of natural cell membranes provides the opportunity to investigate how drugs interact with the cells to carry out their therapeutic activity. A better understanding of the molecular basis of these interactions is a precondition for recognizing and correlating the side effects *in vivo*. The use of phospholipid bilayers as molecular models helps to understand how drugs interact with cell membranes, and how the interaction affects their structure and functions. Because of their simplicity and known structure, such model membranes can become into powerful tools for studying the molecular mechanism of specific functions.[1] Furthermore, the advances in a number of spectroscopic techniques have been employed to address the drug-lipid

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interaction using model membranes reconstituted from lipids and a few membrane-associated proteins.[2]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for their analgesic, anti-inflammatory, and antipyretic properties.[3] They are mainly prescribed for rheumatoid and osteoarthritis, treatment of mild pain and musculoskeletal problems. They are among the most frequently used therapeutic agents all over the world and many of them are available both as prescription and over-the counter drugs.[4]

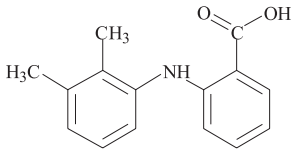

Figure 1. Chemical structure of mefenamic acid.

Mefenamic acid (N-(2,3-dimethylphenyl) anthranilic acid, Figure 1), is an anthranilic acid derivative. It is typically prescribed for oral administration; maximum plasma concentration is reached after 1-4 h, plasma half-life about 2 h, 90% is plasma protein bound and mainly renal elimination.[5] The anti-inflammatory effect of the NSAID comes from the interaction with the enzyme cyclooxygenase (COX-2), while the undesired effects arise from COX independent mechanism.[6] Side effects of mefenamic acid include headaches, nervousness and vomiting.[5] Serious side effects may include diarrhea, blurred vision, skin rash, itching and swelling, sore throat and fever. The most serious side effect is the gastric mucosal damage caused by the mucus secretion inhibition associated to chronic NSAID therapy.[7-9]

In this study we report the interactions of the highly consumed NSAID mefenamic acid in human erythrocyte membranes and lipid bilayers with the aim to better understand the molecular mechanisms of its interaction with cell membranes. Human erythrocytes were chosen because of their only one membrane and no internal organelles, which make them an ideal cell system for studying basic drug-biomembrane interactions.[10] On the other hand, although less specialized than many other cell membranes, they carry on enough functions in common with them such as active and passive transport, and the production of ionic and electric gradients, to be considered representative of the plasma membrane in general. The molecular models of cell membranes consisted of bilayers of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylethanolamine (DMPE), representative of phospholipid classes located in the outer and inner monolayers of cell membranes, respectively.[11,12] The capacity of mefenamic acid to perturb the bilayer structures of DMPC and DMPE was evaluated by X-ray diffraction, FT-IR phase transition measurements and Föster resonance energy transfer (FRET) measurements; intact human erythrocytes were observed by scanning electron microscopy (SEM). These systems and techniques have been used in our laboratories to determine the interaction with mefenamic acid and the membrane-perturbing effects of other anti-inflammatory drugs.[13,14]

Experimental

X-Ray diffraction studies of DMPC and DMPE multibilayers

Mefenamic acid from Sigma (St. Louis, MO; lot. 055K1555, MW 241.3), synthetic DMPC (lot. 14OPC-241, MW 677.9) and DMPE (lot. 14OPE-58, MW 635.9) from Avanti Polar Lipids (AL, USA), were used without further purification. Approximately 2 mg of each phospholipid were suspended in 200 μL of (*i*) distilled water and (*ii*) aqueous suspensions of mefenamic acid in a range of concentrations (equivalent to 0.01-2.0 mmol L^{-1}). Samples were incubated for 30 min at 37 ºC and 60 ºC with DMPC and DMPE, respectively and centrifuged for 10 min at 2500 rpm. Samples were then transferred to 1.5 mm diameter special glass capillaries (Glas-Technik & Konstruktion, Berlin, Germany) and X-ray diffracted. Specimen-to-film distances were 8 and 14 cm, standardized by sprinkling calcite powder on the capillary surface. Ni-filtered CuKα radiation from a Bruker Kristalloflex 760 (Karlsruhe, Germany) X-ray generator was used. The relative reflection intensities were obtained in a MBraun PSD-50M linear position-sensitive detector system (Garching, Germany); no correction factors were applied. The experiments were performed at 18 ± 1 °C, which is below the main phase transition temperature for both phospholipids. Higher temperatures would have induced transitions to more fluid phases making the detection of structural changes more difficult. Each experiment was performed at least in triplicate. X-ray data were analyzed by means of the Origin software 8.0.

Fourier-transform infrared spectroscopy (FT-IR)

Pure lipid suspensions were prepared in order to attain a concentration equivalent to 30 mmol L^{-1}. The corresponding amount of lipid was dissolved in chloroform and then the solvent was removed under N_2 stream in order to obtain a lipid film. To remove the traces of organic solvent the films were heated for 15 min at 40 °C. The multilamellar vesicles (LMV) were hydrated for 30 min in buffer (20 mmol L^{-1} HEPES, pH 7.4) in a sonicator. The LMV were subjected to three cycles of cooling and heating between 5 °C and 60 °C and then stored at 4 °C at least 12 h before measurement.

For the phase transition measurements, 5 μL of the pure lipid dispersions (30 mmol L^{-1}) were placed and squeezed between the CaF_2 windows of a heatable, demountable liquid cell equipped with a 12.5 μm Teflon spacer. The temperature was measured (± 0.05 °C) in one of the CaF_2 plates with a digital thermometer during the measurements. Once mounted in the sample holder of the spectrometer a waiting time of 20 min was allowed for the temperature equilibrium before the spectra were recorded. Samples were heated by an external computer-controlled water bath, and temperature scans were performed automatically between −5 °C and 70 °C with a heating rate of 0.6 °C *per* min. Measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). 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 behavior, 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.[15] Each experiment was performed at least in triplicate.

Föster resonance energy transfer spectroscopy (FRET)

For the FRET measurements liposomes (1 mmol L^{-1}) were double labeled with NBD-PE (N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE, lot. 27198W, MW 956.25) and Rh-PE (-(rhodamine B sulfonyl)-PE, lot. 26177W, MW 1333.81). Labeled lipids were obtained from Molecular Probes (Eugene, OR, USA). Fluorescent dyes were dissolved together with the lipid in chloroform in a lipid:NBD-PE:Rh-PE 100:1:1 molar ratio. The solvent was evaporated under a stream of nitrogen. LMVs were formed by resuspending the dried phospholipid film in buffer (20 mmol L^{-1} HEPES, pH 7.4), mixed thoroughly and sonicated with a Branson sonicator for 1 min (10 mL of solution). Subsequently, the preparation was cooled for 30 min at 4 °C and then heated for 30 min at 60 °C; cycles were repeated three times. Preparations were stored at 4 °C overnight before measurement. The phospholipid final equivalent concentration used in the experiments was 0.01 mmol L^{-1}. Mefenamic acid was added from a stock solution (20 mmol L^{-1}) in DMSO every 50 s to a final concentration of 0.05 mmol L^{-1} FRET experiments were carried out using 1 mL of the diluted double-labeled phospholipid (0.01 mmol L^{-1}). The vesicles were excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE (531 nm) and acceptor Rh-PE (593 nm) were measured simultaneously on a fluorescence spectrometer (SPEX F1T11, SPEX Instruments, Edison, NY). Intercalation was monitored as the increase of the ratio of the donor fluorescence intensity (I_{donor}) at 531 nm to that of the acceptor intensity ($I_{acceptor}$) at 593 nm (FRET signal) independently of time. The experiments were carried out at three different temperatures; below, at and above the phase transition of each phospholipid. DMPE liposomes were not employed due to the low proportion of labeled liposomes obtained following the same procedure of preparation (< 10%). Each experiment was performed at least in triplicate.

Scanning electron microscopy (SEM) studies of human erythrocytes

Blood was obtained from healthy human male donors under no pharmacological treatment. Blood samples (0.1 mL) were obtained by puncturing the ear lobule and mixed with 10 μL of heparin (5000 UI mL^{-1}) in 0.9 mL of saline solution (NaCl 0.9%, pH 7.4). Sample was centrifuged (1000 rpm ×10 min) and the supernatant was discarded and replaced by the same volume of saline solution; the whole process was repeated three times. The sedimented erythrocytes were suspended in 0.9 mL of saline solution and 100 μL aliquots of this suspension were mixed with equal volumes of (*i*) saline suspensions (control), and (*ii*) 100 μL of each mefenamic acid suspension in saline solution. The final mefenamic acid equivalent concentrations were in the range of 10 μmol L^{-1} to 2 mmol L^{-1}. Samples were then incubated for 1 h at 37 ºC. Following incubation, samples were centrifuged (1000 rpm × 10 min) and the supernatant was discarded. Fixation was performed by addition of 500 μL of 2.5% glutaraldehyde and overnight incubation at 4 °C. Fixed samples were washed with distilled water, placed over Al glass cover stubs, air dried at 37 ºC for 30 min to 1 h, and gold-coated for 3 min at 133.32 Pa in a sputter device (Edwards S150, Sussex, England). Resulting specimens were examined in a Jeol SEM (JSM 6380 LB, Japan). Each experiment was performed at least two times.

Results and Discussion

The study of drug-membrane interactions is essential for the understanding of NSAID activity, selectivity, and side effects. However, it is surprising the lack of studies on molecular details of the interaction between mefenamic acid and cell membranes. In the present study, the interaction of mefenamic acid with intact human red blood cells and model membranes made from synthetic phospholipids was investigated by applying a combination of different techniques.

The capacity of the mefenamic acid to perturb the structures of DMPC and DMPE multilayers was evaluated

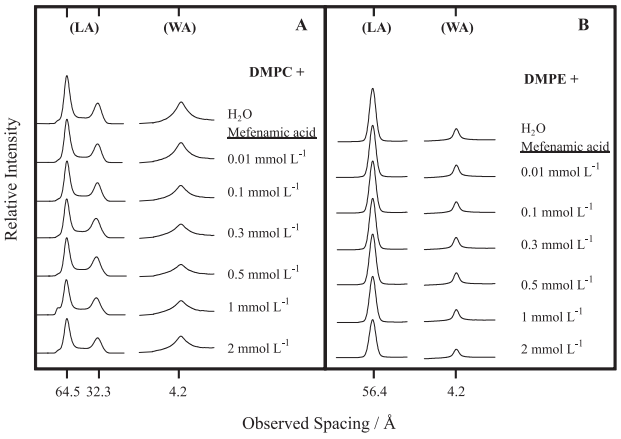

Figure 2. Microdensitograms from X-ray diffraction patterns of (A) dimyristoylphosphatidylcholine (DMPC) and (B) dimyristoylphosphatidylethanolamine (DMPE) in water and aqueous solutions of mefenamic acid; (LA) and (WA) correspond to low- and wide angle reflections, respectively (N=3).

by X-ray diffraction. Figure 2A shows a comparison of the diffraction patterns of DMPC and DMPC incubated with aqueous suspensions of mefenamic acid in a range of concentrations (equivalent to 10 μmol L^{-1} - 2 mmol L^{-1}). As expected, water altered the DMPC structure: its bilayer repeat (bilayer width plus the width of the water layer between bilayers) increased from about 55 Å in its dry crystalline form[16] to 64.5 Å when immersed in water, and its low-angle reflections (indicated as LA in the Figure), which correspond to DMPC polar terminal groups, were reduced to only the first two orders of the bilayer repeat. On the other hand, only one strong reflection of 4.2 Å showed up in the wide-angle region (WA), which corresponds to the average distance between fully extended acyl chains organized with rotational disorder in hexagonal packing. These results were indicative of the fluid state reached by DMPC bilayers in the presence of water. Figure 2A also discloses that the exposure to increasing concentrations of mefenamic acid produced monotonic perturbations of DMPC polar and hydrophobic regions, indicated by the LA and WA reflections intensity decrease, which reached about a 30% with 2 mmol L^{-1} mefenamic acid. The results of the X-ray diffraction patterns of DMPE bilayers incubated in water and mefenamic acid aqueous suspensions are shown in Figure 2B. The diffraction patterns also showed that increasing concentrations of mefenamic acid caused a moderate weakening in DMPE reflection intensities, which are diminished about a 15% at the highest mefenamic acid concentration (2 mmol L^{-1}). These results can be explained by the structural differences of both phospholipids. Under hydrated conditions the $-N(CH_3)_3^+$ polar group of DMPC induces the formation of a clathrate-like hydration shell around the head groups; the gradual hydration leads to water filling the highly polar interbilayer spaces increasing their separation. This condition promoted the incorporation of mefenamic acid molecules with the resulting higher structural perturbation. On the other hand, strong direct intermolecular hydrogen bonds are formed between the $-NH_3^+$, the PO_4^- groups and water molecules of DMPE. This structural difference leads to differences in the packing of the phospholipid head groups. DMPE head groups are packing tighter than those of DMPC due to its small head groups and, therefore higher effective charge.[17]

FT-IR represents a powerful and highly informative tool for studying lipid vibrational modes in model membranes. The technique is sufficiently sensitive and it does not need a probe into the membranes to obtain the spectroscopic information. It can provide detailed information about a variety of specific chemical groups at hydrated conditions. The wavenumber of the peak position of the CH_2 band ($\nu_s CH_2$) as a function of the temperature is a recognized parameter sensitive to lipid order and packing.[18] Insertion of molecules into the bilayer increases the space between the fatty acids causing an increase in their mobility and their frequency. The peak positions of the methylene stretching modes ($\nu_s CH_2$) in each phase of the lipids have different values: in the gel phase ($\nu_s CH_2$) lays at 2850 cm^{-1} and in the liquid crystalline phase around 2852 to 2853 cm^{-1}. At the main phase transition temperature (T_m) the lipid undergoes a conversion from the gel phase to the liquid crystalline phase because of the melting process of the hydrocarbon chain. The T_m has a characteristic value for each phospholipid depending of the length of the acyl chains[19] and the structure of the head groups.[20]

Figure 3A shows the temperature dependence of the wavenumber values of the peak positions of the DMPC acyl chains for the pure lipid and for DMPC:mefenamic acid at different molar ratios. The interaction of the mefenamic acid results in a fluidization of the system; the evidence is the increase in the wavenumbers of the ($\nu_s CH_2$) band at fixed temperature, indicating an interaction of the drug with the phospholipid CH_2 groups, which offers information about the lipid packing. Figure 3B discloses the values of the peak positions of DMPE in dependence of temperature. A fluidization effect is also observed in the structure of DMPE liposomes. The fluidization was evident both below and above the phase transition temperature of DMPC and DMPE. A shift of the wavenumber of the CH_2 symmetric vibration is closely related to the degree of order of the acyl chains and therefore the fluidity of the system.[21] One of the most important properties of membranes that influence on the function and localization of proteins is fluidity. Changes in membrane fluidity can affect proteins and their activities, and also the ability of the drugs to pass through the membrane, which affect their efficacy.[22]

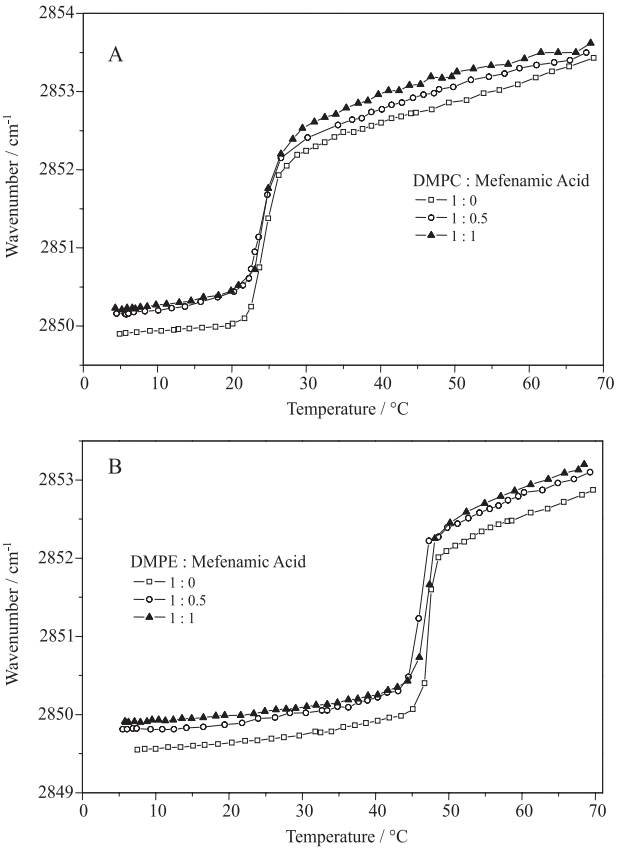

Figure 3. Peak positions of the symmetric stretching vibration bands of the methylene groups depending of temperature determined by FT-IR. (A) DMPC (B) DMPE in the presence of mefenamic acid at –□– 1:0, –○– 1:0.5, –▲– 1:1 molar ratios (N = 3).

Förster resonance energy transfer spectroscopy (FRET) was applied in order to determine a possible intercalation of the mefenamic acid into DMPC liposomes by using liposomes fluorescently labelled by NBD-PE (donor) and Rh-PE (acceptor) dyes. The experiments were carried out at three different temperatures: below, at and above the phase transition of DMPC, 15 °C, 25 °C, and 37 °C, respectively. In Figure 4 five titrations at equimolar steps are presented for the mefenamic acid at the three different temperatures corresponding to 1:1 up to 1:5 DMPC:mefenamic acid, molar ratios. The energy transfer between the two dyes is sensitive to the spatial separation. Intercalation of unlabeled molecules into the labeled liposomes induces the probe gap and therefore the emission intensity of the donor increases as that of the acceptor decreases. In control experiments it was verified that the titration of liposomes with different volumes of DMSO did not affect the liposome stability in the experiments, and the FRET signal was not affected by the several additions of the solvent (Figure 4).

The results of the titration with mefenamic acid show an increase in the values of the FRET signal ($I_{donor}/I_{acceptor}$), which indicated a rapid intercalation of the mefenamic acid

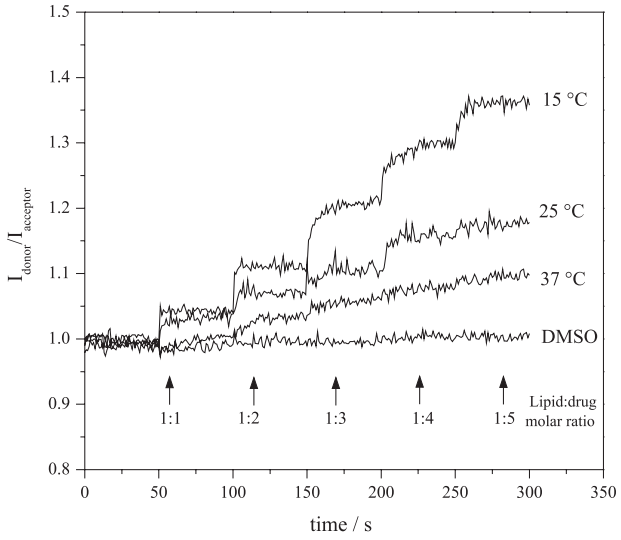

Figure 4. Förster fluorescence resonance energy transfer spectroscopy (FRET) of 0.01 mmol L^{-1} DMPC liposomes and at 25 °C with mefenamic acid. Every 50 s, 0.5 µL of a stock solution of 0.1 mmol L^{-1} mefenamic acid was added to 1 mL of liposomes; the FRET signal $I_{donor}/I_{acceptor}$ was monitored dependently of time (N = 3).

into the DMPC liposomes. These results are in accordance with the FT-IR phase transition measurements where a fluidization of the hydrophobic core of the liposomes was detected. From the drug physicochemical properties it was expected that it would be intercalated into the liposome acyl chains since mefenamic acid is liposoluble (logP 5.1[23]). Molecular hydrophobicity is assumed to be one of the driving forces of passive diffusion through biological membranes. In fact, logP partition coefficient has been successfully used for the determination of the drug distribution in the body, i.e. predicting the transdermal absorption of the molecules.[24] The highest intercalation obtained was at 15 °C (below the phase transition temperature). The explanation can relay in the fact that a temperature increase enhanced the solubility of DMSO in water, thus reducing the possibility of incorporating the drug molecules into the liposomes. Therefore, mefenamic acid could intercalate into the membranes inducing electrostatic repulsions between the phospholipid acyl chains, so the bilayers are able to accommodate the drug molecules in the packing of the lipid molecules. This plasticity of the lipid bilayer environment is not unexpected given the weak interactions between the lipid molecules. Therefore, intercalation of drug molecules would induce changes in the physicochemical properties of the membranes affecting their normal functions.

A commonly used membrane for studying drug-membrane interactions is that one from red blood cells. The reason is based on that their interaction with exogenous molecules induces cell-shape transformations of the normal

erythrocytes, which are caused by changes in the surface area of the monolayers (bilayer-couple hypothesis).[25] Thus, any effect that expands the outer leaflet relative to the inner one produces a tendency to form convex structures on the cell surface (echinocytes); conversely, an expansion of the inner leaflet relative to the outer one promotes concavities (stomatocytes).[26] The explanation of these changes is based on the different composition of the lipids and proteins in both leaflets. The inner monolayer contains a significant fraction of negatively charged lipids (phosphatidylserines), thus making it a more attractive environment for cationic amphipaths, while the outer leaflet is more attractive to neutral, zwitterionic and anionic molecules.[27] As discussed elsewhere,[25] anionic amphipaths that produce echinocytes associate preferentially with the outer monolayer, probably as a result of their inability to cross the bilayer. Conversely, those compounds that induce stomatocytosis do so by interacting with the cell inner monolayer, perhaps by association with inner monolayer lipids or proteins.

The effects of the *in vitro* interaction of mefenamic acid with human erythrocytes were followed by SEM. Under physiological conditions, normal human red blood cells assume a flattened biconcave disc-shaped (discocyte) *ca.* 8 μm in diameter. Erythrocytes were incubated with mefenamic acid in a range of concentrations (equivalent to 10 μmol L^{-1} - 1 mmol L^{-1}), and one sample was incubated with saline solution as a control experiment (Figure 5A). The results are shown in Figure 5B-F. As it can be seen, erythrocyte shape changed in a concentration-dependent manner. At a concentration as low as 10 μmol L^{-1} the drug induced loss of the normal round biconcave shape of erythrocytes changing them into cup-shaped stomatocytes. It must be taken into account that the serum concentration of mefenamic acid after therapeutic treatment is four times higher (40 μmol L^{-1}).[28] Although it is known that mefenamic acid is bound to albumin in *in vivo* human plasma, it should be understood that therapeutic levels are the steady state concentrations necessary to be reached for the drug to exert a significant clinical benefit. Although reports on the interaction of mefenamic acid on human erythrocytes and cell membranes are very scanty, it has been reported that mefenamic acid affected *in vitro* the permeability of gastric mucosal cells in a 1 to 10 mmol L^{-1} concentration.[29] With increasing concentrations of mefenamic acid the stomatocytosis is more evident, and at the highest assayed concentration 60% of the cells suffered this shape change. Morphological effects induced by mefenamic acid on erythrocytes indicate that they are able to interact with the erythrocyte membrane bilayer. These results are supported by FRET experiments, where the compound intercalated into DMPC bilayers. The resulting increase in the inner area supports the explanation that the morphological changes in erythrocytes are due to the interactions of the drugs with the inner monolayer which explain the formation of stomatocytes in SEM experiments. It must be taken into account that alteration of the normal biconcave shape of red blood cells increases their resistance to entry into capillaries, which could contribute to a decreased blood flow, loss of oxygen, and tissue damage through microvascular occlusion.[30,31] Functions of ion channels,

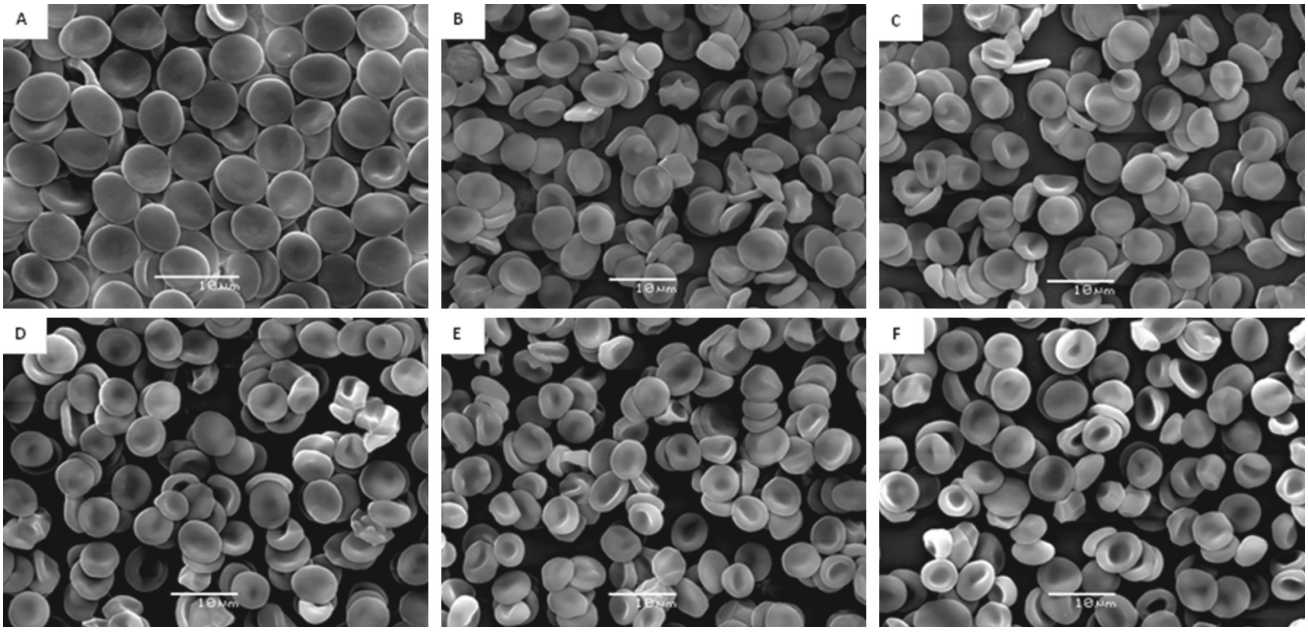

Figure 5. Effect of mefenamic acid on the morphology of human erythrocytes. Images obtained by scanning electron microscopy (SEM) of (A) control; (B) 0.01 mmol L^{-1}; (C) 0.1 mmol L^{-1}; (D) 0.3 mmol L^{-1}; (E) 0.5 mmol L^{-1}; (F) 1 mmol L^{-1} mefenamic acid (N = 3).

receptors and enzymes immersed in cell membrane lipid moieties also might be affected. These findings may also provide a new insight towards the possible mechanism of action of mefenamic acid.

Conclusions

Our results confirm that mefenamic acid is able to interact with phospholipids present in eukaryotic membranes inducing perturbational effects on membrane cells and therefore to users of this anti-inflammatory drug. The analyses of the results allow concluding that the major effects induced by mefenamic acid are based on the high affinity with the lipid matrix of the membrane cells according to X-ray, FRET and FT-IR experiments. Such interactions induced deep effects to phospholipids such as fluidization of the bilayers. Changes in the fluidity of the membrane cells are often involved in the toxic side effects of drugs.

Acknowledgments

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