Biophysical Chemistry 137 (2008) 28–34

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Membrane fusion: A new function of non steroidal anti-inflammatory drugs

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ARTICLE INFO

Article history:
Received 16 May 2008
Received in revised form 16 June 2008
Accepted 16 June 2008
Available online 21 June 2008

Keywords:
NSAIDs
Content mixing
Small Unilamellar Vesicles (SUVs)
Fluorescence spectroscopy
Transmission electron microscopy (TEM)

ABSTRACT

Membrane fusion is an important event in many biological processes and is characterized by several intermediate steps of which content mixing between the two fusing vesicles signals the completion of the process. Fusion induced solely by small drug molecules is not a common event. Non Steroidal Anti-Inflammatory Drugs (NSAIDs), that control pain and inflammation, are also capable of exhibiting diverse functions. In this study we present a new function of NSAIDs belonging to the oxicam group, as membrane fusogenic agents. Small Unilamellar Vesicles (SUVs) formed by the phospholipid, dimyristoylphosphatidylcholine (DMPC), were used as model membranes. Fluorescence assays using terbium/dipicolinic acid (Tb/DPA) were used to monitor content mixing and corresponding leakage in presence of the drugs. Transmission Electron Microscope (TEM) was also used to image fusion bodies in drug treated vesicles as compared to the untreated ones. The results show that the three oxicam NSAIDs viz. Meloxicam, Piroxicam and Tenoxicam can induce fusion of DMPC vesicles and lead the fusion process to completion at a very low drug to lipid ratio (D/L) of 0.045. The oxicam drugs exhibit differential fusogenic behavior as reflected in the kinetics of content mixing and leakage, both of which can be described by a single exponential rate equation. Moreover, not all NSAIDs can induce membrane fusion. Indomethacin, an acetic acid group NSAID and ibuprofen, a propionic acid group NSAID, did not induce fusion of vesicles. This new property of NSAIDs has important applications in biochemical processes.

1. Introduction

Non Steroidal Anti-Inflammatory Drugs (NSAIDs) were originally developed to combat pain and inflammation. Renewed research interest has been generated for the past one decade to study these drugs in order to understand the mechanism behind their new and alternate functions. These alternate functions include chemoprevention and chemo-suppression against many cancer cell lines and in also animal models [1–3], protective effect against neurodegenerative disease like Alzheimer disease etc [4–6]. Our group has been using oxicam NSAIDs as a prototype chemical group to elucidate the mechanism behind alternate functions of NSAIDs and several new functions of oxicam NSAIDs have been identified [7,8]. Induction of apoptosis in cancer cells is an important strategy in chemo-suppression. One of the different ways to do so involves direct permeabilization of mitochondrial membrane leading to the release of cytochrome C in the cytosol that in turn signals the downstream pro-apoptotic events. We have shown that one of the oxicam NSAIDs viz. piroxicam can permeabilize mitochondrial membrane [7] leading to the release of cytochrome C in cytosol, which activates pro-apoptotic caspase 3. As a possible mechanism behind permeabilization of mitochondrial membrane, it has been proposed that piroxicam can affect mitochondrial membrane morphology leading to fusion and rupture. Membrane fusion induced solely by small drug molecules is not a common event and is indeed a new function of NSAIDs. Our previous work [7] has put forward several interesting questions. These include: a) is membrane fusion a property of piroxicam only or can membrane fusion be induced by other drugs belonging to the oxicam group of NSAIDs that are structurally similar to piroxicam? b) If so, can these drugs lead the fusion process to completion? c) Is this a general property of NSAIDs? In this work, we have aimed to address the above questions and establish that membrane fusion is indeed a new and alternate function of oxicam NSAIDs.

Membrane fusion is an integral process in many important biological events like neuronal exocytosis of synaptic vessel [9], viral invasion of cells [10], fertilization of eggs by sperm [11], intracellular protein trafficking [12], etc. It requires the direct or indirect participation of external agents that act as fusogenic agents. Variety of fusogenic molecules exist which include proteins or peptides like SNARE (*S*oluble *N*-ethylmaleimide-sensitive factor *A*ttachment Protein *Re*ceptor) family of proteins [13], viral proteins like Hemagglutinin, HA0, of influenza virus [14] and gp160 of human immunodeficiency virus HIV1 [15]; polymers like poly (ethylene glycol), PEG [16] and metal ions like Ca^{2+} [17]. According to the stalk hypothesis of membrane fusion [18], the fusion can proceed via three main steps, a) initial local membrane contact, b) stalk formation or intermediate hemifusion and finally c) fusion pore formation and mixing of vesicles content. It should be mentioned, that

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doi:10.1016/j.bpc.2008.06.007

there exist many examples where the fusion process do not proceed to completion and can either be locked in the intermediate stage [19,20] or may revert back to the initial contact stage [21,22]. Excepting for the anesthetic drug halothane, which can induce membrane fusion at high drug to lipid (D/L) ratio of 10 [23], no report exist of another drug that can induce membrane fusion and lead the process to completion.

In this report we show that oxicam NSAIDs viz. piroxicam [4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] tenoxicam [[4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2H-thieno (2,3-e)-1, 2 thiazine-3-carboxamide 1,1-dioxide] and meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] (Fig. 1) can induce fusion in membrane mimetics. As a first line of study, we have chosen small unilamellar vesicles (SUV) formed by the phospholipid, dimyristoylphosphatidylcholine (DMPC) that serve as a convenient membrane mimetic system that is easy to prepare and whose size can be more readily controlled. We are aware that large unilameller vesicles (LUV) could be a better model of membranes and the high curvature of SUVs could facilitate spontaneous destabilization of vesicles. Carefully designed controls, both positive and negative have therefore been included to show that within our studied time frame and experimental conditions used, spontaneous membrane fusion does not contribute significantly. DMPC has been used as the phospholipid, since PC (phosphatidylcholine) is a zwitterionic head group and at pH 7.4, it does not have any net charge, thereby providing no electrostatic advantage to the fusion process. It is also the principal lipid component of mitochondria. In addition, all the NSAIDs used in this work are known to interact with DMPC vesicles [24–26].

As has been mentioned before, fusogenic agent may induce membrane fusion, but need not necessarily lead the process to completion. Lipid mixing assays gives information on the initial stages of membrane fusion whereas the final stage of fusion is marked by the mixing of aqueous contents of the vesicles. For a clear demonstration that the three oxicam NSAIDs not only can induce fusion of vesicles, but also lead the fusion process to completion, we have presented results of content mixing assay, since content mixing between fusing vesicles signals the completion of the fusion process. Transmission electron microscope (TEM) images are also presented that clearly exhibit the existence of vesicles in different stages of fusion process in drug treated samples as compared to the untreated ones. NSAIDs belonging to other chemical groups viz. indomethacin (acetic acid

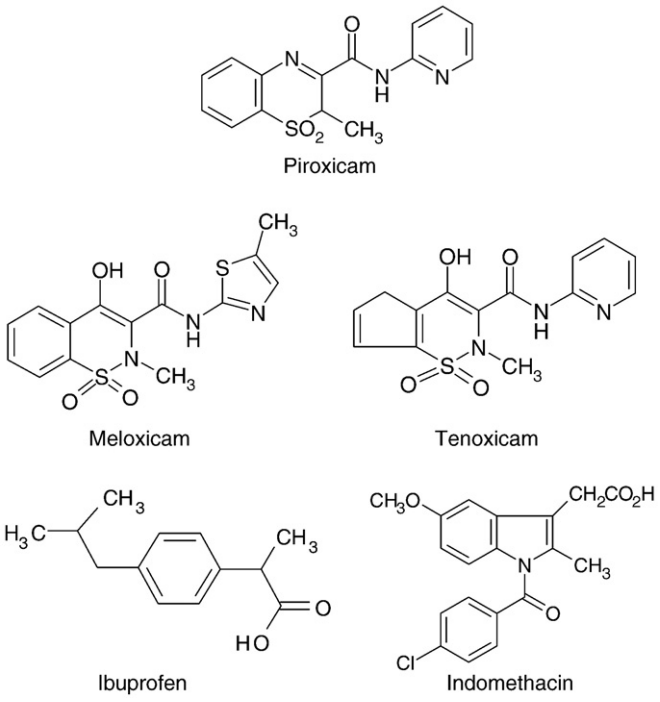

Fig. 1. Chemical structures of piroxicam, meloxicam, tenoxicam, ibuprofen and indomethacin.

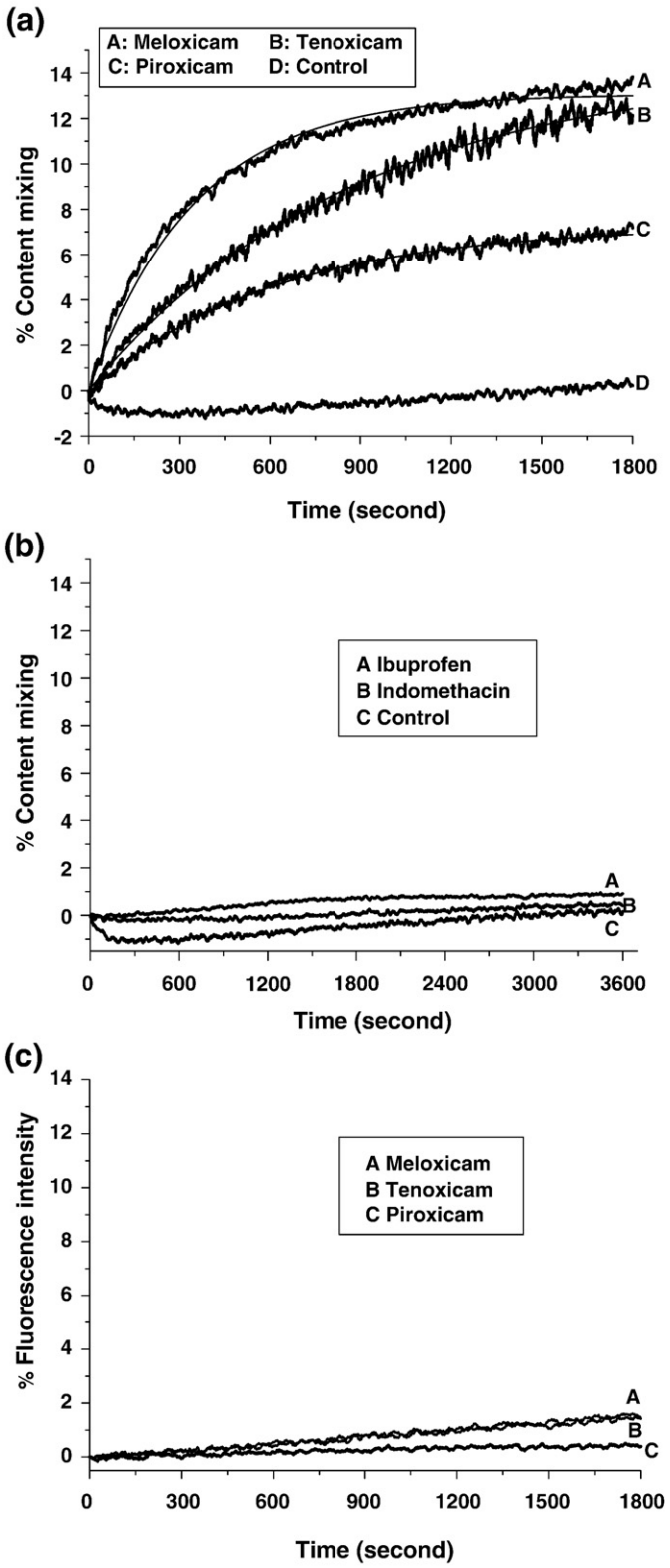

Fig. 2. Drug dependent content mixing of DMPC vesicles followed by Tb^{3+}/DPA assay with time for [a] meloxicam (A), tenoxicam (B), piroxicam (C) and untreated (control) (D) and [b] ibuprofen (A), indomethacin (B) and control (C). Figure [c] describes the same experiment without Tb^{3+} or DPA encapsulation. In all the cases the DMPC concentration was 1.1 mM and the drug concentration is 50 μM. All measurements were carried out in 10 mM TES, 100 mM NaCl, 1 mM EDTA, pH 7.4 at 37 °C.

group) and ibuprofen (propionic acid group) (Fig. 1) were used as negative controls. Both the NSAIDs are known to interact with PC vesicles but did not induce fusion as shown in this study.

2. Material and methods

DMPC, piroxicam, tenoxicam and ibuprofen were purchased from Sigma Chemicals (USA), meloxicam from LKT Laboratories (USA) and indomethacin from Fluka (USA) and were used without further purification. Water was distilled thrice before use. Stock solutions of the NSAIDs were prepared in Dimethyl Sulfoxide (DMSO) (Merck, Germany) and the exact concentration was adjusted by the corresponding buffer. The dilution of the drugs was done in such a way so that each sample contains a maximum of 0.5% (v/v) of DMSO. It should be mentioned that the presence of 0.5% (v/v) of DMSO had no effect on the vesicles and this has been verified from the TEM images of the vesicles in presence and absence of 0.5% (v/v) DMSO (data not shown). To show that DMSO also do not have any effect on membrane fusion, 0.5% (v/v) DMSO was added to all positive controls that were done in absence of the drugs. The temperature was kept constant at 37 °C and drug concentrations were kept constant at 50 μM in all experiments performed.

2.1. Preparation of SUVs

Small unilamellar vesicles (SUVs) of DMPC were prepared by the method of sonication [27]. To prepare SUVs of DMPC, the phospholipid was dissolved in 2:1 (v/v) chloroform:methanol solution and the solvent were evaporated under a stream of Argon. The resultant lipid film was then dried overnight in vacuum desicator at −20 °C. The dried film was hydrated and swelled in 10 mM 2-[tris (hydroxymethyl) methylamine]-1-ethanesulfonic acid (TES) and 60 mM NaCl at pH 7.4 for $TbCl_3$ (8 mM) containing vesicles and 10 mM TES at pH 7.4 for dipicolinic acid (DPA) (80 mM) vesicles. The mixture was vortexed to disperse lipids. The dispersion was then sonicated for about 10 min using dr. Heilscher (Germany) probe sonicator (200 W). The samples were then allowed to stand for 40 min to be hydrated completely. The sonicated samples were centrifuged at 10,000 rpm for 15 min to remove titanium particles and aggregated lipids [26]. The titanium particles were introduced as impurity in the sample from the sonicator probe.

2.2. Estimation of phosphate

The phospholipid concentration was measured by following published protocol [28]. Vesicle sample of 0.2 ml was digested with 0.8 ml perchloric acid at 180°C for 3 h. After cooling to room temperature 5.0 ml distilled water was added. 0.5 ml of 5% ammonium molybdate solution was then added followed by addition of 0.4 ml amminonaphthol sulphonic acid (ANSA) reagent (prepared by dissolving 6 mg sodium metabisulfate, 1.2 mg sodium sulfite and 100 mg of ANSA in 50 ml distilled water). Blue color was allowed to develop for 20 minutes and the amount of phosphate was estimated from the absorbance measured at 660 nm.

2.3. Content mixing assay

The Tb/DPA content mixing was based on those originally proposed and modified by Wilschut et al. [29,30]. Vesicles were prepared in either 80 mM DPA or 8 mM $TbCl_3$ and the untrapped, probes were removed from the external buffer of the vesicles using a Sephadex G-50 (Amersham Biosciences) column equilibrated with assay buffer (10 mM TES, 100 mM NaCl, 1 mM EDTA at pH 7.4). The lipid concentration in all the experiments was kept at 1.1 mM as determined by the method of phosphate estimation (data not shown).

To monitor drug induced content mixing, stock drug solutions were added to a mixture (1:1) of Tb^{3+} and DPA-containing vesicles and the time course of the content mixing were measured in terms of an increase in fluorescence intensity due to the formation of Tb/DPA complex with time. To calibrate the fluorescence scale (100% content mixing), an aliquot of chromatographed Tb-vesicles is rechromatographed on a Sephadex G-50 column, but equilibrated with 10 mM TES, 100 mM NaCl, pH 7.4 to eliminate the EDTA in the first buffer so that the released Tb^{3+} from the vesicles will not be chelated [30]. An aliquot of rechromatographed Tb-vesicles was lysed by 0.1% (w/v) Triton X-100 in presence of adequate amount of DPA, avoiding excess addition. The released Tb^{3+} will be fully complexed with DPA, which was assumed to be 100% content mixing. Then the % of content mixing was calculated in the following way:

$$\%\,\text{Content mixing} = \frac{(F-F_0)/F_0}{(F^l-F_0^l)/F_0^l} \times 100 \quad (1)$$

F Fluorescence intensity of Tb/DPA complex in presence of drug at time 't'

F_0 Fluorescence intensity of Tb/DPA complex in presence of drug at '$t=0$'

F^l Fluorescence intensity of rechromatographed, lysed Tb-vesicles in presence of adequate amount of DPA

F_0^l Fluorescence intensity of rechromatographed, lysed Tb-vesicles

2.4. Leakage assay

The leakage assay was done by using co-encapsulated Tb/DPA vesicles [16]. Tb^{3+} (8 mM) and DPA (80 mM) co-encapsulated vesicles,

Table 1
Exponential analysis of time courses [a] of % content mixing and % leakage a 37 °C for the drugs at D/L ratio of 0.045

Drugs	Partition coefficient (K_p) between lipid and aqueous buffer (pH 7.4)	Lipophilicity (log P)[d] in octanol-buffer (pH 7.4)	Extent of content mixing or leakage a (%)	Rate constant k (s^{-1})	Initial Rate IR (% s^{-1})
Content mixing assay					
Meloxicam	110.2[b]	0.07	13.53±1.38	$(2.86\pm0.34)\times10^{-3}$	38.70×10^{-3}
Piroxicam	25.8[b]	−0.14	7.35±1.01	$(1.68\pm0.25)\times10^{-3}$	11.58×10^{-3}
Tenoxicam	181.2[c]	−0.75	12.46±0.94	$(1.15\pm0.12)\times10^{-3}$	14.33×10^{-3}
Leakage assay					
Meloxicam	110.2[b]	0.07	3.93±0.27	$(2.34\pm0.04)\times10^{-3}$	9.20×10^{-3}
Piroxicam	25.8[b]	−0.14	2.94±0.78	$(2.16\pm0.05)\times10^{-3}$	6.35×10^{-3}
Tenoxicam	181.2[c]	−0.75	2.27±1.31	$(2.04\pm0.09)\times10^{-3}$	4.63×10^{-3}

[a] Time courses were fit to a single exponential [$f=a\{1-\exp(-kt)\}$] curves using non-linear least square fitting provided in software Origin 5.0. The extent of content mixing or leakage at infinite time is given by the pre-exponential factor 'a'; 'k' denotes the time constant referred to as rate constant and the initial rate (IR) is given by 'ak'. Parameter uncertainties are expressed as the standard deviations of data for at least 4 different experiments in contrast to the usual methods of deriving them from the non-linear least square fit procedure.
[b] Values taken from reference [26].
[c] Unpublished data using method as in reference [26].
[d] Values taken from literature, P. Luger, K. Daneck, W. Engel, G. Trummlitz, K. Waguer, Structure and Physicochemical property of meloxicam and a new NSAID, Eur. J. Pharm. Sci. 4 (1996) 175–187.

prepared in 10 mM TES, pH 7.4, were chromatographed on a Sephadex G-50 column equilibrated with 10 mM TES, 100 mM NaCl, 1 mM EDTA, pH 7.4 to eliminate unbound probe in the external buffer. When leakage of contents (co-encapsulated Tb/DPA) occurs, there was a drop in fluorescence intensity due to the quenching of the Tb by EDTA in the external buffer. The loss of remaining contents could be induced by addition of 0.1% (w/v) Triton X-100. 0% leakage was characterized by the fluorescence intensity of vesicles containing co-encapsulated Tb/DPA in buffer with drug at $t=0$; 100% leakage was characterized by the fluorescence intensity of a co-encapsulated Tb/DPA vesicle sample treated with drug and 0.1% (w/v) Triton X-100. Both measurements were made relative to the fluorescence intensity of detergent released vesicles. Thus the % leakage was calculated as:

$$\%\,\text{leakage} = \frac{\left(F_{Co}^{d,t=0} - F_{Co}^{d,\det}\right) - \left(F_{Co}^{d,t} - F_{Co}^{d,\det}\right)}{\left(F_{Co}^{d,t=0} - F_{Co}^{d,\det}\right)} \times 100 \quad (2)$$

$F_{Co}^{d,t=0}$ Fluorescence intensity of Tb/DPA co-encapsulated vesicles in presence of drug at the time t=0 (first data in the kinetics measurement).

$F_{Co}^{d,t}$ Fluorescence intensity of Tb/DPA co-encapsulated vesicles in presence of drug with time

$F_{Co}^{d,\det}$ Fluorescence intensity of Tb/DPA co-encapsulated vesicles in presence of drug after treatment of 0.1% (w/v) Triton X-100.

2.5. Fluorescence measurements

All the fluorescence measurements were performed by Hitachi 4500 spectrofluorimeter (Japan), which was operated in L-format. The excitation and the emission wavelength for Tb/DPA complex are 275 nm and 490 nm respectively. Slits of 5 nm were used in both the excitation and emission side throughout the experiment. Each experiment was repeated at least four times and the standard deviation is quoted as the error in each parameter.

2.6. Transmission electron microscopy (TEM) measurement

TEM was done with a FEI electron microscope model Tecnai G2 20 S Twin operating at 80 KV and 200 KV with a resolution of 0.2 nm. Samples were spread over a copper grid coated with carbon. SUVs were negatively stained with 2% (v/v) phosphotungstic acid (PTA). The magnifications were varied from 15,000× to 19,500× for different samples, which are mentioned on the transmission electron micrographs.

3. Results

The content mixing and leakage of fused vesicles have been followed by the standard Terbium (Tb)/Dipicolinic acid (DPA) assay [29,30]. Fig. 2 shows a plot of % content mixing as a function of time for the drug treated vesicles as compared to the untreated ones (positive control). It is obvious that all the three drugs viz. meloxicam, tenoxicam and piroxicam are capable of inducing significant amount of membrane fusion as compared to the control. Saturation is reached within a time frame of 30 min, which is comparable to membrane fusion induced by other fusogenic agents [31]. Even though we have used SUVs whose high curvature promotes destabilization of vesicles, the control shows that under the experimental conditions used, spontaneous vesicle fusion in absence of the drugs is not significant within the studied time frame. It should be mentioned that the drug to lipid ratio (D/L) for all our experiments were very low ($D/L=0.045$) compared to that seen in case of halothane induced membrane fusion where D/L ratio was 10 [23]. In order to show that membrane fusion is not a general property of NSAIDs belonging to other chemical groups, two standard NSAIDs viz. ibuprofen and indomethacin have been used

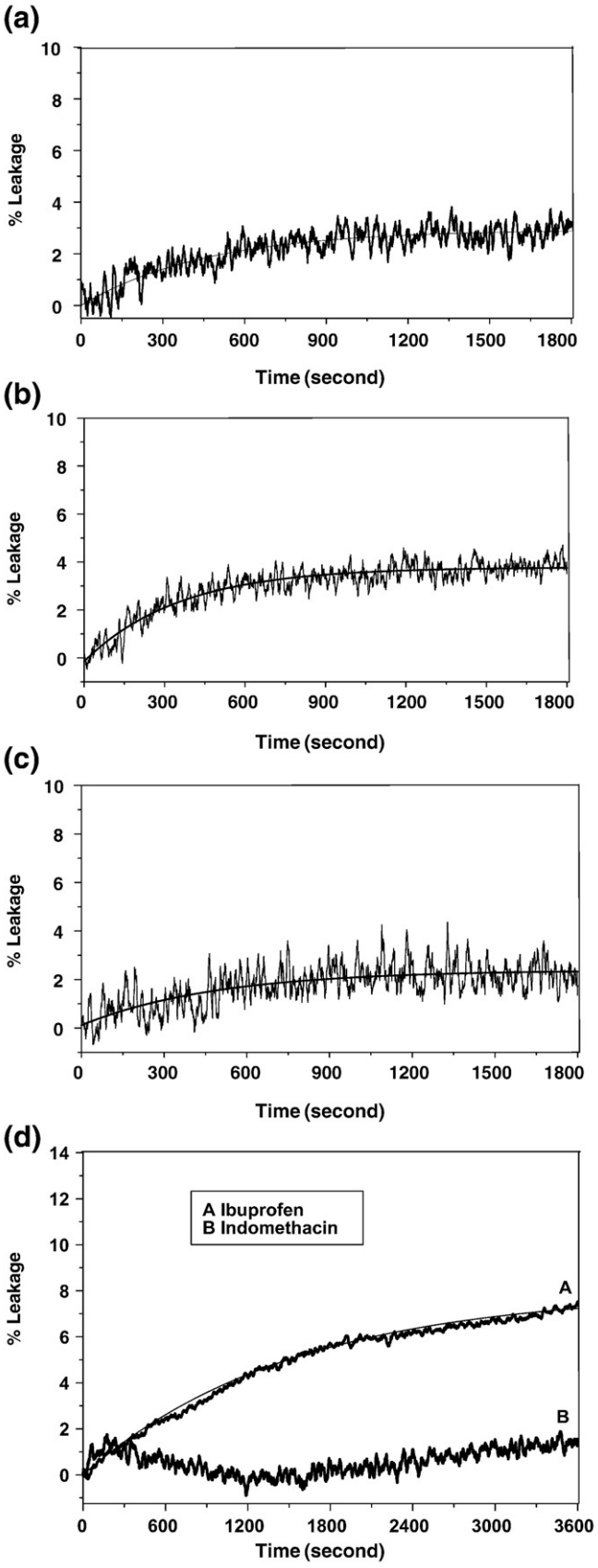

Fig. 3. Leakage of contents with time using Tb^{3+}/DPA in presence of 50 μM drug [a] piroxicam, [b] meloxicam, [c] tenoxicam [d] ibuprofen and indomethacin. In all the cases the DMPC concentration was about 1.0 mM. Measurements were carried out at 10 mM TES, 100 mM NaCl, 1 mM EDTA, pH 7.4 at 37 °C.

as negative controls [Fig. 2b]. Identical experimental conditions have been used as in Fig. 2a and the time courses are compared with the same control (drug untreated). Fig. 2b clearly shows that both ibuprofen and indomethacin do not induce fusion of the DMPC vesicles within the studied time frame and the experimental conditions used. Moreover both these drugs are known to interact with phosphatidylcholine (PC) vesicles [24,25]. It should be mentioned that the small negative value of % content mixing in the control curves arises due to the noise in the fluorescence intensity data which makes $F–F_0$ value of Eq. (1) negative at some points. The same artifact is not seen in the other curves of Fig. 2a since in case of drug induced membrane fusion, the value of F is always greater than F_0 for all $t > t_0$. We prefer to include the data without any manipulation excepting 20-point averaging. Interestingly, the drugs themselves show a differential fusogenic behavior in terms of kinetics, initial rate and extent of fusion. The time courses of % content mixing in presence of oxicam NSAIDs (Fig. 2a) could be fitted to a single exponential [$f = a(1-\exp(-kt))$].The exponential constant '*k*' referred to as 'rate constant', the extent of % content mixing at infinite time given by the pre-exponential factor '*a*' and the initial rate (IR) of '*ak*' are summarized in Table 1. Since the partition coefficient is a measure of drug lipid interaction, Table 1 also shows the partition coefficients Kp of the oxicam drugs, between the DMPC vesicles and the aqueous buffer at pH 7.4. Kp was measured by monitoring the increase in fluorescence emission intensity of the drugs with increasing lipid concentration [26]. The octanol-buffer partition coefficients (log*P*) at the same pH are also included. The log*P* values, a measure of the hydrophobicity of the drug molecules, is an important parameter that guides the location of the molecules inside the vesicles. The Kp values on the other hand, reflect the total interaction of the drugs with the vesicles guided both by the hydrophobic and hydrophilic interaction. Hence the trend in log*P* values for three oxicam drugs do not match the trend seen in the Kp values. No clear correlation is seen between the Kp values and the kinetic parameters. Only piroxicam with a minimum Kp value also show the lowest extent of content mixing and initial rate. The rate 'k' seems to follow the log*P* values, with the highest rate shown by most hydrophobic meloxicam and the lowest rate by the most hydrophilic tenoxicam. Since membrane fusion is a complex process characterized by several intermediate steps, it is not expected that any one physicochemical property of the drug will control the entire process. This could be the reason for the lack of any strong correlation of the kinetic parameters of content mixing with either Kp or log*P* values. In order to determine the contribution of the drugs to the measured fluorescence signal (λ_{ex}=275 nm and λ_{em}=490 nm) of Tb^{3+}/DPA complex of Fig. 2a, same concentration of empty vesicles (not encapsulating Tb^{3+} or DPA) were treated with similar drug concentration as in Fig. 2a. Time courses of the change in fluorescence intensity of the drugs, converted to % content mixing, are included in Fig. 2c. The contribution coming from the drugs are obviously negligible compared to that of Tb/DPA complex, formed due to content mixing.

For proper interpretation of the results of the content mixing assay, it is important to show the extent of content leakage both spontaneous and under the influence of the drugs within the same time frame. This

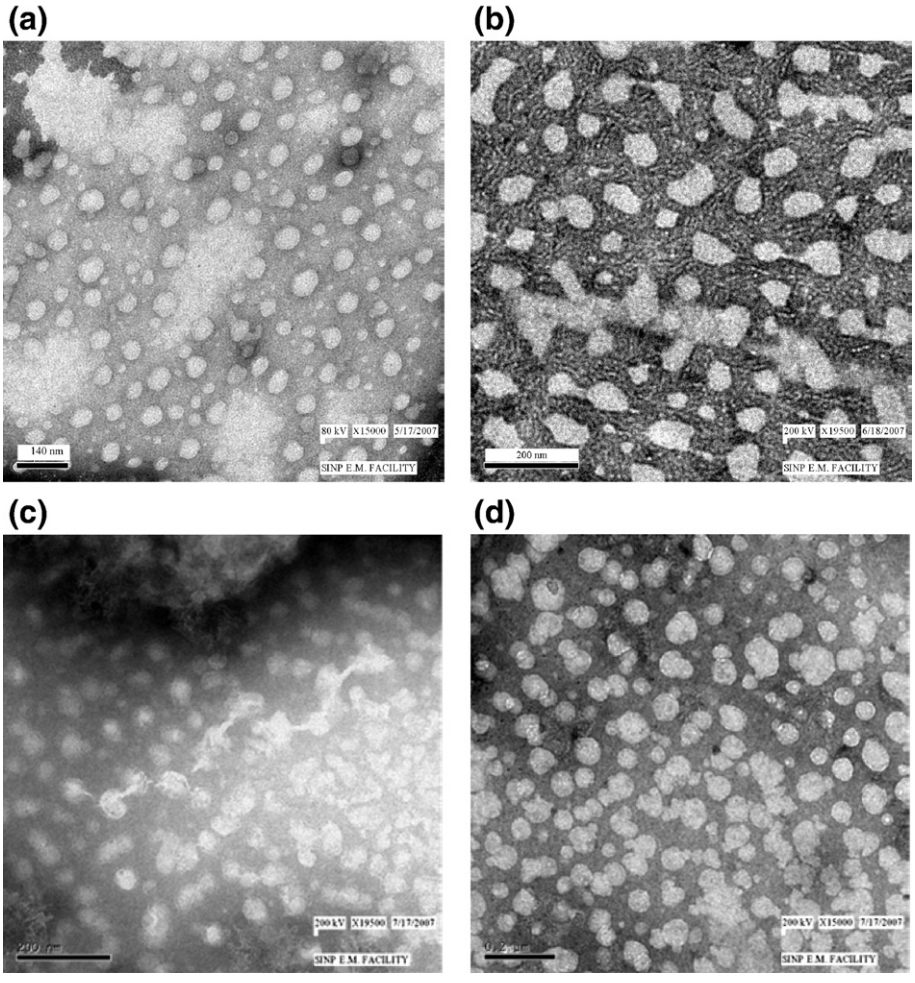

Fig. 4. Transmission Electron Micrographs of [a] untreated DMPC vesicles (control), DMPC vesicles treated with 50 μM of [b] piroxicam [c] meloxicam and [d] tenoxicam. In all cases, grids were prepared after 30 min of drug addition and stained with phosphotungstic acid (PTA).

is because the associated content leakage would result in the release of the Tb^{3+}, DPA, and/ or $[Tb(DPA)_3]^{3-}$ complex into the external buffer containing EDTA (as mentioned in the materials and methods section), which could reduce the fluorescence intensity. EDTA is added to the buffer to chelate the released Tb^{3+} that prevents the formation of $[Tb(DPA)_3]^{3-}$ complex. Vesicles in which Tb^{3+}/DPA are co-encapsulated are used to measure the leakage [16]. Fig. 3(a–c) shows the % of leakage as a function of time under the influence of three drugs within the same time frame as Fig. 2a. As expected, there is a leakage of contents in each case, which is small. The time course of leakage could also be fitted to a single exponential and the parameters are given in Table 1. The leakage rates are higher in case of piroxicam and tenoxicam compared to the rates of content mixing. This is expected since the onset of leakage usually occurs at an earlier time than content mixing. However for meloxicam the leakage rate is comparable to the content mixing rate. Meloxicam also shows a maximum extent of leakage at infinite time compared to the two other drugs. Unlike the content mixing data, the leakage rate and extent follow the log*P* values. Fig. 3d shows the time course of the leakage of ibuprofen and indomethacin. Ibuprofen shows significant leakage but indomethacin does not. To sum up, indomethacin can neither induce fusion nor any significant amount of vesicles leakage whereas ibuprofen cannot induce fusion but shows significant permeabilization of the vesicles.

To further demonstrate that the oxicam NSAIDs can induce membrane fusion, TEM imaging was done. Fig. 4a shows the TEM images of DMPC vesicles not treated with oxicam NSAIDs (control). Fig. 4b–d are the TEM images of DMPC vesicles treated with 50 μM piroxicam, meloxicam and tenoxicam respectively. Clear images of several fusion bodies, arrested at the different stages of the fusion process, are seen in the drug treated vesicles that are absent in the control.

4. Discussion

The results that are presented in this study conclusively establish the following facts:

(a) Three oxicam NSAIDs viz. piroxicam, meloxicam and tenoxicam can induce fusion of membrane mimetic systems, and lead the process to its completion at D/L ratio of 0.045.
(b) Even though oxicam NSAIDs are structurally related, they show differential fusogenic behavior as shown by the kinetics of content mixing and leakage.
(c) This is not a general property of NSAIDs. Drugs belonging to other chemical structural groups viz. ibuprofen (propionic acid group) and indomethacin (acetic acid group) do not have any fusogenic property.
(d) Even though the high curvature of the SUVs are known to facilitate membrane fusion, carefully designed controls show that both spontaneous fusion and fusion induced by other group of NSAIDs are not possible in the SUVs used here.

Membrane fusion induced solely by small molecules, that too by drugs, is not a common event. Cation induced membrane fusion viz. Ca^{2+} induced fusion [32,33] has been observed only in case of anionic liposomes where electrostatic effects play a guiding role. We have therefore used DMPC vesicles that are neutral at the working pH used here. This was done to reduce the advantage of electrostatic interaction between the drugs and the vesicles to the fusion process. However, it should be mentioned that the oxicam NSAIDs, which are in their anionic forms at the working pH, could partition in the DMPC vesicles [26]. As mentioned before, membrane fusion involves the formation of some specific intermediates like stalks and pores [18] that in turn require a great deal of structure-energy balance and each step of membrane fusion is controlled by specific energy barrier [34–36]. Big molecules like proteins and peptides can control some steps of fusion by changing their conformation [15,37,38]. Several examples in literature show that for fusogenic agents that cannot strike the correct energy balance, fusion stops at an intermediate stage [20,22]. It is expected to be difficult for small molecules to control all the crucial energetic steps for the smooth completion of the fusion process which could be the reason why drug induced membrane fusion is not common. To our knowledge, halothane, the only drug that can induce membrane fusion, can do so at a very high D/L ratio of 10 [23]. Thus, the fact that the oxicam NSAIDs can induce membrane fusion at a much lower D/L ratio of 0.045 is unique in itself. The peak plasma concentration of one of the oxicams viz. piroxicam is 24 μM. At such low D/L ratio of 0.045, fusion can be looked upon as an interaction between three partners viz., two liposomes and one drug molecule characterized by several intermediate steps. It is therefore not expected that any one physical property of the drugs will completely guide the fusion process. The difference in the fusogenic behavior of the three drugs depends on the complete physicochemical profile of the molecules. The leakage on the other hand represents the simple binary interaction between the drug and a vesicle leading to permeabilization of the latter. Hydrophobic interaction guides the location of a molecule inside a vesicle. Greater hydrophobicity of a drug dictates deeper penetration leading to enhanced permeabilization of the vesicles. This could be the reason why correlation is seen between the kinetic parameters of leakage with logP values.

As has been mentioned in the introduction, a direct physiological relevance of this new function of oxicam NSAIDs has previously been reported by our group [7] Piroxicam, has been shown to permeabilize mitochondrial membrane leading to the release of cytochrome C in the cytosol that signaled the activation of downstream pro-apoptotic caspase-3 [7]. Piroxicam affected mitochondrial membrane morphology, which resulted in fusion and rupture. This has been proposed by us to be one possible mechanism to permeabilize mitochondria. It should be mentioned that inducing Mitochondrial Outer Membrane Permeabilization (MOMP) is an important strategy for the initiation of apoptosis in cancer cells. Another important application of this new function will be to induce membrane fusion *invitro* experiments using common drugs having well studied ADMET (Adsorption, Distribution, Metabolism, Excretion and Toxicity) profile. This novel function of NSAIDs opens up a vista of using common drug molecules with well-studied ADMET in biotechnology wherever membrane fusion induction plays a key role. Further studies are underway; to elucidate the mechanism behind oxicam NSAIDs induced membrane fusion in a greater detail and effort to parse the effects of different physicochemical property of the drugs on their membrane fusing ability will be the subject of a separate manuscript.

5. Conclusion

Three oxicam NSAIDs viz. piroxicam, meloxicam and tenoxicam can induce fusion of liposomes and lead the process to completion. This is a unique property of oxicam NSAIDs, which is not shared by NSAIDs belonging to other chemical structural groups. The three-oxicam drugs used here show differential fusogenic behavior. Drugs belonging to other structural groups do not induce fusion indicating that the chemical structure plays an important role.

Acknowledgements

We acknowledge the help of Mr. Pulak Ray, Mr. Tapan Kumar Ray and Mr. Ajay Chakrabarti of Biophysics Division of Saha Institute of Nuclear Physics for their help in Transmission Electron Microscopy (TEM).

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