ELSEVIER

International Journal of Pharmaceutics 193 (1999) 123–127

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Note

Enhanced inhibition of platelet aggregation in-vitro by niosome-encapsulated indomethacin

Gopal K. Pillai [a,*], Maher L.D. Salim [b]

[a] *Department of Pharmacy, Faculty of Medical Sciences, The University of the West Indies, Trinidad and Tobago*
[b] *Faculty of Pharmacy and Medical Sciences, Amman University, Amman, Jordan*

Received 21 January 1999; received in revised form 3 September 1999; accepted 7 September 1999

Abstract

In order to achieve sustained antiplatelet effect from indomethacin, it was incorporated in a non-ionic surfactant vesicle (niosome). The objective was to study the effect of niosomal-encapsulated indomethacin on platelet function such as inhibition of aggregation and ATP release induced by a variety of agonists (adenosine 5′-diphosphate (ADP), epinephrine, arachidonic acid, ristocetine) and to explore the feasibility of carrier-mediated drug delivery to the platelets. Multilamellar vesicles (niosomes) were prepared from Tween-60 by the lipid hydration method. Freshly prepared human platelet rich plasma (PRP) was used for aggregation/inhibition studies, the extent of which was observed as a change in light transmission measured by the Chronolog Aggregometer. The percent inhibition of aggregation induced by the agonist ADP ranged from 28.21 ± 0.28 at the 2.0 μmol level to 92.6 ± 1.20 at 12.7 μmol of the encapsulated drug while the same concentrations of the drug inhibited aggregation only to the extent of 13.75 ± 0.13 and 36.82 ± 0.57%, respectively. A 100% inhibition of aggregation induced by arachidonic acid was achieved by niosomal indomethacin while inhibition by the free drug was 41.9% at equimolar concentrations. ATP release study showed that 100% inhibition was achieved by 8 μmol of the encapsulated drug while inhibition by the free drug was 40.00 ± 1.82%. Therefore, at equimolar doses, the niosomal drug proved to be more efficient in inhibiting platelet aggregation than the free drug, probably due to greater quantity of the drug reaching the specific site of inhibition in the interior of the platelets and acting directly on the cyclo-oxygenase system to prevent thromboxane formation.

Keywords: Indomethacin; Niosomes; Platelet aggregation; ATP release

1. Introduction

Platelets aggregation is established as an important factor in the etiology of thrombosis. Platelets play a major role in the clotting process and

* Corresponding author.

0378-5173/99/$ - see front matter
PII: S0378-5173(99)00317-8

therefore in several cardiovascular conditions. Platelets are also implicated in development of occlusions following by-pass surgery and balloon angioplasty. Commonly used anti-platelet agents have met with limited success in the prevention of occlusions being formed again. It is believed that the failure of anti-platelet agents may be due to their inherent instability leading to rapid inactivation in-vivo and hence short duration of action.

Indomethacin is known to inhibit platelet aggregation induced by different agonists. It has been reported that indomethacin incorporated in to liposome inhibited platelet aggregation induced by adenosine 5′-diphosphate (ADP) to a much greater extent than the unencapsulated drug (Gursoy et al., 1988). The main effect of indomethacin is on cyclo-oxygenase enzyme, which converts arachidonic acid to thromboxane A_2. The $t_{1/2}$ of indomethacin is 2–3 h. It is eliminated mainly by hepatic metabolism and less than 15% is excreted unchanged in the urine.

In order to achieve sustained anti-platelet effect from indomethacin, it may be incorporated in to vesicular carriers such as phospholipid vesicles (liposomes) and non-ionic surfactant vesicles (niosomes). The encapsulation of the drug in these vesicles would provide some protection from rapid inactivation while fusion and endocytosis of the drug-loaded vesicle would target and deliver the drug in to the platelets.

The objective of this work was to study the effect of niosome-encapsulated indomethacin on platelet function such as inhibition of platelet aggregation and ATP release induced by agonists such as ADP, arachidonic acid, ristocetine and epinephrine and to explore the feasibility of carrier-mediated drug delivery to the platelets.

2. Materials and methods

Adenosine 5′-diphosphate (ADP), ristocetine, epinephrine, cholesterol, steraylamine, sephadex G-50 and Triton X-100 were obtained from Sigma, MO, USA; Arachidonic acid, ATP and luciferin-luciferase reagent from Chronolog, PA, USA; Tween 60 (polyoxyethylene sorbitan stearate) from Jansen Chimica, Belgium. Indomethacin was a gift sample from Jordanian Pharmaceutical Manufacturing (JPM). All other chemicals were of analytical grade.

The equipment used to measure the degree of aggregation was Chronolog Lumi—aggregometer (Model No.500 CA) provided with thermostated sample chamber and facility for stirring the sample with a magnetic stirrer. The results were recorded on Chronolog chart recorder.

2.1. Preparation of niosomes

Multilamellar (MLV) niosomes were prepared by the lipid hydration method. The method briefly was as follows: to a 10-ml solution of indomethacin in chloroform (1.25 mg/ml) were added 71.25 mg of cholesterol, 71.25 mg of Tween 60 and 7.5 mg of stearylamine. The mixture was vortex-mixed, transferred to a 1-L round bottom flask and the organic solvent was removed under vacuum in a rotary vacuum evaporator on a water bath at 60°C to form a thin film on the wall of the flask. After removal of the last trace of organic solvent, the film was hydrated by 5 ml of phosphate-buffered saline (PBS, pH = 7.4) at 60°C and at a rotation speed of 150 rpm for 1 h. The dispersion was left to cool to room temperature and was sonicated in an ultrasonic bath for 1 h. The un-entrapped drug was removed by size exclusion chromatography on sephadex G-50 column (60×1.5 cm) using PBS as the eluant. The entrapment efficiency was determined by dissolving 500 μl of the niosomes obtained from gel chromatography in 500 μl of Triton X-100 (10%), dilution to 10 ml with PBS, followed by measurement of absorbance of the clear solution at 320 nm (Unicam UV-VIS spectrophotometer). The stability of the niosomes in PBS was monitored by storage of niosomes in PBS for 2 h and measuring absorbance at 320 nm at 0, 30, 60, 90 and 120 min.

2.2. Specimen collection

Human blood specimens were collected fresh for each experiment by venipuncture in to polypropylene tubes containing 3.8% sodium citrate in the ratio of one part anticoagulant to nine

parts of blood. Volunteers did not take any medication for 10 days prior to participation in the study. Blood samples were kept at room temperature and the aggregation test was done within 30 min of the venipuncture.

2.3. Preparation of platelet rich plasma (PRP)

Blood samples were centrifuged at $100 \times g$ for 10 min. PRP was aspirated with a polypropylene transfer pipette in to polypropylene tubes. Platelet poor plasma (PPP) was prepared by centrifugation of the blood samples at $1200 \times g$ for 10 min (10000 rpm).

2.4. Measurement of inhibition of aggregation

Niosomes containing indomethacin (2–12.7 μmol) was added to PRP (440 μmol) and incubated for 30 min. ADP was added to achieve a final concentration of 5 μmol and changes in light transmission was measured by the Aggregometer. The procedure was repeated with each of the agonists (arachidonic acid, 0.5 μmol; ristocetin, 0.6 μmol; epinephrine, 50 μmol) with a fixed concentration of indomethacin. PPP was used as the reference.

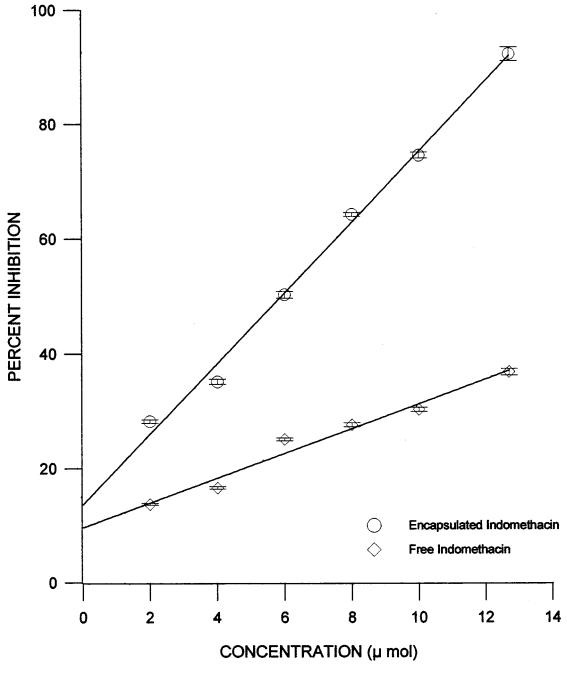

Fig. 1. Inhibition of platelet aggregation (induced by ADP; 5 μmol) by free and encapsulated Indomethacin.

2.5. Measurement of inhibition of ATP release

The above procedure was repeated by the addition of 50 μl of luciferin-luciferase reagent. The final concentration of ADP was 5 μmol. Changes in fluorescence were measured by the lumi-aggregometer. PPP was used as a reference.

3. Results and discussion

Non-ionic surfactant vesicles (niosomes), prepared from synthetic non-ionic surfactants by methods similar to that of liposome preparation, are capable of entrapping a variety of drugs and have been evaluated as alternative to liposomes (Florence and Bailie, 1989; Yoshioka et al., 1994). Non-ionic surfactants form unilamellar and multilamellar vesicles and have similar physical properties as liposomes, and therefore are regarded as a relatively inexpensive drug delivery system. The inclusion of drugs in niosomes has lead to significant changes in their biodistribution such as a greater degree of targeting the drug to selected tissues, sustained release and altered pharmacokinetics (Florence et al., 1990; Chandraprakash et al., 1993; Parthasarathy et al., 1994).

Niosomes were prepared from Tween 60 (polyoxyethylene sorbitan stearate) and were assayed for indomethacin content (the encapsulation efficiency was $50 \pm 3\%$). Stability of indomethacin under the experimental conditions used for encapsulation procedure was also checked and no degradation was observed. Fig. 1 shows the effect of encapsulation of indomethacin in niosome on platelet aggregation induced by ADP. The percent inhibition of aggregation, measured as changes in light transmission by the Aggregometer ranged from 28.21 ± 0.28 at the 2 μmol level to 92.16 ± 1.20 at 12.7 μmol while the same concentrations of free indomethacin inhibited aggregation only to the extent of 13.75 ± 0.13 and $36.82 \pm 0.57\%$, respectively. The difference in the inhibition of ag-

Table 1
Inhibition of platelet aggregation (induced by different agonists) by free and encapsulated indomethacin (12.7 μmol)

ADP (5 μmol)		Arachidonic acid (0.5 μmol)		Ristocetine (0.6 μmol)		Epinephrine (50 μmol)	
Free	Niosomal	Free	Niosomal	Free	Niosomal	Free	Niosomal
36.82 ± 0.5	92.1 ± 1.20	41.9 ± 1.2	100 ± 0	37.8 ± 0.3	96.7 ± 0.4	33.7 ± 1.2	88.8 ± 0.4

gregation between the free and encapsulated drug was significant ($P < 0.001$). Empty niosomes were used as the control, which did not have any effect on aggregation. Table 1 shows the inhibition of platelet aggregation induced by ADP, arachidonic acid, ristocetin and epinephrine by free and niosomal indomethacin. Incubation with ADP resulted in a decrease in light transmission initially followed by a rapid increase whereas the other agonists caused an immediate increase in light transmission. It was possible to achieve 100% inhibition of aggregation induced by arachidonic acid by the encapsulated drug while only 41.9 ± 1.2% inhibition was obtained with the free drug. Table 2 shows the inhibition of ATP release (induced by ADP) by the free and encapsulated drug. At the 8.0-μmol level of the encapsulated drug 100% inhibition was observed while that of the free drug was 40.00 ± 1.82%. When the results are compared at equimolar doses, niosomal indomethacin proved to be more potent in inhibiting platelet aggregation than the free drug, probably due to the possibility of greater quantity of the drug reaching the specific site of inhibition in the interior of the platelets.

The agonists in general, exert their stimulatory effect by binding to specific plasma membrane receptors on the platelets. The agonist-receptor complex then initiates further steps in the intracellular stimulus-response-coupling cascade, which produces different physiological responses. ADP, for example, interacts with its receptors to initiate an increase in Ca^{2+} resulting in a rearrangement of membrane glycoprotein to combine with fibrinogen (Philips et al., 1980). This process lead to primary aggregation which cannot be inhibited by cyclo-oxygenase inhibitors (Hallam et al., 1985a). Ca^{2+} also activates phospholipase A_2 leading to liberation of arachidonate and consequent formation of thromboxane (Siffert and Akkerman, 1988). The released thromboxane initiates a series of sequential reactions such as activation of phospolipase C, phosphorylation of protein kinase C and the platelet contractile proteins leading to a quick exocytosis followed by an irreversible aggregation (Hallam et al., 1985b). Therefore, in addition to the action of indomethacin on the platelet receptor, some of the niosomal indomethacin may enter the platelets by fusion and endocytosis and act directly on the cyclo-oxygenase system to prevent thromboxane formation.

Table 2
Inhibition of ATP release (induced by ADP, 5 μmol) by free and niosomal-indomethacin

Indomethacin conc. (μmol)	% Inhibition free drug	% Inhibition niosomal drug
2.0	18.25 ± 0.95	39.25 ± 1.71
4.0	24.75 ± 1.71	75.25 ± 0.95
6.0	31.50 ± 1.29	88.50 ± 1.29
8.0	40.00 ± 1.82	100.0 ± 0.00

References

Chandraprakash, K.S., Uduppa, N., Umadevi, P., Pillai, G.K., 1993. Effect of niosome encapsulation of methotrexate, macrophage activation and tissue distribution of methotrexate and tumor size. Drug Deliv. 1, 333–337.

Florence, A.T., Bailie, A.J., 1989. Non-ionic surfactant vesicles: alternatives to liposomes in drug delivery? In: Prescot, L.F., Nimmo, W.S. (Eds.), Novel Drug Delivery and its Therapeutic Applications. Wiley, Chichester, pp. 281–296.

Florence, A.T., Cable, C., Cassidy, J., Kaze, S.B., 1990. Non-ionic surfactant vesicles as carriers of doxorubicin. In: Gregoriadis et al. (Eds.), Targeting of Drugs. Plenum, New York, pp. 117–126.

Gursoy, A., Akbuga, J., Eroglu, L., Ulutin, S., 1988. The inhibitory effect of liposome-encapsulated indomethacin on inflammation and platelet aggregation. J. Pharm. Pharmacol. 40, 53–54.

Hallam, T.J., Simpson, A.W., O'Connor, N., Rink, T.J., 1985a. Control and inter relation of aggregation and secretion; the roles of Ca^{2+}, diacyl glycerol and thromboxane with particular reference to ADP stimulation. Adv. Exp. Med. Biol. 192, 145–162.

Hallam, T.J., Daniel, J.L., Kendrick-Jones, J., Rink, T.J., 1985b. Relationship between cytoplasmic free calcium and myosin light chain phosphorylation in intact platelets. Biochem. J. 232, 373–377.

Parthasarathy, G., Uduppa, N., Umadevi, P., Pillai, G.K., 1994. Niosome encapsulation of vincristine sulphate: improved anticancer activity with reduced toxicity in mice. J. Drug Targeting 2, 173–182.

Philips, D.R., Jennings, L.K., Edwards, N.H., 1980. Identification of membrane proteins mediating the interaction of human platelets. J. Cell. Biol. 86, 77–86.

Siffert, W., Akkerman, J.W., 1988. Na^{+}/K^{+} exchange as a modulator of platelet activation. Trends Biochem. Sci. 13, 148–151.

Yoshioka, T., Sternberg, B., Florence, A.T., 1994. Preparation and properties of vesicles (niosomes) of sorbitan monoesters (span 20, 40, 60 and 80) and a sorbitan triester (span 85). Int. J. Pham. 105, 1–6.