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LETTER TO THE EDITOR

ENHANCED ANTI-INFLAMMATORY EFFECTS OF NANOENCAPSULATED DICLOFENAC

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This study was conducted to compare the anti-inflammatory efficacy of nanoencapsulated and free-form diclofenac in rat. Diclofenac-loaded liposomes were prepared using the proliposome method. The anti-inflammatory effects of nanoencapsulated and free diclofenac were evaluated using the carrageenan-induced paw edema, formalin-induced paw licking and cotton-pellet-induced granuloma tests *in vivo*. For carrageenan-induced paw edema, 2 and 20 mg/kg liposome-encapsulated diclofenac showed significant paw volume reduction compared to free form diclofenac of equivalent dosage groups. In the formalin test, significant reduction in paw-licking time was observed in late phase for both liposome-encapsulated and free-form diclofenac (2 and 20 mg/kg) with the percentage of inhibition of 28.62, 60.17% for free-form diclofenac and 31.45, 78.84% for liposome-encapsulated diclofenac, respectively. In cotton-pellet-induced granuloma test 20 mg/kg free-form diclofenac showed significant reduction in the size of granuloma in both transudative and granuloma weight with percentage of inhibition of 42.93 and 49.26%, respectively, when compared to controls. Interestingly, 20 mg/kg nanoencapsulated diclofenac showed a larger reduction of the parameter with percentage of inhibition of 48.43 and 63.55%, respectively. Collectively, these results indicated that nanoencapsulated diclofenac exhibited statistically higher efficacy than free-form diclofenac when orally administered. Hence, clinical dosage may be reduced thereby reducing the drug's adverse effects.

Diclofenac, 2-(2-(2,6-dichlorophenylamino) phenyl) acetic acid (Fig. 1), is a well known non-steroidal anti-inflammatory drug (NSAID) used to treat inflammation and as an analgesic agent in conditions such as acute injury or arthritis (1). Diclofenac is classified under Biopharmaceutics Classification System (BCS) class II active pharmaceutical ingredients (APIs), with high permeability but low solubility (2). NSAIDs show similar pattern of adverse drug reactions (ADRs) in different organ systems whereby the variation is mainly due to quantitative differences and types of NSAIDs administered (3).

Administration of diclofenac may lead to adverse effects such as gastrointestinal damage and disorders, stomach ulceration, epigastric pain, platelet dysfunction and thrombotic cardiovascular adverse event due to the inhibition of prostaglandin

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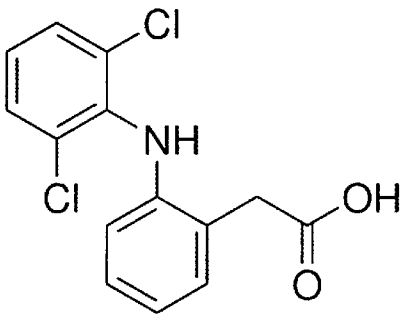


Fig. 1. *Chemical structure of diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl) acetic acid). $C_{14}H_{11}C_{l2}NO_2$ MW = 296.148 g/mol.*

synthesis by binding to cyclo-oxygenase (COX) enzyme (1). Diclofenac has been reported to cause induction of apoptosis in various cultured cell lines, oxidative injury towards hepatocytes due to disruption of intracellular ROS level and mitochondrial permeability which will eventually lead to idiosyncratic hepatotoxicity and hepatic injury (4).

Considerable attention has been focused on designing dosage forms of drugs which fulfil the therapeutic needs of the patients effectively. Various forms of lipid have been used as carriers to enhance drug gastrointestinal solubilisation and absorption. This aids in improving the therapeutic efficacy and reducing the adverse drug reactions of low bioavailability drug by reducing dosage of the drug administered (5).

Liposomal drug delivery systems have been used to improve the delivery of therapeutic agents to specific sites in the body. By loading action-specific drugs on the appropriate organs, liposomes aid in avoiding undesirable drug actions on normal organs and tissues thus minimizing the side effects. Reformulation of liposomes is constantly in progress to improve the targetability, therapeutic efficacy and safety (6). As nanoparticulate carriers, liposomes demonstrate great biological properties, such as good longevity in blood, enabling the attachment of targeting ligands on surfaces, contrast properties and stimuli-sensitivity which make them an excellent option in pharmaceutical delivery systems (7).

The present study describes the liposomal delivery system in forming liposome-encapsulated diclofenac to improve its therapeutic effects using a commercially available preparation (6).

MATERIALS AND METHODS

Commercial Pro-lipo™ Duo from Lucas Meyer Cosmetics (France), were used. Diclofenac sodium salt was purchased from Sigma® (USA), dimethyl sulfoxide (DMSO) 99.5% from Sigma®Life Science (USA), carrageenan, Commercial Grade, Type I from Sigma® (USA), and formaldehyde solution 37-38 % w/w stabilized with methanol from HmbG® Chemicals.

Animals

Healthy adult male Sprague Dawley rats weighing 180–200 g were used in this study. Rats were maintained in groups of 6 per cage in a 12-hour light-dark cycle at a controlled ambient temperature (27±2°C) with *ad libitum* food and water. Six rats were used for each group. All research and animal care procedures were approved by the Animal Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia and were performed in accordance with the National Institute of Health's Guide for Care and Use of Laboratory Animals.

Liposome sample preparation

Preparation of loaded liposomes with the model drug, diclofenac and blank were carried out in accordance with the manufacturer's (Lucas Meyer Cosmetics, France) instructions with some modifications. The preparation was carried out at room temperature (25±2.0°C). The details of the preparation have recently been published.

Initially, stock solution of diclofenac (60 mg/ml) was prepared fresh daily by dissolving a pre-determined quantity of diclofenac powder in dimethyl sulfoxide (DMSO). Next, 1 ml stock of diclofenac solution (60 mg/ml) was added gradually to 5 g prolipo-Duo in a beaker with moderate stirring (± 100 rpm) for 1 h. The mixture was then hydrated with 9 ml of distilled water which was added gradually with moderate stirring (±100 rpm) for 10 h to form a concentrated liposome suspension. 25 ml of distilled water was then added for the purpose of dilution, with moderate stirring (±100 rpm) for 30 min. All the procedures were conducted at room temperature. Drug-loaded liposome (1.5 mg/ml, DMSO 2.5 %) prepared can be stored at temperature 2-8°C for one month. Blank liposomes were prepared with the same procedure by replacing stock diclofenac solution with DMSO.

Carrageenan-induced paw edema test

The Carrageenan-induced paw edema was assessed by using methods described by Zakaria et al. (2008) with some

slight modification. The rats were orally administrated with liposome-encapsulated or free-form diclofenac, (0, 0.2, 2, and 20 mg/kg). 30 min post-treatment, intraplantar administration of 0.05 ml of 1% carrageenan suspension was injected into the right hind paw of each rat. Paw volume was measured using UgoBasile 7140 plethysmometer (Italy) before (Vo) and at 1, 2, 3, 4 and 5 hours (V_T) following the administration of carrageenan. The degree of inflammation was than quantified by measuring water volume displaced by the paw between final volume (V_T) and initial volume (Vo).

Percentage of inhibition =

$$\frac{(V_T - V_0)_{control} - (V_T - V_0)_{treated}}{(V_T - V_0)_{control}} \times 100$$

Cotton pellet-induced granuloma test

Cotton pellet-induced granuloma test was employed using the method of Singh et al. (2011) with slight modifications. Cotton pellets weighing around 50 mg each were autoclaved for 2 h. The autoclaved cotton pellets were then implanted aseptically at the interscapular distance under the skin on the shaved backs of anesthetized rats. The rats were orally administrated with liposome-encapsulated or free-form diclofenac, (0, 0.2, 2, and 20 mg/kg) once a day for a period of seven consecutive days. The rats were sacrificed on day eight. The moist cotton pellets surrounded by granuloma tissues were dissected out and weighed, then dried overnight in an oven at 60°C before they were weighed again for their final dry mass. The percentage of inhibition was indicated by the ability to reduce the development of granuloma tissues.

$$\text{Percentage of inhibition} = \frac{T_c - T_t}{T_c} \times 100$$

where T_c is the weight of granuloma tissue of control group and T_t is the weight of granuloma tissue of the treated group.

Formalin-induced paw licking test

The formalin-induced paw licking test was carried out using the method of Sulaiman et al. (2008), with slight modifications. The rats were orally administrated with liposome-encapsulated or free-form diclofenac, (0, 0.2, 2, and 20 mg/kg). 30 minutes post-treatment, intraplantar administration of 0.05 ml of 2.5 % formalin solution was injected into the right hind paw of each rat.

Two spontaneous behaviors which serve as the indication of pain (licking/biting and flinching/shaking of injected paw), both of which can be quantified objectively, were observed. The time animals spent licking/biting and flinching/shaking of injected paw were recorded as total licking time (s) per observation period. Observations were made between 0-5 minutes (early/acute phase) and 15-30 min (late/tonic phase) following intraplantar injection of 0.05 ml 2.5 % formalin solution into the right hind paw. The percentage of anti-inflammation (inhibition of paw-licking and biting response) was calculated as the cotton pellet test.

RESULTS

Table I shown results obtained from the carrageenan-induced paw edema test. Doses of free-form diclofenac 0.2, 2 and 20 mg/kg and liposome-encapsulated diclofenac with the doses of 0, 0.2, 2 and 20 mg/kg exhibited significant anti-inflammatory activity at $P<0.05$. The minimum significant inhibitory properties with paw volume 0.34±0.02 ml and percentage of inhibition of 4.0% was achieved in 0.2 mg/kg of diclofenac at the third hour and gradually increased with the increase in concentration of drug dosage. The first effect of liposome-encapsulated diclofenac could be seen at the third hour followed by treatment with 0 mg/kg of liposome-encapsulated diclofenac. 20 mg/kg of liposome-encapsulated diclofenac showed the greatest swelling inhibitory effect compared to free-form diclofenac. The effect could be seen from the third to the final hour of the experiment. In terms of paw edema inhibition percentage treatment with 2 and 20 mg/kg of liposome-encapsulated diclofenac showed a significant higher inhibition at the third hour with 51.3% and 70.2% of inhibitory effect, respectively, compared to 42.2% and 55.9% of free diclofenac. Similar results could be observed at the fourth hour. Significantly different inhibitory action of liposome-encapsulated diclofenac of 20 mg/kg could be seen from the third hour to the fifth hour. The inhibitory effects of 20 mg/kg liposome-encapsulated diclofenac was 70.2%, 64% and 54.3%, compared to free diclofenac with only 55.9%, 45.2% and 42%.

Fig. 2 shows the anti-inflammatory effects of free-form and liposome-encapsualted diclofenac against a chronic inflammation model. The significant anti-inflammatory inhibitory activity can only be seen in the higher treatment dose. Treatment with diclofenac at a dose of 20 mg/kg and liposome-encapsulated diclofenac at doses of 2 and 20 mg/kg showed significant anti-inflammatory activity when compared with the control, indicated by the increment in inhibition percentage. Therefore, the liposome-encapsulated diclofenac showed significantly greater anti-inflammatory properties

Table I. *Carrageenan-induced paw edema test: mean hind paw volume.*

Treatment	Dose (mg/kg)	Edematous Paw Volume, ml, mean ± SEM (n = 6)					Anti-inflammation, %				
		1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
Dx	0 (control)	0.19 ±0.03	0.24 ±0.03	0.31 ±0.03	0.35 ±0.15	0.29 ±0.09	-	-	-	-	-
	0.2	0.18 ±0.06	0.23 ±0.03	0.28 ±0.03*	0.34 ±0.02*	0.28 ±0.03	5.15	5.26	9.68	4	5.14
	2	0.17 ±0.04	0.19 ±0.04	0.18 ±0.03*	0.29 ±0.04*	0.21 ±0.05	11.50	18.88	42.15	18.54	27.37
	20	0.13 ±0.04	0.11 ±0.03	0.14 ±0.05*	0.19 ±0.05*	0.17 ±0.07	35.28	53.33	55.91	45.18	42
LDx	0	0.18 ±0.05	0.24 ±0.05	0.29 ±0.03*#	0.35 ±0.06	0.28 ±0.06	6.01	0.70	6.18	0.71	4.29
	0.2	0.18 ±0.02	0.21 ±0.06	0.28 ±0.06*	0.33 ±0.06*	0.26 ±0.05	8.58	10.88	9.41	8.24	11.43
	2	0.14 ±0.04	0.15 ±0.03	0.15 ±0.02*#	0.24 ±0.03*	0.26 ±0.07	29.61	35.44	51.34	32.24	10.29
	20	0.14 ±0.03	0.14 ±0.03	0.09 ±0.04*	0.13 ±0.03*#	0.13 ±0.04	26.18	42.95	70.16	64	54.29

** P<0.05, statistically significant from control*
 # P<0.05, statistically significant from free drug in same dosage group

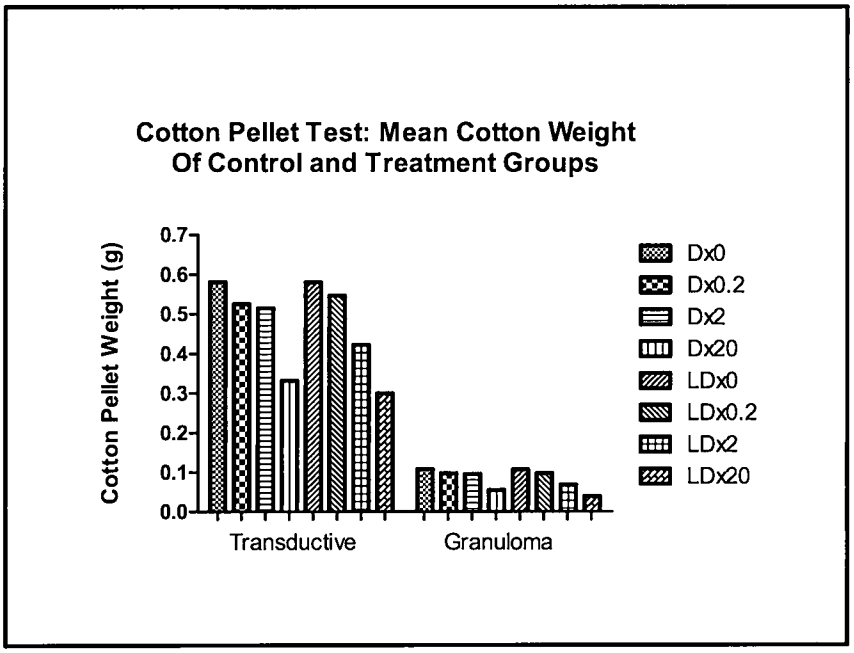


Fig. 2. *Cotton pellet induce granuloma model test: increase in mean weight of pellet. * P<0.05, statistically significant from control. # P<0.05, statistically significant from free drug in same dosage group.*

when compared to the free-form diclofenac at the same doses. Treatment with liposome-encapsulated diclofenac 2 mg/kg showed significant 27.4% and 36.9% (0.42±0.08, 0.07±0.02 g) of inhibition for both transudative and granuloma weight, respectively, compared to 11.3% and 11.4% (0.52±0.1, 0.1±0.01 g) of free-form diclofenac. The effect of liposome-encapsulated diclofenac at the dose of 20 mg/kg showed significantly greater anti-inflammatory properties with 48.4% and 63.6% for transudative and granuloma weight inhibition when compared to 42.9% and 49.3% of free-form diclofenac.

Fig. 3 shows the results obtained from the formalin-induced paw licking test. No significant result was observed in any of the treatment groups for both free-form and liposome-encapsulated diclofenac at the

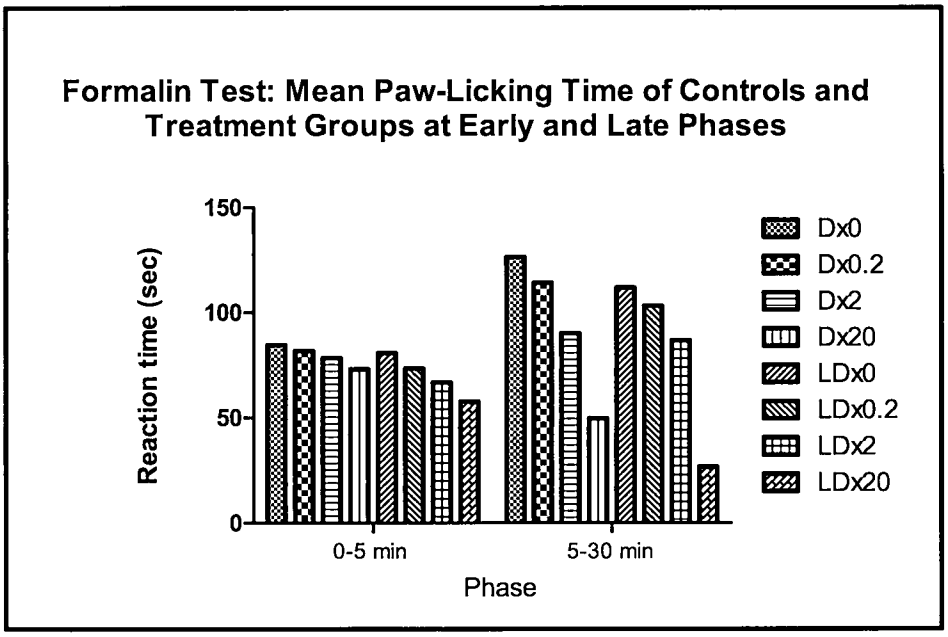


Fig. 3. *Formalin-induced paw licking test: mean paw licking time at early and late phase. * P<0.05, statistically significant from control. # P<0.05, statistically significant from free drug in same dosage group.*

early phase (0-5 min). In the late phase (15-30 min), treatment with free-form and liposome-encapsulated diclofenac at the dose of 2 and 20 mg/kg showed significant anti-inflammatory activity with total paw licking time of 90.21±15.85, 49.65±20.49 sec for free-form diclofenac and 86.64±8.94, 26.74±9.24 sec for liposome-encapsulated diclofenac when compared with the control. In addition, an increment in the anti-inflammation percentage was observed. Liposome-encapsulated diclofenac showed greater anti-inflammatory properties when compared to the free-form diclofenac at the same doses and a significant result was shown in the group with the highest dose, 20 mg/kg, at the late phase.

DISCUSSION

The anti-inflammatory efficacy of nanoencapsulated and free-form NSAID was investigated in the present study using three experimental models: the carrageenan-induced paw edema test, cotton pellet granuloma model test and formalin-induced paw licking test. Liposomes possess both hydrophilic and hydrophobic regions which ensure the dissolution of sparingly water-soluble molecules within the lipid bilayer (8). Liposomes are able to serve as micro-reservoirs and improve the solubility of less water-soluble drugs such as diclofenac due to its controllable leakage characteristic, thus improving bioavailability and therapeutic index of encapsulated drug (9).

The results obtained from the study showed that liposomes had the ability to improve the therapeutic effect of diclofenac by increasing the drug efficacy. The nanoencapsulation of diclofenac in liposome produced an enhancement of the anti-inflammatory properties compared with free-form drug. Carrageenan-induced paw edema test is suitable for evaluating anti-inflammatory effect of drugs by estimating the volume of edema form which is pathologically or artificially induced, as the method is precise and efficient (10). This study revealed that the oral administration of liposome-encapsulated diclofenac reduced carrageenan-induced paw edema in a dose-dependent manner. The improvement in therapeutic efficacy of liposome-encapsulated dicofenac can be correlated with the ability of liposomal delivery system in alteration of drugs' biopharmacological properties,

such as drug solubilisation enhancement, absorption enhancement via lymphatic transport pathway, gastric transit time prolongation, and additional protective effects which prevent undesired drug metabolism and gastric efflux (11).

The immediate inflammatory responses in rats are elicited by histamine and serotonin and the prolonged delayed-onset responses are mediated by kinins and prostaglandins (12). The anti-inflammatory effect demonstrated by the inhibition of edema volume at the delay phase of carrageenan-induced paw edema test therefore is mainly due to the inhibitory effect of diclofenac on cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzyme which are responsible for prostaglandin synthesis (13). The greater anti-inflammatory effect exhibited by liposome-encapsulated diclofenac when compared with free-form diclofenac indicates that the encapsulation of diclofenac by liposome aids in increasing efficacy of the drug and improving its therapeutic effect (14).

The cotton pellet granuloma test model is widely employed to assess the transudative, exudative and proliferative components of chronic inflammation. It has been proven that the inflammatory granuloma form is a typical feature of chronic inflammation (15). Cotton pellet implanted at the dorsal neck region of a rat acts as a foreign agent to induce inflammation. Granuloma formation is due to the proliferative phase of the inflammation of the microphages, neutrophils, fibroblasts and collagen formation which are the basic sources of granuloma formation; therefore, decrease in granuloma formation indicates the suppression of the proliferative phase (16). Results from the experiment indicate that only the higher dose showed significant ($P<0.005$) anti-inflammatory inhibitory activity. The suppression may be due to the inhibition of migration, aggregation of neutrophil and macrophage and suppression of these cell functions (17).

The formalin-induced paw licking test is useful to determine the involvement of central or peripheral pathway of inducing pain. The formalin test possesses two distinctive phases, each reflecting different types of pain. The earlier phase reflects direct effect of formalin on nociceptors (pain sensory receptor) which suggests non-inflammatory pain, whereas the late phase reflects pain from inflammation (18). Treatment with free-form diclofenac at 2 and 20 mg/kg and Liposome-encapsulated diclofenac at 2 and 20 mg/kg showed significant anti-inflammatory activity with reduction in licking time when compared with the controls in the late phase (15-30 min). These indicate that the inflammatory response took place only in the late phase and can be inhibited by non-steroidal anti-inflammatory drugs (19). The percentage of anti-inflammation activity in the late phase for liposome-encapsulated diclofenac was higher than free diclofenac of the same dose. This suggests that liposomes are able to increase the efficacy of drug therapeutic effects by improving the delivery of drugs to the target site, which was done by increasing the solubility of diclofenac by liposome (8).

In conclusion, results from this current study revealed that nanoencapsulated diclofenac exhibits greater anti-inflammatory effects than free-form diclofenac. The encapsulation of diclofenac helps improve the drug efficacy and enable the reduction of the dose, thus reducing the drug's adverse reactions. Indeed, recently we published reports showing improved efficacy and reduced cytotoxic effects of two other drugs (20, 21). The findings indicated that the anti-inflammation action was mediated via the inhibition of action on inflammatory mediators such as prostaglandins, histamine, serotonin, and bradykinin.

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