FORMULATION OF LIPOSOME-ENCAPSULATED DICLOFENAC FOR IMPROVED ANTI-INFLAMMATORY EFFICACY AND REDUCED SYSTEMIC TOXICITY

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By

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FORMULATION OF LIPOSOME-ENCAPSULATED DICLOFENAC FOR IMPROVED ANTI-INFLAMMATORY EFFICACY AND REDUCED SYSTEMIC TOXICITY

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March 2016

Chair: Professor Muhammad Nazrul Hakim Abdullah, PhD
Faculty: Medicine and Health Sciences

Diclofenac, a frequently prescribed nonsteroidal anti-inflammatory drug for the treatment of various musculoskeletal disorders and pain management therapies, is frequently associated with low bioavailability and delay onset of therapeutic activities. A prolonged administration of diclofenac also leads to multiple undesired adverse drug reactions such as gastrointestinal, hepatic and renal complications. In the present work, liposomal drug delivery system, a promising lipid-based nanoparticle technology, was exploited with the aim to improve the therapeutic efficacy and reduce toxicity of diclofenac. Different *in vitro* and *in vivo* experimental models were employed to attain a proof of concept, as well as to gain insight into the mechanisms underlying present diclofenac liposomal oral formulation. A validated, rapid and reproducible proliposomes method for the preparation of liposomes-encapsulated diclofenac was successfully developed. The optimized liposomal formulation (Pro-Lipo™ Duo from Lucas Meyer, France; DMSO solvent; 16 mg diclofenac per 1 g Pro-Lipo™; 10 hours hydration time; no size reduction treatment) yield a homogenous liposomes population (polydispersity index = 0.15) with small particle size (244.3 nm), the formulation also shown highest drug entrapment (581.4 µg/g Pro-Lipo™) and exhibited a satisfactory entrapment efficacy of 91.2 %. The prepared liposomes-encapsulated diclofenac was stable in refrigerated temperature (2-8 °C) for at least 4 weeks. In Lipopolysaccharide-induced RAW 264.7 murine macrophage model, the potential of present optimized liposomal diclofenac formulation in reducing both cytotoxicity and *in vitro* inflammatory responses were demonstrated. Liposomes-encapsulated diclofenac exhibited a significantly ($P<0.05$) stronger inhibition of proinflammatory mediators (NO, TNF-α, IL-1β, IL-6 and PGE_2) than the conventional diclofenac formulation of equivalent dosage. Present research work also demonstrated that intragastrically administered liposomes-encapsulated diclofenac was able to improve the drug's therapeutic effects in various *in vivo* experimental models with percentage inhibition of inflammation up to 78.7 %. Paw edema test of multiple inducers (carrageenan, histamine, serotonin and formalin), carrageenan-induced granuloma pouch test and cotton pellet-induced granuloma test showed that

liposomes-encapsulated diclofenac possessed a significantly ($P<0.05$) stronger acute and chronic anti-inflammatory activities than diclofenac, even if lower drug dosage were used to treat animals, percentage inhibition of inflammation ranging from 51.1 % to 61.9 % were observed. The improved *in vivo* drug therapeutic efficacy were attributed to the enhanced inhibition of pro-inflammatory mediators, including TNF-α, IL-1β, IL-6 and COX mediated PGE_2 synthesis cytokines (improved in percentage of inhibition up to 63.7 %), as well as suppression of NO production (46.6 %) in animals. In addition, sub-acute toxicity study revealed that animal models treated with liposomal formulation exhibited less signs of toxicity. Biochemical analysis (liver and kidney function tests) as well as histopathology assessment indicated that present liposomal formulation was able to significantly ($P<0.05$) reduce diclofenac-induced organ (stomach, liver and kidney) toxicity, with reduced macroscopic and microscopic gastric lesion in repeatedly treated rats. The highest percentage of improvement for stomach, liver and kidney lesion score were observed to be at 45.1 %, 25.7 % and 22 %, respectively. In conclusion, present research work successfully developed a practical liposomal diclofenac formulation with improved therapeutic efficacies and reduced systemic toxicities.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

FORMULASI LIPOSOM-BERKANDUNGAN DIKLOFENAK UNTUK PENINGKATAN EFIKASI ANTI-INFLAMASI DAN PENGURANGAN KETOKSIKAN SISTEMIK

Oleh

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Diklofenak, sejenis ubat anti-keradangan bukan steroid yang sering dipreskripsikan untuk rawatan pelbagai penyakit muskuloskeletal dan terapi pengurusan kesakitan kerap dikaitkan dengan tahap biokeperolehan yang rendah dan kelewatan dalam aktiviti terapeutik. Penggunaan diklofenak bagi jangka masa yang panjang juga mengakibatkan pelbagai kesan sampingan yang tidak diingini seperti masalah gastrousus, hepatik dan ginjal. Dalam kajian ini, sistem penyampaian ubat liposomal, sejenis teknologi nanopartikel berasaskan lipid yang memberangsangkan, telah dieksploitasi dengan tujuan untuk meningkatkan Indeks terapeutik diklofenak. Pelbagai model eksperimen *in vitro* dan *in vivo* telah digunakan untuk memperolehi bukti konsep, serta untuk mendapat pemahaman mekanisme yang berkaitan dengan formulasi liposom-berkandungan diklofenak tersebut. Kaedah proliposomeas yang disahkan, ringkas dan mudah diulangi bagi penyediaan formulasi liposom-berkandungan diklofenak telah berjaya dibangunkan. Formulasi liposom optima (Pro-Lipo™ Duo; Pelarut DMSO; diklofenak 16 mg setiap 1 g Pro-Lipo™; 10 jam masa penghidratan; Tiada rawatan pengurangan saiz) menghasilkan populasi liposom yang homogenus (Indeks polidispersiti = 0.15) dengan saiz zarah kecil (244.3 nm), kaedah tersebut juga menunjukkan kecekapan pemerangkapan yang tinggi (581.4 µg/g Pro-Lipo™) dan memuaskan (91.2 %). Formulasi liposom-berkandungan dikolofenak yang dihasilkan adalah stabil pada suhu peti sejuk (2-8 °C) untuk tempoh sekurang-kurangnya 4 minggu. Formulasi liposom-berkandungan dikolofenak yang optima ini menunjukkan potensi dalam pengurangan respon sitotoksik dan keradangan *in vitro* dalam model sel makrofaj RAW 264.7 yang dirangsangkan oleh lipopolisakarida. Formulasi liposom-berkandungan diklofenak menunjukkan perencatan mediator prokeradangan yang lebih tinggi dan ketara ($P<0.05$) berbanding formulasi konvensional diklofenak pada dos yang sama. Kajian penyelidikan ini turut menunjukkan formulasi liposom-berkandungan dikolofenak berjaya

meningkatkan kesan terapeutik ubatan tersebut dalam pelbagai model eksperimen *in vivo* dengan peratus antikeradangan sebanyak 78.7 %. Ujian kebengkaan tapak kaki yang dirangsangkan oleh pelbagai perangsang (carrageenan, histamin, serotonin dan formalin), ujian kantung granuloma yang diaruh oleh carrageenan serta ujian granuloma yang diaruh oleh pelet kapas menunjukkan formulasi liposom-berkandungan diklofenak memberi kesan antikeradangan akut dan kronik yang lebih kuat secara ketara ($P<0.05$) berbanding dengan diklofenak, walaupun dos yang lebih rendah digunakan. Peratus antikeradangan direkod dari 51.1 % sehingga 61.9 %. Keberkesanan terapi ubat-ubatan *in vivo* yang lebih baik adalah disebabkan oleh penigkatan kesan perencatan sitokin pro-radang, termasuk TNF-α, IL-1β, IL-6 dan PGE_2 (penigkatan peratus perencatan sebanyak 63.7 %), serta perencatan NO (46.6 %) dalam model eksperimen. Di samping itu, kajian ketoksikan sub-akut mendedahkan bahawa model haiwan yang dirawat dengan formulasi liposom mempamerkan pengurangan tanda-tanda ketoksikan. Analisis Biokimia (ujian fungsi hati dan ginjal) serta penilaian histopatologi menunjukkan bahawa formulasi liposom kini mampu mengurangkan secara ketara ($P<0.05$) keracunan organ perut, hati dan ginjal yang berpunca dari diklofenak dengan peratus pengurangan sebanyak 45.1 %, 25.7 % dan 22 % bagi organ perut, hati dan ginjal masing-masing. Kesimpulannya, kerja-kerja penyelidikan ini berjaya menghasilkan formulasi liposom-berkandungan diklofenak yang praktikal dengan efikasi terapeutik yang lebih baik dan pengurangan keracunan sistemik.

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I certify that a Thesis Examination Committee has met on 11th March 2016 to conduct the final examination of Goh Jun Zheng on his thesis entitled "Formulation of liposome-encapsulated diclofenac for improved therapeutic efficacy and reduced systemic toxicity" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of Supervisory Committee were as follows:

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ACUC	Animal Care and Use Committee
AD	Anno Domini (In the year of the Lord)
ADR	Adverse drug reaction
ALP	Alkaline phosphatase
AL	Active liposome
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APIs	Active pharmaceutical ingredients
AST	Aspartate aminotransferase
BC	Before Christ
BCS	Biopharmaceutical Classification System
BDDCS	Biopharmaceutics Drug Disposition Classification System
BUN	Blood urea nitrogen
cGMP	Cyclic guanosine monophosphate
ChL	Charged liposome
CL	Conventional liposome
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
CV	Coefficient of variation
dH_2O	Distilled water
DMARD	Disease-modifying antirheumatic drugs
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPC	Dioleoyl phosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DRV	Dehydration-rehydration vesicle
DSC	Differential scanning calorimetry
DSPC	Disteroyl phosphatidylcholine
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alia* (and others)
etc.	*et cetera* (and other things)
e.g.	*exempli gratia* (for example)
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FMHS	Faculty of Medicine and Health Sciences
GC	Gas chromatography
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GUV	Giant unilamellar vesicle
HPLC	High performance liquid chromatography
H&E	Hematoxylin and eosin staining
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
i.e.	*id est* (that is)
IL	Interleukin

INT	Tetrazolium salt
IUPAC	International Union of Pure and Applied Chemistry
K+	Potassium
LAL	Limulus Amebocyte Lysate
LCL	Long-circulating liposome
LDH	Lactate dehydrogenase
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
LUVET	Large unilamellar vesicles by extrusion
LUV	Large Unilamellar vesicle
LYM	Lymphocytes
MLV	Multilamellar vesicle
MPS	Mononuclear phagocyte system
mRNA	Messenger Ribonucleic acid
MV	Multivesicular vesicle
Na+	Sodium
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NO_2	Nitrite
NSAID	Nonsteroidal anti-inflammatory drug
N/A	Not applicable
N/D	Not determined
OECD	Organisation for Economic Co-operation and Development
OLV	Oligolamellar vesicle
PC	Phosphatidylcholine
PDI	Polydispersity index
PE	Phosphatidylethanolamine
PG	Prostaglandin
PLs	Proliposomes
PPAR	Peroxisome proliferator activated receptor
PS	Phsphatidylserine
REV	Reverse evaporation vesicles
RSD	Relative standard deviation
SEM	Standard error of the mean
SMEDDS	Self-microemulsifying drug delivery system
SPLV	Stable plurilamellar vesicles
SPSS	Statistical Package for the Social Sciences
SUV	Small unilamellar vesicle
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
TRL	Targeted-release liposome
TrRL	Triggered-release liposome
TX	Thromboxane
UK	United Kingdom
UPM	Universiti Putra Malaysia
US	The United States of America

UV	Ultraviolet
UVs	Unilamellar vesicles
WBC	White blood cell
WHO	World Health Organization
w/w	Weight to weight

CHAPTER 1

INTRODUCTION

1.1 Introduction

Drug development research is a constant effort of disease amelioration in the discovery and development of new therapeutic entities. The primary goal is to identify entities or drug delivery formulations which capable of enhancing drug therapeutic efficacy while reducing the toxic side effects (Xiang and Anderson 2006). Drug delivery system is a process or attempt to direct the pharmaceutical compound administered in order to achieve a desirable therapeutic effect in humans or animals (Tiwari *et al.* 2012). Among the variety of systems attempted to overcome the limitation of conventional therapeutic formulations, liposomal drug delivery system, an artificial phospholipid nanovesicles have received intensive attention over the past few decades due to its unique physicochemical characteristics (Elbayoumi and Torchilin 2010).

Liposomes are spherical nanosized phospholipid vesicles composed of one or more concentric lipid bilayers with an enclosed aqueous core, obtained by the dispersion of phospholipids in water (Torchilin 2005). Liposomes have shown great potential as the pharmaceutical carrier of choice for various clinical applications due to its attractive biological properties and unique physicochemical characteristics (Elbayoumi and Torchilin 2010). For instance, liposomes are capable of encapsulating both hydrophilic and lipophilic active agents, altering drug pharmacokinetics and pharmacodynamics, versatile in physicochemical behaviors, biodegradable and biocompatible (Dwivedi *et al.* 2014).

Since the discovery of liposome by British hematologist Alec D Bangham in 1961, the vesicular nanocarrier has played an essential role in various scientific disciplines inclusive of the enhancement of therapeutic effects in pharmaceutical and clinical aspects. Studies have shown that liposome-encapsulated drugs exhibit a significant reduction in adverse drug reactions in addition to the improvement in therapeutic efficacy when compared to the conventional formulation (Allen and Cullis 2013). The present focus of liposomal drug delivery systems is in cancer therapy, antimicrobial therapy, immunology and gene therapy. With some of the liposomal formulations are now available commercially in the market or in advance clinical trials, the potential of liposome technology and clinical applications is enormous (Goyal *et al.* 2005). The interest of development of liposomes as pharmaceutical carries remains high and new improved formulations designed for different therapeutic areas, including the treatment of inflammatory diseases may soon be expected (Immordino *et al.* 2006).

The chief drugs used in inflammation treatment can be divided into three major groups, namely the cyclo-oxygenase (COX) inhibitors, antirheumatoid drugs, and the new anticytokine and other biological agents, with Nonsteroidal anti-inflammatory drugs (NSAIDs) being the most widely used of all agents (Rang *et al.* 2012). NSAIDs represent a large class of therapeutic agents with anti-inflammatory, analgesic and antipyretic (fever-reducing) effects. They are most widely used to treat inflammation, mild to moderate pain and fever, specifically in the treatment of arthritis, soft tissue injuries, headache, dental and menstrual pain (Day and Graham 2013).

In the United States of America (US) alone, 70 million NSAIDs prescriptions and more than 30 billion over-the counter tablets sold annually, making NSAIDs the most widely used classes of drug worldwide. Sharing similar mechanism of action, the use of NSAIDs are commonly associated with several side effects such as gastrointestinal (GI) adverse effects (e.g. ulceration, bleeding, dyspepsia), hepatotoxicity, renal failure, hypocoagulability, allergic reactions and increase in risk of cardiovascular thromboembolic events (Khatchadourian *et al.* 2014). Annually, 1 to 2 % of conventional NSAIDs users were hospitalized due to severe GI complications. Report of US Food and Drug Administration (FDA) estimated conservatively that over 100,000 NSAID-related hospital admissions, 16,500 deaths occur every year, with a hospitalized mortality rate of 5 to 10 % and 0.08 % expected annual death rate (Pountos *et al.* 2011; Singh 2000). In Malaysia, data on NSAIDs adverse drug reactions remains limited, a study conducted through community pharmacies across the country reported 21.2 % of a total number of 368 respondents experienced some adverse effects after using NSAIDs, with GI complication being the most common side effects follow by headache, dizziness, rashes, itchiness and fever. Several other side effects such as numbness, tingling sensations, sore throats and insomnia were also reported (Chua and Paraidathathu 2005).

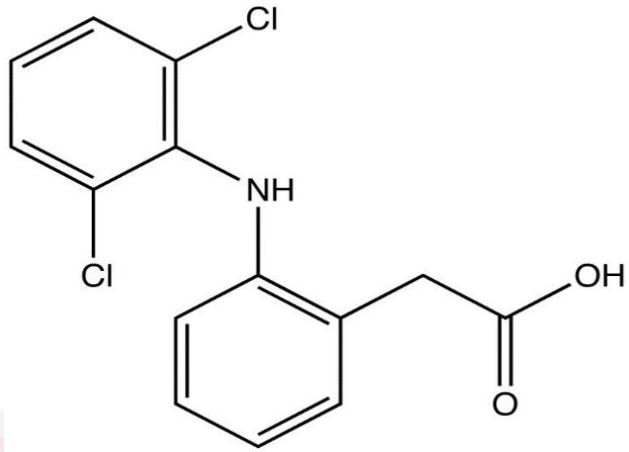

Figure 1.1: Chemical structure of diclofenac
(Source: Chuasuwan *et al*. 2009)

Diclofenac, 2-[2,6-dichlorophenyl)amino] phenyl acetic acid, is one of the most widely prescribe member in the acetic acid group of NSAID. Figure 1.1 illustrated the structural formulation of diclofenac. It was first introduced in the United Kingdom (UK) in 1979 and currently marketed under multiple trade names. Diclofenac is commonly supplied as either sodium or potassium salt, which used in the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, degenerative joint diseases, fever and pain management therapy due to its anti-inflammatory, antipyretic and analgesic properties (Cole 2011). Diclofenac possess high potency and it is the most potent NSAID on a molar basis. Some evidence shows that besides the inhibition of COX pathway, diclofenac might exert its action by inhibiting the lipooxygenase pathways, there is also speculation that diclofenac may inhibit phospholipase A2 as part of its mechanism of action, which contributes toward the high potency (Gan 2010). However, both diclofenac sodium and diclofenac potassium are classified as class II active pharmaceutical ingredients (APIs) under the Biopharmaceutics Classification System (BCS), which characterize by its high permeability but low solubility (Chuasuwan *et al.* 2009).

The orally administered diclofenac has also been associated with several common adverse effects of NSAIDs such as gastrointestinal complication (ulceration, bleeding), hepatotoxicity and nephrotoxicity, with severe liver toxicity observed compared to other drugs of the same group which eventually limit its clinical utilization (Van Leeuwe 2011). Apart from the concerns of diclofenac induced GI, renal and hepatotoxicity, studies in recent years have revealed that diclofenac demonstrated an increased risk of cardiovascular events, with numerous heart and stroke related adverse events associated with diclofenac use has been recorded in the World Health Organization (WHO) Global Individual Case Safety Reports Database System (VigiBase) (Perry *et al*. 2014; TGV 2014).

Liposomal drug delivery system appears to be promising in improving NSAIDs efficacy and safety profile. Several studies which utilized diclofenac as a model compound for lipid encapsulation have shown promising results in improving drug distribution, vascular permeation, retention time and other pharmacological properties, with topical formulation for transdermal drug delivery and ophthalmic formulation being the major areas of focus (Fujisawa *et al.* 2012; Jukanti *et al.* 2011; Manconi *et al.* 2011). However, the potential of liposomal drug delivery system in enhancing the therapeutic effects of intragastrically administered diclofenac, while simultaneously reducing the drug induced organ toxicity has remained obscure due to the absence of elaborative study. With an approach in mind to prepare a novel liposomal diclofenac formulation with enhanced pharmacological properties and improved drug safety have therefore led to the development of present research hypothesis.

1.2 Research hypothesis

Nanoencapsulation of diclofenac sodium by liposomal drug delivery system is able to improve the therapeutic efficacy of drug by enhancing diclofenac sodium anti-inflammatory properties and reducing drug induced cytotoxicity.

1.3 Objectives of study

The present research works were conducted in order to achieve the following objectives:

a) General objective
To produce a novel liposomal diclofenac formulation and to investigate the therapeutic efficacy as well as toxicity effects of nano-encapsulated diclofenac sodium as compared to the conventional diclofenac sodium formulation.

b) Specific objectives
1. To develop a simple, optimized and reproducible procedure for the preparation of liposome-encapsulated diclofenac.
2. To evaluate the potential of liposomes in reducing cytotoxicity and enhancing anti-inflammatory properties of liposomes-encapsulated diclofenac *in vitro*.
3. To investigate the ability of liposomes in enhancing the anti-inflammatory effects of intragastrically administered diclofenac in animal models.
4. To determine the role of liposomes in reducing gastric, hepatic and renal toxicities of intragastrically administered diclofenac in animal models.

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1. **Goh JZ**, Tang SN, Zuraini A, Zakaria ZA, Kadir AA, Chiong HS, Fauzee MSO, Nazrul Hakim M. Enhanced anti-inflammatory effects of nanoencapsulated diclofenac. European Journal of Inflammation 2013;11(3):855–861.

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3. **Goh JZ**, Tang SN, Chiong HS, Yong YK, Zuraini A, Nazrul Hakim M. Evaluation of antinociceptive activity of nanoliposome-encapsulated and free-form diclofenac in rats and mice. International Journal of Nanomedicine 2015;10:297–303.

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1. **Goh JZ**, Chiong HS, Nazrul Hakim M. Validated spectrophotometric determination of diclofenac sodium. In: Acta Pharmacological Sinca, 2013 July 9-13, Shanghai International Convention Center, Shanghai, China. p.140.

2. Chiong HS, **Goh JZ**, Nazrul Hakim M. Improved anti-nociceptive activities by liposomes-encapsulated diclofenac. In: Acta Pharmacological Sinca, 2013 July 9-13, Shanghai International Convention Center, Shanghai, China. p.140.

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