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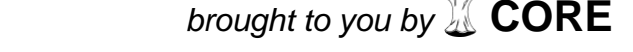
brought to you by 

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UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Liposomes as Nanosystems for the Transport and Delivery of Bioactive Agents

Manuela Colla Carvalheiro

DOUTORAMENTO EM FARMÁCIA
(TECNOLOGIA FARMACÊUTICA)

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The cover and back cover pages are freeze-fraction electron micrographs of liposomes obtained from Dr. Eugénia Cruz at the Max-Planck Institut für Biophysik, Frankfurt/Main, Germany

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Liposomes as Nanosystems for the Transport and Delivery of Bioactive Agents

Lipossomas como Nano-Sistemas para o Transporte e Entrega de Agentes Bioactivos

(com resumo em Português)

PhD thesis supervised by Doctor Maria Eugénia Meirinhos da Cruz, Prof. Doctor João Nuno Sereno de Almeida Moreira and Prof. Doctor Rogério Paulo Pinto de Sá Gaspar.

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Liposomes as Nanosystems for the Transport and Delivery of Bioactive Agents

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para prestação de Provas de Doutoramento em Farmácia, Tecnologia Farmacêutica

"Whether or not your efforts are smiled upon by fate,
what really matters in the end is to be able to say,
I did what I was able to"

Louis Pasteur

Ao meu pai, *"alla mia nonna"*

(In memoriam)

À minha mãe e aos meus irmãos

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David e Carolina

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Abstract

The concept of pharmaceutical nanobiotechnology was originated in the 1970s when liposomes started to be used as nano-drug delivery systems (NanoDDS) to incorporate lipophilic and hydrophilic drugs. Since then liposomes have been the most widely investigated nano-carrier system aiming to achieve controlled drug delivery. The inability of several conventional therapies to deliver the therapeutic dose of the active agents to the diseased tissues at the desired time and concomitantly avoid causing severe toxic effects to healthy tissues or organs has brought considerable attention to the development and clinical use of NanoDDS.

In the work developed in this thesis we intended to use liposomes as NanoDDS for cytosolic delivery of bioactive agents aiming to target either diseases of the Mononuclear Phagocytic System (MPS) or cancer. Simultaneously we explored the flexibility and the multifunctional nature of liposomes in different aspects. Special attention was given on the potential of liposomes to carry new bioactive agents with distinctive physicochemical features: small molecules (anti-parasitic drugs) or macromolecules (oligonucleotides) and their ability to target different types of cells, such as macrophages (phagocytic cells) and tumour cells (non-phagocytic). In order to achieve our aims we chose two disease models: Leishmaniasis and Small Cell Lung Cancer (SCLC).

The development of liposomal formulations of dinitroanilines for the treatment of leishmaniasis was addressed in Chapter II. Dinitroanilines have proved in vitro anti-leishmanial activity but they are not used in clinical practice as chemotherapeutics for the treatment of leishmaniasis. Nevertheless, they hold great potential in the treatment of this disease due to a selective mechanism of action against parasite tubulins and to the absence of toxicity to mammals. To reach this aim we chose two complementary strategies. The first (Part A) consisted in the association of one dinitroaniline, trifluralin (TFL) with conventional liposomes. The second (Part B) consisted in the incorporation of chemical derivatives of TFL (TFL-D) prepared by organic chemistry hemi-synthesis methods, in order to further improve the chemical stability and biological activity. Conventional liposomes were used as solvents for these hydrophobic and difficult to handle dinitroanilines (either the TFL or the TFL-D) and also because they are naturally cleared from the circulation by the MPS favouring their choice to target intracellular infections of this system, such as leishmaniasis.

In Part A, after achieving an efficient incorporation and stabilization of TFL in liposomal formulations (stability on storage up to 2 years in lyophilized form) their therapeutic activity in appropriated animal models of visceral and cutaneous leishmaniasis was evaluated. All TFL liposomal formulations were active against different strains of *Leishmania*, showing significant reduction in the levels of visceral and cutaneous infections in mice. A superior activity (at least 2-fold) was observed for liposomal TFL as compared to the free drug. A selected TFL liposomal formulation also improved the clinical condition of experimentally infected dogs and reduced the parasite load.

In Part B, two new dinitroaniline derivatives were used. This approach was pursued mainly to circumvent several disadvantages of TFL such as unfavourable physicochemical properties and difficulties on handling. Selected conventional liposomes were optimised for the incorporation of these TFL-Ds. The anti-leishmanial activity of TFL-D liposomal formulations was evaluated both *in vitro* and *in vivo*. The *in vitro* biological evaluation of the TFL-D liposomal formulations has demonstrated their activity against *Leishmania* parasites in culture without revealing signs of toxicity. In addition, extensive parasite load inhibition (> 90%) was observed after treatment with one of the TFL-D liposomal formulations in a murine model of zoonotic visceral leishmaniasis.

The use of liposomes as NanoDDS in cancer therapy was addressed in Chapter III. The association of conventional anti-cancer drugs with liposomes has been particularly investigated not only because it increases their concentration in the tumour tissue and reduces their negative side effects, but also because of the extensive application of gene therapy protocols in the treatment of cancer. In fact, antisense oligonucleotides (asODN) or other nucleic acid molecules are considered a new class of anti-cancer drugs since they are able to selectively inhibit the expression of a gene. They act by binding to a complementary region of the mRNA causing its degradation with the consequent down-regulation of the corresponding protein. However, nucleic acids molecules need adequate NanoDDS to be efficiently delivered into the cytosol of the tumour cells due to their poor stability in physiological fluids, high susceptibility to nuclease degradation and limited ability to penetrate through cellular membranes. Based on this rationale, Chapter III is focused on the development of a targeted-liposome delivery system containing an asODN for the treatment of SCLC. For this purpose, long circulating (PEG-grafted) cationic liposomes were used for the encapsulation of the asODN. The attachment of a targeting ligand for selective

cellular delivery, on the outer surface of this long circulating formulation, makes it a specific delivery system for SCLC cells. Two different cationic liposomal formulations, Coated Cationic Liposomes (CCL) and Stabilized Antisense Lipid Particles (SALP), were selected for the encapsulation of an asODN that inhibits the expression of *c-myc* oncogene, associated with SCLC cell proliferation. The hexapeptide antagonist G was chosen as the targeting ligand to promote internalization of these formulations. The effect of the peptide coupling method, conventional and post-insertion, on the loading capacity and size of both formulations was assessed. The post-insertion coupling method applied both to CCL and SALP liposomes containing *as(c-myc)*, developed in Chapter III resulted in antagonist G-targeted formulations with the necessary characteristics for evaluation of *in vitro* delivery of asODN to SCLC. The strategy of using antagonist G as the targeting ligand proved to be successful as it increased the uptake of both formulations as compared to the non-targeted counterparts, in particular in a variant SCLC cell line characterised by being resistant to conventional chemotherapy. The presence of the antagonist G at the surface of SALP did not affect the long circulation characteristics of the SALP liposomes as shown in pharmacokinetic studies. In addition, the preferential accumulation of this formulation in the lungs, substantiate the rationale behind the design of these targeted liposomes for *in vivo* intracellular delivery of nucleic acids.

Overall, the main objectives of this work were reached. Throughout its experimental development new and important issues were identified and remain open. These issues may be an interesting starting point for future research.

Resumo

O conceito de nanobiotecnologia no campo farmacêutico teve a sua origem na década de 1970 quando os lipossomas foram, pela primeira vez, utilizados como sistemas de transporte e entrega de fármacos (NanoDDS, do inglês *Nano Drug Delivery Systems*) para a incorporação quer de moléculas hidrófobas quer hidrófilas. Desde essa altura e tendo como objectivo a entrega de fármacos de forma controlada, os lipossomas têm sido os sistemas de transporte *in vivo* mais estudados. O desenvolvimento de NanoDDS tem sido alvo de grande interesse como alternativa a algumas terapias convencionais nos casos em que se demonstra incapacidade de entrega da dose terapêutica de fármacos nos seus locais de acção e em tempo útil, sem que se verifiquem, em simultâneo efeitos adversos nos tecidos sãos.

O trabalho aqui desenvolvido refere-se à concepção e desenvolvimento de nano-formulações, via incorporação em lipossomas, para transporte intracelular de agentes bioactivos não convencionais e de natureza diversa, quer nas suas características físico-químicas, quer nos alvos terapêuticos a que se destinam: moléculas de baixo peso molecular, comercializadas ou obtidos por hemi-síntese, dirigidas a macrófagos infectados por *Leishmania* e uma macromolécula (oligonucleótido) destinada a células do cancro das pequenas células do pulmão (SCLC, do inglês *Small Cell Lung Cancer*).

O Capítulo II refere-se ao desenvolvimento de formulações lipossomais de dinitroanilinas para o tratamento da leishmaniose. As dinitroanilinas são uma nova classe de compostos que, embora evidenciem actividade anti-parasitária, não fazem parte da prática clínica do tratamento desta doença. No entanto, possuem um mecanismo de acção selectivo nas tubulinas dos parasitas que, juntamente com ausência de toxicidade para os mamíferos, lhes conferem grande potencial como fármacos leishmanicidas. Para atingir este objectivo foram utilizadas duas abordagens complementares, a primeira das quais (Parte A) consistiu na incorporação de uma dinitroanilina, a trifluralina (TFL), em lipossomas convencionais. A segunda abordagem (Parte B) consistiu na incorporação em lipossomas de moléculas hemi-sintéticas derivadas da TFL (TFL-D). Esta estratégia teve como objectivo melhorar a estabilidade química e a actividade biológica da TFL. Os lipossomas convencionais foram utilizados como solventes para as dinitroanilinas (TFL e TFL-D), que são compostos hidrofóbicos e de difícil manuseamento. Além disso e uma vez que este tipo de lipossomas é

naturalmente eliminado da circulação sanguínea através do sistema fagocitário mononuclear (MPS, do inglês *Monoculear Phagocytic System*) a sua utilização para o tratamento de infecções de células e órgãos desse sistema, tal como a leishmaniose, parece pertinente.

Na Parte A deste estudo, foram obtidas formulações lipossomais de TFL com elevada estabilidade (até 2 anos na forma liofilizada) e que apresentam parâmetros de incorporação apropriados. Estas formulações foram avaliadas em termos da sua actividade terapêutica em modelos animais de infecção de leishmaniose visceral e cutânea (*Leishmania donovani* e *L. major*). Observou-se uma acentuada redução dos níveis de infecção nos animais tratados com as formulações lipossomais de TFL, as quais demonstraram ser, pelo menos, 2 vezes superiores à do fármaco na forma livre. Do mesmo modo, verificou-se uma acentuada melhoria do quadro clínico, devida à grande redução da carga parasitária em cães experimentalmente infectados com leishmaniose e tratados com uma formulação lipossomal de TFL.

Na Parte B deste trabalho foram utilizados dois novos derivados da TFL. Esta abordagem constitui uma forma de ultrapassar as propriedades físico químicas da TFL, tais como a sua baixa solubilidade aquosa e a dificuldade de manuseamento deste fármaco. Após optimização das formulações de lipossomas convencionais contendo dois TFL-D foi avaliada a sua actividade leishmanicida quer em ensaios *in vitro* quer em modelos animais. A avaliação biológica *in vitro* das formulações lipossomais de TFL-D demonstrou serem activas contra parasitas de *Leishmania* em cultura, sem revelarem sinais de toxicidade em relação a culturas de células de mamífero. Por outro lado, em estudos num modelo animal de leishmaniose visceral zoonótica, foi observada uma elevada redução (> 90%) da carga parasitária após tratamento com estas mesmas formulações.

A utilização de NanoDDS tem sido investigada com particular interesse no tratamento do cancro, com o objectivo de a associação de fármacos anti-tumorais com lipossomas possa aumentar a concentração daqueles no tecido tumoral, reduzindo simultaneamente, a sua acumulação em tecidos normais. Esta estratégia tem sido seguida não só com fármacos anti-tumorais convencionais como também e com maior incidência nas últimas décadas, em protocolos de terapia génica. De facto moléculas como os oligonucleótidos antisentido (asODN, do inglês *antisense oligonucleotides)* ou outros ácidos nucleicos são considerados uma nova classe de fármacos anti-tumorais uma vez que são capazes de inibir a expressão de um gene. Os asODN actuam através da ligação a uma região complementar de um determinado mRNA provocando a sua

degradação e consequente inibição da expressão da proteína correspondente. No entanto, para os ácidos nucleicos atingirem os seus alvos intracelulares necessitam de NanoDDS uma vez que são pouco estáveis em fluidos biológicos, apresentam uma elevada susceptibilidade à degradação por nucleases e não atravessam facilmente as membranas biológicas. Assim o Capítulo III refere-se ao desenvolvimento de formulações lipossomais apropriadas para a encapsulação de asODN e direccionadas por ligandos acoplados à superfície. Neste sentido, foram utilizados lipossomas catiónicos de longo tempo de circulação aos quais foi acoplado um ligando capaz de promover a internalização celular selectiva deste sistema por parte das células do SCLC. Dois tipos de lipossomas catiónicos designados por CCL (do inglês *Coated Cationic Liposomes*) e SALP (do inglês *Stabilized Antisense Lipid Particles*) foram seleccionados para a encapsulação de um asODN, designado por *as(c-myc)* que inibe a expressão do oncogene *c-myc*, associado à elevada proliferação celular que caracteriza o SCLC. O ligando responsável pelo direccionamento e internalização utilizado foi o hexapéptido designado por antagonista G. Dois métodos de acoplamento do péptido (convencional e pós-inserção) foram comparados e foi avaliado o efeito de cada um em parâmetros como a eficácia de encapsulação e o tamanho médio dos lipossomas. Os lipossomas CCL e SALP direccionados com o antagonista G possuem as características necessárias para a avaliação da entrega de *as(c-myc)* a células de SCLC em cultura. A estratégia da utilização do antagonista G como ligando para a entrega dirigida de *as(c-myc)* mostrou um aumento da internalização de ambas as formulação, em particular no caso de uma linha celular de SCLC caracterizada por ser resistente a fármacos convencionais. Por outro lado, a presença do antagonista G à superfície dos SALP não afectou negativamente as características de longo tempo de circulação no sangue apresentadas por esta formulação. Para além disso, a acumulação preferencial desta formulação direccionada nos pulmões, sustenta esta estratégia de desenho de sistemas especificamente dirigidos para a entrega intracelular de ácidos nucleicos neste órgão.

Dum modo geral, os principais objectivos deste trabalho foram atingidos. Ao longo do seu desenvolvimento experimental novas e importantes questões foram identificadas que permanecem em aberto e que constituem um ponto de partida interessante para trabalhos futuros.

Keywords

Liposomes

Cell targeting

Leishmaniasis

Dinitroanilines

Small cell lung cancer

Antisense Oligonucleotides

Gene silencing

Palavras Chave

Lipossomas

Direccionamento celular

Leishmaniose

Dinitroanilines

Cancro das pequenas células do pulmão

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Silenciamento de genes

Table of Contents

Abstract	xiii
Resumo	xvii
Keywords/ Palavras Chave	xxi
Index	xxiii
List of Figures	xxix
List of Tables	xxxi
Abbreviations	xxxiii
Aims and organisation of the thesis	xxxvi

CHAPTER I

General Introduction	1
I-1 - Overview	3
I-2 - Liposomes as platforms for drug delivery	5
I-2.1 – Basic properties of liposomes	5
I-2.2 - Liposome Classification	7
I-2.2.1 - Conventional liposomes	8
I-2.2.2 - Long-circulating liposomes	9
I-2.2.3 - Targeted liposomes	11
I-2.2.4 - Cationic liposomes	12
I-2.3 - Routes of administration of liposomes *in vivo*	14
I-3 - Liposome Cell Interactions and cytosolic delivery	15
I-3.1 - Liposome interactions with macrophages	18
I-3.2 – Liposome interactions with cancer cells	20
I-4 - Liposomes therapeutic applications	23
I-4.1 - Leishmaniasis	24
I-4.2 - Small Cell Lung Cancer	26
Chapter I - Reference List	29

CHAPTER II

Dinitroanilines Liposomal Formulations for the Treatment of Leishmanial Infections	37
II - Introduction	39

CHAPTER II – Part A

Trifluralin liposomal Formulations active against Leishmania infections 47

II-A.1 - Abstract 49

II-A.2 – Materials and Methods 51

II-A.2.1 - Lipids and Chemicals 51

II-A.2.2 - *Leishmania* strains and animals 51

II-A.2.3 - Preparation of Trifluralin liposomal formulations 52

II-A.2.3.1 – For scale-up studies 53

II-A.2.3.2 – For animal studies 54

II-A.2.4 - Characterisation of Trifluralin liposomal formulations 54

II-A.2.4.1 – TFL quantification 55

II-A.2.4.2 – Total lipid quantification 56

II-A.2.4.3 – Mean particle size and zeta potential determinations 56

II-A.2.5 - Stability evaluation of Trifluralin liposomal formulations 57

II-A.2.5.1 - Stability of TFL liposomes in suspension 57

II-A.2.5.2 - Stability of TFL liposomes as freeze-dried cakes 57

II-A.2.5.3 - Stability of frozen TFL liposomes 58

II-A.2.6 - In vivo studies in animal models of infection 58

II-A.2.6.1 - Visceral murine model of infection 58

II-A.2.6.2 - Cutaneous murine model of infection 59

II-A.2.6.3 - Experimental canine model of infection 60

II-A-2.7 - Statistical Analysis 61

II-A.3 - Results 63

II-A.3.1 - Development of trifluralin liposomal formulations 63

II-A.3.1.1 - Effect of lipid composition on the incorporation of TFL in liposomes 63

II-A.3.1.2 - Stability of TFL liposomal formulations under different storage conditions 68

II-A.3.1.2.1 - In suspension 68

II-A.3.1.2.2 - Freeze-dried 69

II-A.3.1.2.3 - Frozen at low temperature 76

II-A.3.1.3 – Laboratory Large-Scale preparation of TFL liposomal formulations 78

II-A.3.2 - Evaluation of the anti-leishmanial activity of TFL liposomal formulations in animal models of *Leishmania sp* infections 80

II-A.3.2.1 - Therapeutic activity in a murine visceral model 80
II-A.3.2.2 - Therapeutic activity in a murine cutaneous model 83
II-A.3.3.3 - Therapeutic activity in a canine model of *L. infantum* 88
II-A.4 - Discussion 91

CHAPTER II – Part B

Liposomal Formulations of Trifluralin synthetic derivatives active against *Leishmania* infections 99

II-B.1 - Abstract 101
II-B.2 - Materials and Methods 103
II-B.2.1 – Lipids and Chemicals 103
II-B.2.2 - Cell lines, *Leishmania* strains and mice 104
II-B.2.3 - Preparation of TFL-D liposomes 104
II-B.2.3.1 – Freeze-dried TFL-D liposomes 105
II-B.2.4 - Characterisation of TFL-D liposomal formulations 106
II-B.2.5 - Evaluation of the in vitro biological behaviour of Lip TFL-D 107
II-B.2.5.1 - Cellular Association Studies 107
II-B.2.5.2 – Anti-leishmanial activity against intracellular *L. infantum* 108
II-B.2.5.3 - Antileishmanial activity against promastigote cultures 109
II-B.2.5.4 - Cytotoxicity on THP1 cell line 110
II-B.2.5.5 - Haemolytic activity on human whole blood 110
II-B.2.6 - *In vivo* therapeutic activity studies 111
II-B.2.7 - Statistical analysis 112
II-B.3 - Results 113
II-B.3.1 - Incorporation of TFL-D in conventional liposomes 114
II-B.3.1.1 - Preparation of TFL-A6 liposomes with different lipid composition 114
II-B.3.1.2 - Optimization of TFL-D liposomal formulations 116
II-B.3.1.3 - Effect of initial TFL-D to lipid ratio on incorporation parameters 119
II-B.3.1.4 - Stability of freeze-dried TFL-D formulations 121
II-B.3.2 - *In vitro* biological evaluation of TFL-D liposomal formulations 123
II-B.3.2.1 - Cellular association studies 123
II-B.3.2.2 - Cytotoxicity assays of TFL-D liposomes 127
II-B.3.2.3 - Haemolytic activity of TFL-D liposomes 128
II-B.3.2.4 - Activity of TFL-D liposomes *in vitro* against the intracellular amastigote form of *L. infantum* 129

II-B.3.3 - Evaluation of the therapeutic effect of free and liposomal TFL-D in a model of visceral leishmaniasis 132
II-B.4 - Discussion 135
Chapter II - Reference List 143

CHAPTER III

Peptide-targeted cationic liposomes for the delivery of *c-myc* antisense oligodeoxynucleotides to small cell lung cancer cells 151
III-1 - Introduction 153
III-2 – Materials and Methods 159
III-2.1 – Reagents and Materials 159
III-2.2 – Cell lines and animals 160
III-2.3 – Preparation of asODN-containing liposomes 161
III-2.3.1 – Coated cationic liposomes 161
III-2.3.2 – Stabilized antisense lipid particles 162
III-2.4 – Antagonist G-targeted CCL and SALP 163
III-2.4.1 – Conventional coupling method to prepare antagonist G-targeted CCL and SALP 163
III-2.4.2 – Post-Insertion method to prepare antagonist G-targeted CCL and SALP 164
III-2.5 – Characterization of CCL and SALP formulations 165
III-2.5.1 – asODN quantification 165
III-2.5.2 – Total lipid quantification 166
III-2.5.3 – Vesicle size determinations 166
III-2.5.4 – Zeta potential determinations 166
III-2.5.5 – Antagonist G quantification 167
III-2.5.6 – Encapsulation parameters 167
III-2.6 – Cellular Association Studies 168
III-2.7 – Tissue Distribution Studies 169
III-3 – Results 171
III-3.1 - Physicochemical characterisation of non-targeted and antagonist G-targeted CCL and SALP liposomes 171
III-3.2 - Optimisation of the PI(G-SALP) formulation 173
III-3.2.1 - Effect of the preparation method on the *as(c-myc)* encapsulation parameters 173
III-3.2.2 - Effect of PEG-DSPE versus PEG-CerC_{16} 174

III-3.2.3 - Effect of the ratio between PEG-$CerC_{16}$ and Mal-PEG on the *as(c-myc)* encapsulation 175

III-3.3 - Cellular association of antagonist G-targeted liposomes 177

III-3.3.1 - Effect of total PEG on the extent of cellular association to SCLC cell lines 177

III-3.3.2 - Effect of the presence of antagonist G on the extent of cellular association of CCL and SALP liposomes 179

III-3.4 - Biodistribution studies of targeted and non-targeted SALP liposomes 184

III-4 - Discussion 187

Chapter III - Reference List 195

CHAPTER IV

General Conclusions 201

IV-Conclusions and Prospects 203

Chapter IV - Reference List 209

List of Figures

Figure I.1	Different uptake pathways for cytosolic delivery mediated by NanoDDS	17
Figure II.1	The *Leishmania* life cycle	40
Figure II-A.3.1	Stability of TFL liposomal formulations as suspensions	69
Figure II-A.3.2	Effect of sugars on TFL retention, mean vesicle size and zeta potential variation in freeze-dried TFL liposomes	71
Figure II-A.3.3	Short term stability of freeze-dried TFL liposomal formulations	74
Figure II-A.3.4	Long-term storage stability of freeze-dried TFL liposomes	75
Figure II-A.3.5	Stability of TFL liposomes stored at low temperature	77
Figure II-A.3.6	Incorporation parameters obtained for TFL liposomes, prepared as a SLS or as LLS	79
Figure II-A.3.7	In vivo activity of TFL liposomal formulations, free TFL and Glucantime® (Sb^{V}) against *L. donovani* (MHOM/ET/67/L82) infections in livers of BALB/c mice	81
Figure II-A.3.8	Dose and treatment-response activity of free and liposomal TFL *against L. donovani* infections in the liver of BALB/c mice	83
Figure II-A.3.9	Antiparasitic activity of TFL liposomes against *L. major* (MHOM/ET/67/L82) infections in the skin of BALB/c mice	84
Figure II-A.3.10	*In vivo* activity of TFL liposomes against *L. major* (MHOM/ET/67/L82) infections in the skin of BALB/c mice	86
Figure II-A.3.11	*In vivo* activity of TFL liposomes against *L. major* (MHOM/ET/67/L82)	87
Figure II-A.3.12	External clinical signs of leishmaniasis shown by dogs after infection (a to d) and general clinical recovery with remission of symptoms observed after treatment (e to i)	88
Figure II-B.3.1	Saturation profiles for DMPC:DMPG liposomal formulations containing TFL-D	120
Figure II-B.3.2	Stability of the TFL-A6 and TFL-A3 liposomal formulations in the freeze-drying process	122
Figure II-B.3.3	Cellular association of empty DMPC:DMPG (7:3) liposomes with THP-1 cells as a function of lipid concentration and incubation time	126
Figure II-B.3.4	Cellular association of TFL-A6 containing DMPC:DMPG (7:3) liposomal formulations by THP 1 cells as a function of temperature and lipid concentration	127
Figure II-B.3.5	Cytotoxic activity of free and liposomal TFL-D	128

List of Figures

Figure II-B.3.6	Haemolytic activity induced by free and liposomal TFL-D in RBCs	129
Figure II-B.3.7	Anti-leishmanial activity of free and liposomal TFL-D against intracellular *L. infantum* amastigotes in THP-1 infected cells	130
Figure II-B.3.8	*In vivo* anti-leishmanial activity of free and DMPC:DMPG liposomal TFL-D against *L. infantum* 1 (MHOM/PT/89/IMT151) amastigotes in the spleen of BALB/c mice	133
Figure II-B.3.9	*In vivo* therapeutic effect of glucantime (Sb^V), empty and TFL-A3 DMPC:DMPG liposomes against *L. infantum* in comparison to untreated mice in the spleen of BALB/c mice	134
Figure III.3.1	Cellular association of [^{3}H]CHE-labelled PI(G-SALP) formulations with SCLC H82 cells	178
Figure III.3.2	Cellular association of PI(G-CCL) and non-targeted CCL liposomes to SCLC cells	180
Figure III.3.3	Cellular association of PI(G-SALP) and non-targeted SALP formulations to SCLC cells	182
Figure III.3.4	Effect of pre-treatment with an endocytosis inhibitor and competitive inhibition of the cellular association of [3H]CHE labelled PI(G-SALP) with SCLC H82 cells	183
Figure III.3.5	Blood clearance of PI(G-SALP) and SALP, in naïve BALB/c mice	185
Figure III.3.6	Tissue distribution of PI(G-SALP) and SALP, in naïve BALB/c mice	186

List of Tables

Table II-A.2.1	Abbreviations and equations of TFL incorporation parameters	55
Table II-A.3.1	Incorporation parameters for TFL liposomal formulations	64
Table II-A.3.2	Incorporation parameters of various TFL formulations composed of PG and/or PC with or without DSPE-PEG_{2000}	67
Table II-A.3.3	Evaluation of the protective effect of trehalose on TFL recovery and size variation in freeze-dried liposomes	73
Table II-A.3.4	TFL retention and mean vesicle size stability of reconstituted freeze-dried TFL liposomal cakes	76
Table II-A.3.5	Viable *L. infantum* parasites detected in one of the dogs before and after treatment with liposomal TFL (F_{15})	89
Table II-B.2.1	Abbreviations and equations of TFL-D incorporation parameters	107
Table II-B.3.1	Chemical structures of TFL and of the two new synthetic derivatives and their solubility in water	113
Table II-B.3.2	Characteristics of two liposomal formulations containing TFL-A6	115
Table II-B.3.3	Evaluation of the freeze-drying process of different TFL-A6 liposomal formulations	116
Table II-B.3.4	Incorporation parameters of TFL-D containing liposomes as a function of lipid composition and $[TFL D/Lip]_i$	118
Table II-B.3.5	Effect of the presence of Rh-PE in the incorporation of TFL-A6 and TFL-A3 in liposomes	124
Table II-B.3.6	*In vitro* cytotoxicity, haemolytic activity and anti-leishmanial activity of free and liposomal TFL-D	131
Table III.3.1	Encapsulation parameters for non-targeted and antagonist G-targeted CCL and SALP liposomes	172
Table III.3.2	Characterization parameters of PI(G-SALP) containing *as(c-myc)* prepared by modifications of the original SALP procedure in comparison with G-SALP	174
Table III.3.3	Characterization parameters of PI(G-SALP) prepared with two different PEG-derivatized lipids (PEG-DSPE and PEG-$CerC_{16}$).	175
Table III.3.4	Characterization parameters of PI(G-SALP) liposomes prepared with different amounts of PEG-derivatized lipids: (mol% PEG-$CerC_{16}$: mol% Mal-PEG-DSPE).	176
Table III.3.5	Characteristics of the $[^3H]$CHE-labelled PI(G-SALP) and SALP liposomes	184

Abbreviations

[^{3}H]CHE	[1, 2-^{3}H(N)]cholesteroyl hexadecyl ether
asODN	Antisense Oligodeoxynucleotide
CCL	Coated Cationic Liposomes
CHOL	Cholesterol
DDS	Drug Delivery System
DMPC	Dimiristoylphosphatidylcholine
DMPG	Dimiristoylphosphatidylglycerol
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOPG	Dioleoylphosphatidylglycerol
DPPC	Dipalmitoylphosphatidylcholine
DPPG	Dipalmitoylphosphatidylglycerol
DSPC	Distearoylphosphatidylcholine
DSPE-PEG	Distearoylphosphatidylethanolamine-poly(ethylene glycol) 2000
EDTA	Ethylenediaminetetraacetic acid
e.g.	for example
EPR	Enhanced Permeation and Retention
FBS	Fetal Bovine Serum
G-CCL	Conventional Antagonist G-targeted CCL liposomes
G-SALP	Conventional Antagonist G-targeted SALP liposomes
h	hour
HBS	HEPES Buffered Saline
HEPES	4-(2-Hydroxyethyl)-1-Piperazine ethane sulfonic Acid
HSPC	Hydrogenated Soy Phosphatidylcholine

Abbreviations

i.p.	intraperitoneal
i.v.	intravenous
Lip	Total Lipid
min	minute
mRNA	messenger RNA
MPS	Mononuclear Phagocytic System
NanoDDS	Nano Drug Delivery System
NTD	Neglected Tropical Disease
ODN	Oligodeoxynucleotide
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PEG	poly(ethylene glycol)
PEG-CerC$_{16}$	N-palmitoyl-sphingosine-1-succinyl(metoxypolyethylene glycol)2000
PG	Phosphatidylglycerol
PI	Propidium Iodide
P.I.	Poly Dispersion Index
P_{oct}	Partition coefficient octanol/water
RBCs	Red Blood Cells
Rh-PE	Rhodamine-DOPE
SA	Stearilamine
SALP	Stabilized Antisense Lipid particles
Sb^{V}	Pentavalent antimonials (e.g. meglumine antimoniate)
s.c.	subcutaneous
SCLC	Small Cell Lung Cancer
S.D.	Standard Deviation

Abbreviations

SEM	Standard Error of the Mean
SLP	Stabilized Lipid Particles
T_c	Phase Transition Temperature
VET	Vesicles obtained through extrusion techniques

Aims and organisation of the thesis

The aim of the work described here is the development of liposomal formulations as NanoDDS for bioactive drugs having different therapeutic targets. One of the NanoDDS consists of liposomal formulations containing dinitroanilines for the treatment of Leishmaniasis. After intravenous administration conventional liposomes are rapidly cleared from the circulation by the MPS where leishmania parasites reside, making these NanoDDS appropriate for natural intracellular targeting of MPS infections. Another NanoDDS consist of sterically stabilized cationic liposomes for the delivery of an antisense oligonucleotide (asODN) complementary to a specific portion of the mRNA coding for the *c-myc* protein. These liposomes are specifically targeted to the cells of the Small Cell Lung Cancer (SCLC), via a peptide (antagonist G), covalently attached to the liposome surface.

The thesis is organised as follows:

Chapter I provides a brief overview on the delivery of drugs to cells mediated by nano-delivery systems, in particular liposomes.

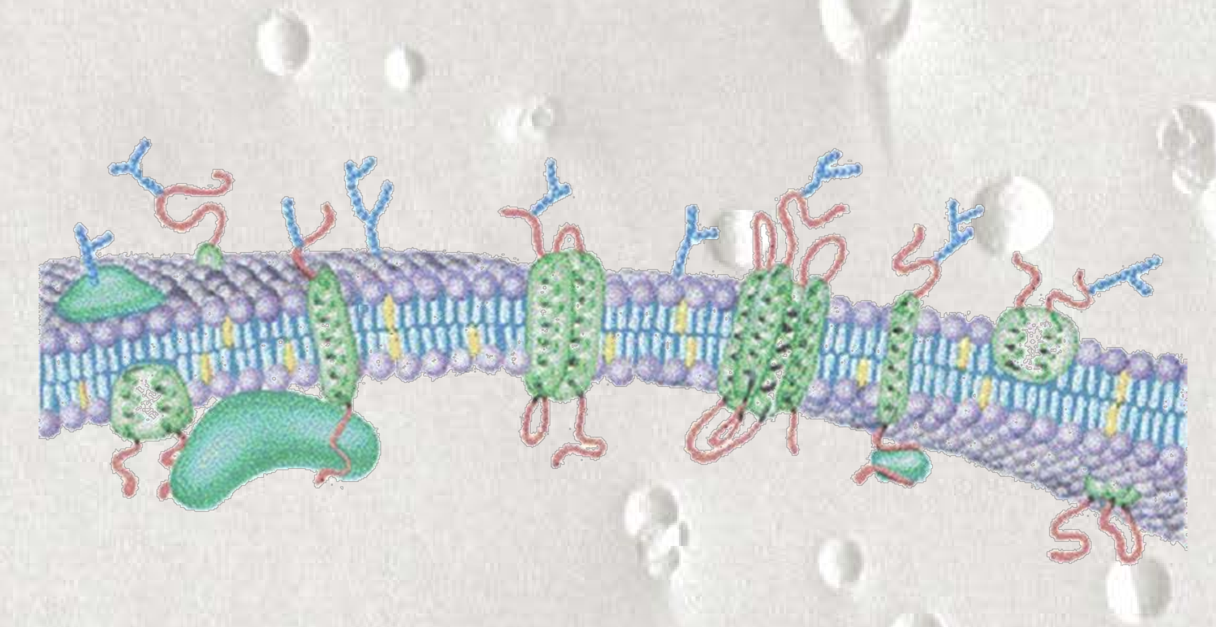
Chapter II describes the development of liposomal formulations for the incorporation of non-conventional drugs (dinitroanilines) for the treatment of Leishmaniasis. This chapter is divided into two parts, Part A and Part B. Part A is focused on the development of several conventional liposomal formulations of one dinitroaniline, trifluralin (TFL), in order to obtain a NanoDDS suitable for parenteral administration of TFL and targeted to the MPS cells. The therapeutic activity of TFL liposomal formulations was evaluated in animal models of Leishmania infections. In Part B is described a complementary strategy consisting on the chemical modification of the TFL molecule to modulate its physical and chemical properties. The resulting synthetic TFL-derivatives were formulated in liposomes. Their biological behaviour was assessed against Leishmania parasites both through in vitro systems and in vivo animal models.

Chapter III describes the encapsulation of *as(c-myc)*, an asODN molecule, in cationic liposomes with long circulation properties and specifically targeted to SCLC cell lines via antagonist G covalently attached to their surface.

Chapter IV offers a general discussion of the results and conclusions.

CHAPTER I

General Introduction



I-1 - Overview

Nanotechnology is a dynamic field, multidisciplinary in nature, which employs knowledge from physics, chemistry, biology, material science and engineering [1]. Over the last decades, it was observed an increased application of nanotechnologies in different areas, such as optics, electronics, pharmaceutical, food and agriculture [2].

In the field of pharmaceutical sciences the application of nanotechnologies for the construction of pharmaceutical delivery systems is of particular interest since it had a positive impact in the treatment of several diseases, such as cancer, metabolic and infectious diseases, inflammation and autoimmune disorders [3]. The concept of pharmaceutical nanobiotechnology was originated in the 1970s when liposomes were used as Nano-Drug Delivery Systems (NanoDDS) to encapsulate lipophilic and hydrophilic drugs. Since then, many noteworthy NanoDDS have been developed as platforms for controlled drug delivery *in vivo* [1]. A multiplicity of NanoDDS are available, including micelles, nanoemulsions, different polymeric and metal nanoparticles, nanocapsules, nanogels, liposomes, solid lipid nanoparticles, quantum dots, dendrimers, lipoproteins, nanotubes, nanofibres, polymer therapeutics and nanodevices [4]. Although these NanoDDS are constructed with materials of different nature and thus present different specific characteristics, they are all prepared to have between 1 and 1000 nm in size making them nanosized DDS, according to the nanoscale definition for the pharmaceutical field [5].

The considerable interest on the development and use of NanoDDS in clinical medicine is due to the inability of several conventional therapies to deliver the therapeutic dose of the biologically active molecules (hereinafter referred to as bioactive agents or drugs) to the diseased tissues at the desired time and concomitantly avoid causing severe toxic effects to healthy tissues or organs [1]. The failure of a significant number of new bioactive agents to live up to their potential in the clinic due to poor

physicochemical properties such as lack of blood solubility, metabolic stability or bioavailability [6, 7] has also lead to their association with NanoDDS. Thus, the main goal of a NanoDDS is to carry the bioactive agent specifically and safely from the site of administration to the desired therapeutic target in a controlled manner [8]. To achieve this goal the NanoDDS can act by different, alternative or complementary mechanisms. These can include: modification of the pharmacokinetic and tissue distribution profile of the bioactive agent, enhancement of its intracellular penetration and protection from degradation [3, 8]. However the design and development of NanoDDS able to deliver each particular bioactive agent precisely and safely to its target site at the right period of time to have a controlled release and achieve the maximum therapeutic effect remains a challenge [9].

In the field of drug delivery, nano-liposomes, a type of lipid-based systems represent one of the most advanced and promising class of NanoDDS. As therapeutic tools they possess great potential to effectively deliver bioactive agents to the site of action and to control their release at a predetermined rate and time [3, 10, 11].

The expression "nano-liposomes" has recently gained particular attention due to the growing importance given to nanotechnologies and nanomedicines, still the term "liposomes", which derives from the Greek words "lipos" meaning fat and "soma" meaning body, has been used to designate these vesicles since their discovery in 1965 [12]. For practical reasons through this dissertation the term liposome will be preferably used.

A number of liposome-based formulations carrying conventional drugs are available in the market [13]. Some examples include: Doxil® (Alza Pharmaceuticals, San Bruno, CA, USA) approved in the U.S., Caelyx® (Schering-Plough Corporation, Kenilworth, NJ) approved in Europe and Myocet® (Elan Pharmaceuticals, Inc., Cedar Knolls, NJ, USA), all liposomal formulations of doxorubicin, and Ambisome® (Astellas Pharma US, Inc.,

Deerfield, IL) a liposomal formulation of the anti-fungal drug Amphotericin-B. Many other liposomal formulations are already approved or in advanced clinical trials for the treatment of cancer and other life-threatening diseases.

I-2 - Liposomes as platforms for drug delivery

In 1965, Bangham et al. [12] reported that phospholipids in aqueous solutions form small closed highly ordered vesicular structures. Three years later Gerald Weissman named these structures as liposomes [14]. Liposomes are microscopic vesicular colloidal particles composed of self-assembled amphiphilic molecules (phospholipids) that are arranged in one or several concentric lipid bilayers surrounding an equal numbers of aqueous compartments [15]. The hydrophilic moieties or polar portions (head) of the phospholipids are oriented towards the extra-vesicular solution and interlamellar aqueous spaces while the hydrophobic chains or nonpolar tails form the bilayer [16, 17]. The possibility of incorporating numerous classes of molecules in liposomes, irrespective of molecular weight, electric charge or solubility, and the structural versatility of liposomes as well as their biocompatible, biodegradable and non-immunogenic nature led Gregoriadis *et al.* [18] in 1972 to first propose their use as DDS. Since then, liposomes have been investigated as biocompatible carriers for various bioactive agents for pharmaceutical, cosmetic, and other purposes [19].

I-2.1 - Basic properties of liposomes

The *in vivo* behaviour and ultimately the success of liposomes as NanoDDS is strictly dependent on physicochemical properties like particle size, lamellarity, surface charge, sensitivity to pH changes and bilayer rigidity. These liposomes properties can be adjusted throught the controlled preparation of an almost unlimited number of

liposomes types [20, 21]. The modulation of structural aspects like the phospholipids polar head charge, the saturation and length of their acyl chain, the presence of cholesterol, the inclusion of non-lipidic molecules and the ratio of lipid components will result in different liposomes with distinct properties (Box 1).

Box 1: Parameters used for the characterisation of liposomes

- Number of lipidic bilayers (lamellarity): unilamellar, multilamellar;
- Size (20 nm to 1 μm); and size distribution (P.I.);
- Surface charge: neutral, positive or negative (determined by the zeta potential);
- Captured volume: aqueous volume sequestered per amount of lipid;
- Drug to Lipid ratio: entrapped drug per amount of liposomal lipid;
- Entrapment efficiency: the quotient between the final and initial liposomal Drug to Lipid ratio, expressed in percentage;

Liposomes have the ability to entrap a great variety of bioactive agents. According to the physicochemical properties of these bioactive agents, namely their solubility, they can be distributed in the different compartments of the liposomes (Box 2) [7].

Box 2: Drug association and retention in liposomes

- Hydrophobic drugs (Log $P_{oct} > 5$) are inserted into the lipid matrix. They are chemically stable due to the establishment of van der Walls interactions; the drug "loading" that can be achieved is high. They present excellent retention in liposomes. In this case the entrapment is called incorporation;
- Hydrophilic drugs (Log $P_{oct} <1.7$) are entrapped in the inner and interlamellar aqueous spaces during the liposome preparation. They do not establish chemical interactions with the liposome; they achieve a smaller "loading" and present a slow release profile over a period of hours/days (dependant of the lipid composition). In this case the entrapment is called encapsulation;
- Amphipatic drugs (Log P_{oct} 1.7-5) are partitioned between the lipid matrix and the aqueous spaces. Electrostatic and van der Walls interactions are established between the drugs and the lipids. This type of drugs shows a fast release from the liposomes.

Polar drugs can establish electrostatic interactions with the polar head groups of the phospholipids and be adsorbed at the external and internal liposome surface [22].

Liposomes as NanoDDS present several advantages over conventional formulation therapies. They offer suitable means for delivering bioactive agents combined with the potential of improving the therapeutic activity while greatly reducing the side effects. In Box 3 are presented the main advantages of liposomes [7, 23, 24, 25].

Box 3: Main advantages of liposomes

- Biocompatibility;
- Modification of the bioavailability of the entrapped drug. Protection from degradation by the immune system, enzymes and unfavourable conditions;
- Modulation of pharmacokinetics and biodistribution of entrapped drugs by masking their physicochemical properties;
- Help in the diffusion of entrapped drugs through biological membranes due to their lipophilic nature;
- Reduction of toxicity of the entrapped drug. Reduction of drug exposure to sensitive tissues and organs;
- Manipulation of drug release rate by a sustained release mechanism.

I-2.2 - Liposome Classification

Liposomes can be classified according to different criteria (Box 4). The most common are: based on the preparation methods; according to structural parameters; or according to composition and *in vivo* application (or phylogenetic scheme) [26, 27, 28]. Of these classifications, the more adequate to understand the rationale behind the choice of appropriated liposomes for use as NanoDDS is the one based on the composition and application. This classification accounts for the functional characteristics of liposomes that are closely related to their *in vivo* behaviour. This type of liposome classification has been adopted by several authors and will also be followed throughout this thesis.

Box 4: Liposomes classification

Structural Parameters (lamellarity and size) [29]

- Multilamellar large vesicles (MLV); > 0.5 μm
- Oligolamellar vesicles (OLV); 0.1 - 1 μm
- Unilamellar vesicles (UV); all size range
- Small unilamellar vesicles (SUV); 20 - 100 nm
- Large unilamellar vesicles (LUV); > 100 nm
- Giant unilamellar vesicles (GUV); > 1 μm
- Multivesicular vesicles (MVV); usually > 1 μm

Preparation Method [29]

- Reverse-phase evaporation (REV)
- Freeze / thawing (FAT)
- Extrusion methods (VET)
- French press (FPV)
- Fusion (FUV)
- Dehydration-rehydration (DRV)

Composition and Application [16]

- Conventional liposomes (Non-modified Surface)
- Long-circulating liposomes (Modified Surface; sterically stabilised)
- Targeted liposomes (Modified Surface; Reactivity to specific sites)
- Cationic liposomes (Modified Surface)

I-2.2.1 - Conventional liposomes

Conventional liposomes represent the first generation of liposomes used as NanoDDS [30]. They are typically composed by mixtures of charged or uncharged naturally occurring phospholipids (e.g. egg or soy phosphatidylcholine (PC)) with or without cholesterol and charged amphipatic molecules. Although they can be prepared with a widely variety of physicochemical properties in order to modulate, to a certain extent, their *in vivo* behaviour (i.e. stability, clearance and distribution), they are characterised by a relatively short blood circulation time. In fact, after i.v. administration conventional liposomes become covered by plasma proteins (opsonins) and are rapidly removed from the blood by phagocytic cells of the mononuclear

phagocyte system (MPS), mainly by Kupffer cells from liver and macrophages from spleen. These organs with fenestrate endothelium are the major organs of accumulation of conventional liposomes. They can act as liposomes reservoirs and condition the sustained release of the drug after the liposomal rupture [26, 31]. The accumulation of liposomes in those organs is ruled by a dose dependent pharmacokinetics, where accumulation increases with dose, until reaching saturation [32]. This MPS-directed transport mechanism of conventional liposomes is usually called passive targeting. A logical therapeutic benefit of this type of behaviour is the use of conventional liposomes as candidate NanoDDS for the treatment of intracellular infections of the MPS, like Tuberculosis, *M. Avium* infections and Leishmaniasis [reviewed in: 25, 33, 34, 35].

I-2.2.2 - Long-circulating liposomes

Uptake of liposomes into tissues other than liver and spleen increases with increasing circulation times of the liposomes [36]. Long-circulating liposomes, capable of avoiding capture by the MPS and persisting for prolonged periods of time in the bloodstream were developed at the end of the 1980s and led to a renewed interest in the use of liposomes as delivery systems.

The first successful strategies increased the liposomes half-life in the blood from a few hours to up to 24 hours. These involved the use of phospholipids with a high phase transition temperatures (T_c) like dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphocholine (DSPC) prepared in small sized vesicles (<100 nm) or the inclusion in the lipid composition of charged phospholipids like phosphatidylinositol (PI) or glycolipids like monosialo ganglioside (GM1). In the nineties several authors reported that the inclusion of GM1 led to an

increase uptake of these liposomes by solid tumours widening the possible therapeutic applications of liposomes [37, 38, 39].

Later on a different and more popular strategy was developed to easily produce long-circulating liposomes. It consisted in coating the outer surface of liposomes with the hydrophilic polymer poly(ethylene)glycol (PEG). This is done by using PEG-derivatized lipids like distearoylphosphoethanolamine (PEG-DSPE) where the polymer is covalently bound to the lipid anchor. These liposomes that have a half-life circulation time of more than 24 hours can be easily prepared by any pre-selected preparation method. They were first called "Stealth®" (registered trademark of Liposome Technology Inc., Menlo Park, CA, USA) because they were not identified by the MPS cells and thus escaped their capture. In general, they are more commonly referred to as "sterically stabilized" as a result of an enhanced *in vivo* stabilisation and reduction of the interactions with plasma proteins. This stabilisation results from the formation of a steric barrier produced by the highly hydrated PEG groups at the surface of liposomes that avoids recognition by opsonins, and is responsible for the induction of long circulation times (half-life in humans >24 h). The substantial increase in the circulation time of these liposomes gives them the opportunity to extravasate at body sites like solid tumours, and sites of infection and inflammation where the permeability of the vascular wall is increased and where new blood vessels are formed (angiogenesis) [29]. In these types of tissues, the accumulation of long-circulating liposomes occurs through the so called Enhanced Permeability and Retention (EPR) mechanism. This mechanism offers the opportunity to design long circulating liposomes with appropriate size to cross these fenestrations [31].

Due to the fact that long-circulating liposomes are not easily captured by the MPS they can also be seen as slow release circulating micro-reservoirs of the entrapped bioactive agent. Thus, through the use of PEG-coated liposomes, high concentrations

of the drug can be found in the sites of action without the need of concomitant high doses of lipids. These liposomes have a dose independent pharmacokinetics in a wide variety of concentrations. This allows a prediction of the plasma levels of the drug making it easier to determine the correct dosage [37].

The versatility in lipid composition, the prolonged circulation half-lifes, the pharmacokinetics properties and the lack of MPS saturation are the essential advantages of long-circulating liposomes [40].

I-2.2.3 - Targeted liposomes

In an attempt to enhance the specificity of the interaction between the liposomes and the target cells and to increase the drug accumulation in the desired tissues, the use of targeted liposomes has been suggested. These liposomes can be conventional, long-circulating or cationic as long as they have, at their surface, targeting moieties or ligands capable of recognizing specific cell populations, binding to them, and inducing internalization of the entire liposomes or only the encapsulated drugs [24, 41]. The targeting moieties include antibodies or antibody fragments (in this particular case liposomes are referred to as immunoliposomes), glycoproteins, peptides, growth factors, carbohydrates, or specific receptor ligands [42, 43, 44]. The success of these liposomes as delivery systems is mainly dependent on the selectivity of the targeting moiety attached to the liposome surface towards the receptors in the target tissue or cell. When choosing the targeting moiety, variables like the degree of receptor expression; whether the ligand is internalized or not and the binding affinity of the ligand, are of crucial importance and must be considered [6]. In addition, for the targeting to occur, the ligands must always be exposed to the liposome exterior. Moreover, to allow the establishment of the ligand/receptor interaction is of convenience for these liposomes to be long circulating. These two features can be

combined through the development of long-circulating targeted liposomes. This type of liposomes can be obtained by different methods that allow the attachment of a specific targeting moiety at the surface of pre-formed long circulating liposomes containing the active drug. The coupling can be accomplished by covalently attaching the ligands directly to the terminus of end-functionalized PEG-derivatized phospholipids in the pre-formed liposomes. A more flexible method consists in the transfer of the targeting moiety coupled to PEG-derivatized phospholipids in a micellar phase into the bilayers of pre-formed long-circulating liposomes containing the active drug. This method called "post-insertion" allows the preparation of a variety of targeted liposomes by a simple mixing procedure from a small number of starting products [45]. In fact, any drug already entrapped in long circulating liposomes can be further targeted to different tissues according to the specific moiety attached at the liposome surface.

Targeted liposomes offer various advantages over individual drugs targeted by means of polymers or antibodies. One of the most compelling advantages is the comparable increase in drug amount that can be delivered to the target. Furthermore, the number of ligand molecules exposed on the liposome surface can be increased, improving ligand avidity and degree of uptake [41].

I-2.2.4 - Cationic liposomes

Cationic liposomes represent the youngest generation of the liposome family. They consist of lipidic mixtures containing synthetic positively charged lipids and were first developed by Felgner *et al.* [46] to improve the delivery of negatively-charged genetic macromolecules or nucleic acids (DNA, siRNA, asODN, plasmid or other) *in vitro* and *in vivo* [47, 48]. The cationic lipid components interact and neutralize the

negative-charges of those macromolecules, thereby condensing them into more compact structures [49, 50].

The association between cationic liposomes and nucleic acids is usually designated as lipoplexes [49]. These lipoplexes, present many types of morphologies, various sizes, and several models of association of the nucleic acids have been proposed [reviewed in 51]. These methodologies of associating cationic liposomes to genetic material can be considered as "active" methods of association of those macromolecules that otherwise present poor encapsulation efficiency in passive encapsulation strategies using neutral liposomes [52, 53]. In association models or encapsulation protocols where the nucleic acids are encapsulated inside a bilayer envelope the term "cationic liposomes" can be applied to the complex formed (cationic liposome/nucleic acid). These cationic liposomal carriers are usually smaller in size and stable *in vivo* [53, 54, 55].

The lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP); N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleyl-3-dimethylammonium-propane (DODAP) are typical examples of cationic lipids [16]. The modification of the surface of cationic liposomes with the inclusion of PEG-derivatized phospholipid is expected to avoid their tendency to form large aggregates. The PEG-coating increases the blood circulation of these cationic liposomes upon i.v. administration, enhancing their chance of reaching their therapeutic targets [56]. To further improve their *in vivo* targeting, cationic liposomes are usually associated to specific ligands.

In addition to their ability to spontaneously condense negatively charged macromolecules, cationic liposomes possess a number of advantages as delivery vehicles, including their natural ability to interact with negatively charged cell surface membranes and promote cellular uptake, and their ability to partially or fully protect associated macromolecules from degradation by serum nucleases [8, 57].

I-2.3 - Routes of administration of liposomes *in vivo*

The administration route by which a medicine is administered *in vivo* plays an important role on the success of the treatment. In general it is chosen according to the purpose of the treatment, the accessibility of the diseased tissue and the toxicity of the injected bioactive agent or of the NanoDDS [58]. Accordingly the route of administration by which liposomes are delivered into the body also modulates their fate and can contribute to the success of their application in different types of treatments, e.g. local or systemic.

Liposomes can be administered by several routes, such as oral, intravenous, intraperitoneal, intramuscular, subcutaneous, dermal, transdermal and aerogenic. The parenteral routes have been the most commonly used and among these the i.v. administration of liposomes is the more relevant. When injected by this route, liposomes are delivered directly into the blood circulation, where they remain confined to the blood vessels. Once there, liposomes come into contact with different plasma proteins. Opsonins, which absorb to the liposome surface, are recognised by macrophages facilitating their uptake of liposomes. High density lipoproteins (HDL) are responsible for the degradation of the liposomal bilayer and consequent release of liposomal content [59]. The degree or extent of the interaction between these proteins and the liposomes depends on the liposomes structure, composition and other formulation characteristics as discussed before and plays a critical role in the liposomes fate in the blood circulation [59, 60]. This is one of the advantages of liposomes, as these characteristic can be modulated according to the final therapeutic target and administration route.

When liposomes are administered by intraperitoneal, intramuscular or subcutaneous routes, their access to the blood circulation is not immediate and depends largely on their size. In these cases liposomes are injected in the interstitial/extracellular space

and subsequently they are cleared by the lymphatic system. Liposomes with diameters smaller than 100 nm are able to enter the lymph capillaries and reach the lymph nodes where they are taken up by macrophages, only a small fraction is drained into blood circulation. Larger liposomes remain in the extracellular space, forming a local liposomal depot. From this depot and depending on the lipid composition, the incorporated drug can be released more or less slowly. This mechanism, called sustained release, has been studied for antigen release aiming at vaccination [60, 61].

Non conventional administration routes, such as dermal and transdermal routes are currently receiving increasing attention for the local or systemic delivery of liposomal associated bioactive agents. A modified liposomal composition (presence of liposomes membrane softeners) provides the carrier with the ability to deform and increase skin permeability with consequent increase on therapeutic efficacy [62].

Similarly, the administration of liposomes by pulmonary route has received an increasing interest not only for the treatment of lung diseases, such as asthma, chronic obstructive pulmonary diseases, cystic fibrosis or tuberculosis, but also because several advantages were observed for systemic delivery [63].

I-3 – Liposome-Cell Interactions and cytosolic delivery

NanoDDS have been envisaged and developed to deliver bioactive agents with therapeutic properties to their site of action. Liposomes in particular are able to provide protection and site specific delivery of the encapsulated drug, liposomes may also facilitate the cytosolic delivery according to the intended target. To allow a liposomal drug to exert its pharmacological activity at an intracellular target several biological barriers must be overcome before achieving efficient delivery [64]. These barriers include crossing the cell membrane which is naturally impermeable to

complexes larger than 1 kDa; and penetrating in the phagolysosomes (e.g. infectious diseases); or avoiding lysosomes and find its sub-cellular target site (e.g. the nucleus in some cases of gene delivery) [65]. All these barriers oblige liposomes to interact with cellular membranes providing several mechanisms for the delivery of their contents to cells, including extracellular release, membrane absorption and fusion, and endocytosis [66]. The adsorption of liposomes onto the cell surface can be either specific or non-specific and liposomes may remain bound to the outer cell membrane where they probably destabilize, releasing their entrapped bioactive agent in the extracellular matrix. The subsequent diffusion of the bioactive agent over the plasma membrane is possible for molecules that are able to cross the plasma membrane via micropinocytosis. Alternatively, the surface absorbed liposomes may fuse with cell membrane, delivering their contents directly to the cytosol [66]. If this is the desired mechanism of drug release, cationic moieties can be introduced in liposomes so that electrostatic interactions between liposomes and the negatively charged cell membrane are reinforced [23]. However, the main mechanism by which all eukaryotic cells actively internalize large molecular complexes, including NanoDDS, and retain them in transport vesicles which traffic along the endolysosomal scaffold is called endocytosis [67]. Endocytosis consists in the formation of a cell membrane invagination that engulfs the extracellular particles with the surrounding fluid, forming an intracellular membrane-bound vesicle, or endosome [68]. A number of different endocytic pathways can be distinguished, these include: phagocytosis, exclusive of specialized cells called phagocytes, which comprise macrophages, monocytes, dendritic cells and granulocytes; and pinocytosis, common to all cell types. (Figure I.1). Pinocytosis can be further divided into macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin/caveolae-independent endocytosis [65]. It is believed that such a diversity of mechanisms is

used by the cells to accomplish different tasks and might have an effect on the intracellular trafficking of the liposomes and thereby on the success of drug delivery [67].

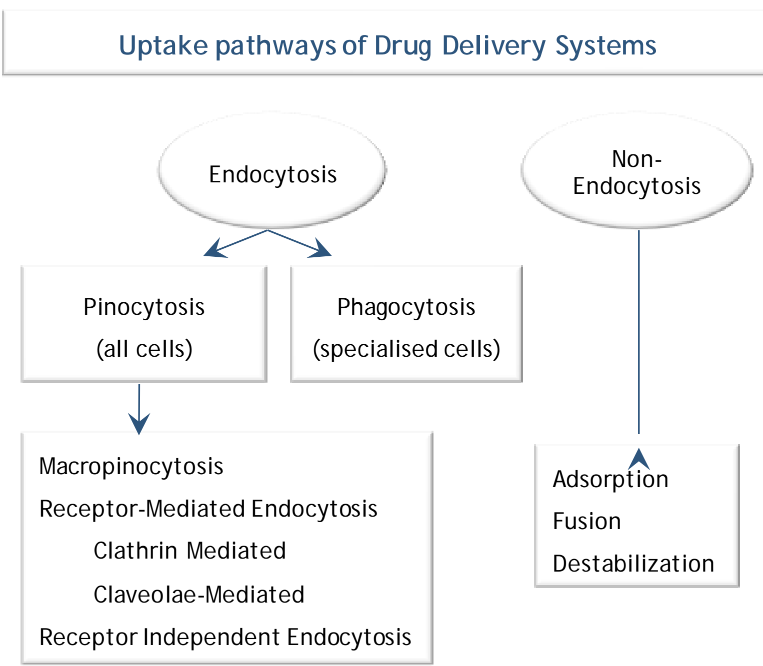


Figure I.1 - Different uptake pathways for cytosolic delivery mediated by NanoDDS. Adapted from [65]

The major and best characterized endocytic pathway is the so called clathrin-mediated endocytosis, while the clathrin independent pathway, such as caveolae-mediated internalisation has recently gained attention [70]. Clathrin-mediated endocytosis serves as the main mechanism of internalization for macromolecules including, proteins, pathogens and particles in general (e.g. NanoDDS). The first step of internalization is the strong binding of a ligand, attached to the internalising particle, to a specific cell surface receptor. Then, through a sequence of events that include the formation of clathrin-coated vesicles and their fusion into endosomes, the endocytosed NanoDDS will end up mainly in the lysosomal

compartment [65, 67, 68]. In caveolae-mediated endocytosis, small, hydrophobic membrane micro-domains rich in cholesterol and glycosphingolipids and the protein caveolin-1 form flask-shape pits called caveolae. The uptake of extracellular particles is also believed to be specifically mediated via receptors in caveolae.

The specific endocytic mechanism, by which NanoDDS are taken up by the cell, will depend on several factors, like the size of the system, the presence and type of targeting ligand and cell type [68, 69]. For example, according to Rejman *et al.* [69], particles with a diameter less than 200 nm are preferentially endocytosed via the clathrin-mediated mechanism whereas larger particles (250-500 nm) enter the cell through caveolae-derived vesicles. Thus understanding the cellular endocytic pathways that allow for the intracellular site specific delivery of NanoDDS may contribute to the appropriate design of specific systems for each particular target. The mechanism of liposomes internalization may be modulated e.g. by targeting them into distinct cellular internalization pathways, considering that not every pathway may be equally effective in releasing a bioactive agent in the cytosol.

I-3.1 - Liposome interactions with macrophages

Macrophages are the major differentiating cell of the MPS. These specialized cells possess numerous functions in the human body. Among other functions, macrophages play an important role in the defence against many pathogens by acting as host cells for parasites, bacteria, viruses and other infectious agents. Macrophages recognise these pathogens in circulation or in infected tissues as foreign structures and capture them by phagocytosis [71]. Most of these pathogens are then killed by mechanisms of the macrophage. In some diseases however, the infections agent lives inside the macrophage and uses it as a place to multiply and disseminate the infection (e.g. *Leishmania* parasites).

Liposomes and other particles with sizes below 5 µm are also captured by macrophages and to a much larger amount than other cell types. Studies involving macrophages from different anatomical sites have demonstrated that after an initial binding step of the liposomes to the macrophage surface, they are internalised and delivered to the endosome/lysosome system as intact entities. Once they reach the phagolysosomes, liposomes are degraded by enzymes releasing their contents inside the cells [72]. The probable co-localisation of the infectious agent and the NanoDDS able to bring to the site of action bioactive agents has brought increasing interest in the development of macrophage-specific DDS.

As discussed before, the capture of conventional liposomes by macrophages is an illustration of passive targeting [73]. Although no specific liposomal coating is required for this type of targeting, the inclusion of negatively charged lipids such as phosphatidylglycerol (PG) enhances their phagocytosis [reviewed in 71]. The phagocytised liposomes are not immediately located in the same cellular compartment as their target parasites and a fraction of the drug-loaded liposomes is recycled back to the extracellular matrix. However, the normal intracellular trafficking of the endosome carrying the liposomes will lead to the fusion with the phagolysosomes and the consequent destabilisation of the liposomal membrane will release the drug at its site of action [60]. Conventional liposomes carrying anti-infectious drugs have been successfully directed to macrophages with a consequent improvement of their therapeutic activity. Numerous *in vivo* animal models have shown improvements in the treatment of intracellular infections using liposomal formulations of several antibiotics such as clofazimine [74], streptomycin, [75], gentamicin [76], amikacin [77], kanamycin [78], rifabutin [79] and primaquine [80].

I-3.2 - Liposome interactions with cancer cells

The rationale to use liposomes as NanoDDS in cancer chemotherapy is to increase the selective toxicity of the anticancer drug towards the tumour tissue and the drug concentration in that tissue, thus increasing its therapeutic index. In other words the application of liposomes in cancer treatment is aimed at altering the tissue distribution and some pharmacokinetic parameters of the drug [81, 82]. In order to reach tumour cells, liposomes loaded with the anticancer drug have to avoid capture by sensitive tissues and more important increase their longevity in the circulation. In fact, an increased circulation time in plasma gives the liposomes an opportunity for selective localization and accumulation in solid tumours. For example, Stealth^{TM} liposomes of about 100 nm in size are able to passively target these tumours upon i.v. administration [83, 84] through the EPR mechanism [85]. This increased permeability at tumours sites allows extravasation of macromolecules and particles (including liposomes carrying anti-cancer drugs) from the leaky blood vessels into the nearby tissue. In addition, the lymphatic drainage system malfunctions in tumour tissue, causing prolonged retention of the particles in the tumour interstitial space [86]. This passive targeting mechanism is thought to result in the sustained release of the anticancer drug from liposomes and diffusion throughout the tumour interstitial fluid being taken up by tumour cells [43]. This strategy is suitable when the anticancer drug is a low molecular weight compound that is able to cross the cell membrane as free drug. A good example of passive targeting strategy is the clinically approved doxorubicin (DXR) in long-circulating PEG-coated liposomes (Doxil®/Caelyx®). DXR is an anthracycline that presents potent anticancer activity in a wide range of human cancer including lymphomas, leukaemia and solid tumours [10]. However its use is constrained by highly problematic systemic toxicities [87]. The incorporation of DXR in PEG-coated liposomes demonstrates the maintenance or enhancement of the

anti-tumour activity while reducing non-specific toxicity by limiting exposure to critical sites such as the myocardium [87].

On the downside, passive targeting is limited because solid tumours often have an heterogeneous blood supply mainly confined to peripheral regions. Therefore, it is unlikely that liposomes can diffuse to central poorly vascularised regions with high interstitial pressure and deliver cytotoxic levels of the anticancer drug [86]. Other factors like pharmacokinetic properties of the liposomes, their size and the sizes of the pores in the blood vessels also influence the level of accumulation of liposomes in tumours [86].

To further improve cellular specificity, to increase the amount of drug delivered to tumour cells and to facilitate cellular uptake of liposomes, recent efforts in the liposome field have been focusing on the attachment of specific ligands to the surface of liposomes [reviewed in 43, 88]. These ligand-targeted liposomes interact with cells by a mechanism known as active targeting. The targeting moieties can be specifically selected based on the unique receptors or epitopes that are exclusively expressed or overexpressed on the tumour target cells. Factors like the density and the homogeneous expression of the target antigen or receptor throughout the tumour tissue must be considered [89]. Some examples of receptors overexpressed by cancer cells and that can be exploited by actively targeted DDS are the folate receptor [90], the transferrin receptor [91] and the epidermal growth factor receptor [92]. Initially, the targeting moieties (mainly antibodies) were directly coupled to the phospholipid bilayer of conventional liposomes; this strategy resulted in an enhanced uptake of the immunoliposomes by the MPS, preventing the targeted liposome to reach their target cells [93]. The development of PEG-coated liposomes, made the active targeting of liposomes a real possibility for cancer therapy. The combination of liposomes with long circulating and targeting properties resulted in the production of various

liposomal formulations in which specific ligands were attached to the distal end (water exposed) PEG chains [4, 43, 94, 95]. Both *in vitro* and *in vivo* results have shown that such ligand-targeted Stealth® liposomes increased the therapeutic efficacy of encapsulated drugs compared to non targeted liposomes. Iden *et al* [96] compared the *in vitro* binding and cytotoxicity and the *in vivo* therapeutic efficacy (murine model of human B-lymphoma) of DXR encapsulated both in Stealth® liposomes and in Stealth® liposomes targeted with an anti-CD19 monoclonal antibody. The *in vitro* studies revealed a significantly higher uptake and cytotoxicity of the targeted liposomes in a CD19-expressing B-cell lymphoma cell line. In the *in vivo* studies, the targeted formulation showed a significant increase in the mean survival time in tumour-bearing mice as compared to the non-targeted formulation. Using similar DXR Stealth® formulations, Moreira et al. [97, 98] showed that a hexapeptide known as antagonist G could be used as a targeting moiety to prepare ligand-targeted Stealth® liposomes in human Small Cell Lung Cancer (SCLC). Antagonist G-targeted liposomes proved to have a long-circulating profile in blood, they were also internalized to a higher extent than the non-targeted formulation with a consequent increase in the DXR cytotoxicity mediated by these liposomes.

The first step in the intracellular delivery of anticancer drugs mediated by targeted-liposomes involves the specific binding of the ligand attached to the liposome carrier to the corresponding receptor at the surface of the target cell. After binding, the drug has two possible options to enter the cell. One possibility is the release of the bioactive agent from the cell-bound liposomes into the extracellular space, followed by subsequent diffusion of the released agent though the plasma membrane, as mentioned before for non-targeted liposomes. The other is the internalisation of the liposomal package via endocytosis [19]. Receptor-mediated endocytosis is a more specific active event where the cytoplasm membrane folds inward to form coated

vesicles or pits [67]. When targeted-liposomes are loaded with hydrophilic macromolecules like proteins, peptides and nucleic acids, their physiochemical properties do not allow them to cross the plasma membrane and the receptor mediated endocytosis mechanism is the only option to carry these anticancer drug molecules to their target site, which is often intracellular [67].

One of the most promising therapeutic applications of targeted-liposomes is as NanoDDS for nucleic acid delivery. As discussed above cationic liposomes are able to complex with the negatively charged nucleic acid molecules being extensively used as NanoDDS. Ligand-targeted cationic liposomes carrying nucleic acid molecules are extensively investigated in anticancer therapy. The literature presents many examples of such NanoDDS [reviewed in 99]. However, after endocytosis, these systems still have to avoid lysosomal degradation and in some cases enter the nucleus. Several strategies have been investigated to overcome these obstacles and include the incorporation of active lipids and peptides in the liposomes to enhance the endosomal release, and nuclear localization signals to enhance nuclear delivery [67].

I-4 - Liposomes therapeutic applications

Liposomes have been extensively investigated as NanoDDS for application in the treatment of a variety of different diseases ranging from cancer chemotherapy, enzymatic and antimicrobial therapy to immunization, diagnostics and topical therapy [reviewed in 100].

In the work developed in this thesis we intended to use liposomes as NanoDDS for cytosolic delivery of drugs aiming to target either diseases involving MPS cells or cancer. Simultaneously we want to explore the flexibility and the multifunctional nature of liposomes in different aspects. The aspects explored were the capability of

liposomes to carry either small molecules (anti-parasitic drugs) or macromolecules (oligonucleotides) and their ability to target different types of cells, such as macrophages (phagocytic cells) and tumour cells (non phagocytic). In order to achieve our aims we have chosen two disease models: Leishmaniasis and SCLC.

I-4.1 - Leishmaniasis

Leishmaniasis is one of the most important vector-borne diseases of humans. It results from infection by various species of *Leishmania*, a protozoan parasite of the family *Trypanosomatidae* (order *Kinetoplastida*). In humans, different species of the parasite are associated with different forms of the disease. Cutaneous leishmaniasis can be caused by many *Leishmania spp*. and the clinical syndromes include skin ulcers and nodules. A few of these pathogens are also responsible for mucocutaneous leishmaniasis that affects the mucous membranes, and may cause disfiguring lesions of the nasal, oral and pharyngeal cavities. Other species are involved in the severe parasitisation of the liver, spleen, and bone marrow causing human visceral leishmaniasis. Visceral leishmaniasis is the most overwhelming type of leishmaniasis which is associated with the poverty of developing countries and is usually mortal if untreated [101, 102]. All forms of leishmaniasis are transmitted to mammals by the bite of certain species of female sand fly during blood meal [103]. The promastigote form infects the sand fly vector and exists as an extracellular parasite. On the contrary, in the mammalian host they infect the mammalian mononuclear phagocytic cells and exist as obligate intracellular amastigotes [104, 105].

The control of leishmaniasis remains a problem, especially in the case of a zoonotic infection. The chemotherapy of leishmaniasis has not changed much in the past decades. Pentavalent antimonial drugs are still the first choice for current antileishmanial regimes although they possess well-known toxicity. In addition, the

emergence of resistant strains in some areas is a major problem. Thus these compounds can only be used in regions where the parasites are sensitive. In cases of resistance to antimonials, recommended secondary treatment include drugs such as Amphotericin B, pentamidine, paromomycin and allopurinol [106]. Regrettably all these alternative drugs present serious limitations such as toxicity, the need of long term treatments and lack of efficacy in endemic areas [107]. The more recent antileishmanial drug, miltefosine, has the advantage of being an oral drug; however severe signs of toxicity, e.g. teratogenicity, restrict its use (e.g. in women of childbearing age). In addition, parasite resistance to miltefosine seems to be easily induced. The most sophisticated treatment available for leishmaniasis consists in lipid-based formulations of amphotericin B. These include AmBisome® (a liposomal formulation), the most widely used; Amphocil™ (a colloidal dispersion); and Abelcet® (a lipid complex). All these formulations have minimized the negative side effects of the otherwise most effective free drug so far with the highest cure rate reported [108]. Although these lipid-based formulations require a shorter course of therapy (3–5 days), are highly effective, and exhibit lower toxicity when compared with the free drug, the cost of these formulations is a barrier to widespread use [109].

In spite of the battery of drugs available for treatment of leishmaniasis, there is no drug that can conjugate high efficacy, acceptable toxicity, at affordable prices. This panorama, and in the absence of an available efficient vaccine, opens room for the search of new drugs, and/or new therapeutics that can help combat this disease.

One proposal for the use of a new class of compounds as antileishmanial agents, formulated in nonconventional systems (NanoDDS) will be presented in chapter II of this thesis.

I-4.2 - Small Cell Lung Cancer

Lung cancer has become the leading cause of cancer-related death in men and women in the last decades [110], accounting for 1.3 million deaths worldwide annually, as of 2004 [111]. The most common symptoms are shortness of breath, coughing (including coughing up blood), and weight loss. A direct association between tobacco smoking and various type of lung cancer has been observed [112]. The vast majority of lung cancers are carcinomas; malignancies that arise from epithelial cells. These can be classified according to their histological type (i.e. size and appearance of the tumour cells). The two major histopathological groups of lung carcinoma are Small Cell Lung Cancer (SCLC), and Non-Small Cell Lung Cancer (NSCLC), the later accounts for about 80% of lung cancers and can be subdivided into three major subtypes; adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [110, 113].

SCLC is a neuroendocrine histological subtype of lung cancer that represents 15% of all pulmonary cancers [110]. Clinically, SCLC is particularly aggressive with a rapid doubling time, presenting an early and widespread metastatic behaviour as well as a rapid development of resistance to cytotoxic agents [114, 115]. Indeed, although a newly diagnosed SCLC is chemosensitive and radiosensitive with response rates of up to 80%, most patients relapse and eventually die from chemotherapy resistant disease. The overall five-year survival rate for SCLC is only 5% [115, 116].

Standard treatment of SCLC patients with limited stage disease (defined as a disease that is confined to one lung and nearby lymph nodes) consists of platinum-based combination chemotherapy, with early, concurrent thoracic irradiation [117]. Surgery can also be considered, but only when there is just one tumour that has not spread. Chemotherapy or radiation will be needed after surgery. In patients with extensive-stage disease (defined as a disease that has spread to both sides of the chest and with metastasis beyond the chest) or relapsed SCLC only combination chemotherapy and

radiation can be considered. The most commonly used chemotherapeutics drugs are cisplatin or carboplatin (DNA cross-linking agents) in combination with etoposide (topoisomerase II inhibitor) [118, 119]. Patients with extensive-disease frequently relapse, and relapsed/refractory SCLC has a poor prognosis (median survival time of 8-10 months) [117]. In relapse SCLC, several newer chemotherapeutic drugs have been investigated as second-line therapy and have shown modest efficacy. These agents include the topoisomerase I inhibitors, topotecan (approved by the FDA) and irinotecan; the taxanes, paclitaxel and docetaxel; the pyrimidine analogue, gemcitabine; the anthracycline antibiotic doxorubicin; and the vinca alkaloids, vinorelbine and vincristine. Combination chemotherapy of these agents has also been investigated [reviewed in 120].

New alternative therapeutic strategies have been focussed on the identification of potential tumour-specific molecular targets involved in the pathogenesis and proliferation of SCLC. New SCLC targeted therapies make use of new classes of drugs that block the growth of cancer cells by interfering with specific molecules needed for carcinogenesis and tumour growth. These consist of therapeutic antibodies or small molecules and have made treatment more tumour-specific and less toxic to normal cells [116]. The identification of the molecular targets that play a key role in cancer cell growth and survival is the basis for the development of targeted therapies. Proteins involved in cell signalling pathways that govern basic cellular functions such as cell division, cellular response to external stimuli and cell death are examples of such molecular targets. Blocking the signals that tell cancer cells to grow and divide uncontrollably, can help stop cancer progression and may induce cancer cell death. Other targeted therapies are focused in cancer cell death, either directly by the induction of apoptosis, or indirectly, stimulating the immune system to recognize and destroy cancer cells [121]. Targeted therapies are being studied to be used alone, or

in combination either with other targeted therapies, or with other cancer treatments, such as chemotherapy. Examples of new target agents such as angiogenesis inhibitors and regulators of apoptosis, currently undergoing pre-clinical and clinical evaluation for treatment of SCLC are reviewed by Abiddin *et al.* [122]. The FDA-approved targeted therapies are also available for consult [121].

In chapter III of this thesis will be presented one proposal for the development of a nonconventional formulation of an antisense oligonucleotide (asODN) against SCLC and their biological evaluation.

Chapter I - Reference List

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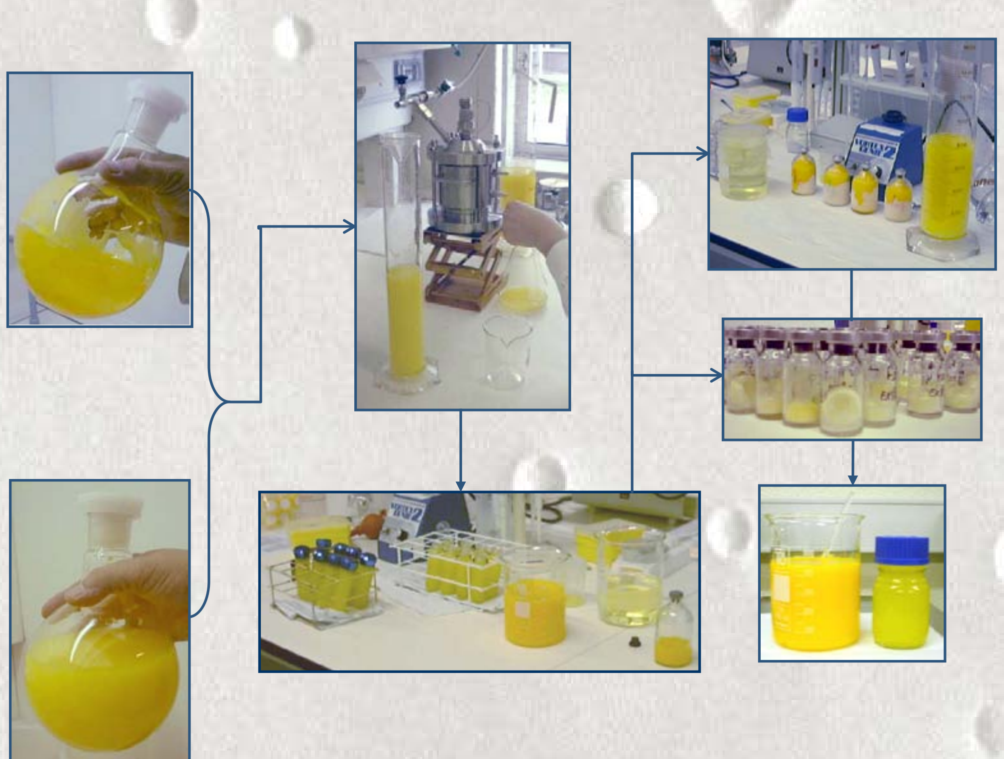
CHAPTER II

Dinitroanilines Liposomal Formulations for the Treatment of Leishmanial Infections



"Scale-up of TFL liposomes", Mundo Farmacêutico, Março - Abril 2006

II - Introduction

The World Health Organization (WHO) estimates that one sixth of the world's population is suffering from neglected tropical diseases (NTDs). The common denominator of all the NTDs is that they are invariably the diseases of the poorest in low-income countries [1, 2]. Malaria and leishmaniasis are the most prevalent neglected diseases caused by protozoan parasites. It is estimate that half of world's population is at risk of malaria while leishmaniasis threatens 350 million people around the world [1].

Leishmaniasis is the collective name of a broad spectrum of human and animal diseases. It represents an immense public health problem in large tropical and subtropical areas throughout the world. Currently, it is endemic in 88 countries on 4 continents. In Europe, the Mediterranean region belongs to the endemic areas of leishmaniasis and the disease is recently emerging in the United States [3, 4]. Leishmaniasis has a prevalence of at least 12 million infections with about 2 million new cases every year. An increased risk of infection among the immunosuppressed patients has been reported [3, 4, 5]. Some leishmaniasis is a widespread serious zoonotic disease with a great impact on public health. Leishmaniasis is caused by over 20 different species and subspecies of the protozoan parasite genus *Leishmania* that live as elongated (10-20 μm), flagellate forms called promastigotes in the gut of the sand fly vector and as round, non-motile aflagellated forms called amastigotes (3-7 μm in diameter) in the macrophages of mammalian hosts. The sand fly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host. The infected macrophages are ingested by the fly during the blood-meal and the amastigotes are released into the stomach of the insect. The amastigotes transform into the motile promastigote form that replicates actively. When the sand fly next feeds on a mammalian host, the leishmania promastigotes are transferred to the host

along with the saliva. Once in the host the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form and multiply leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages are infected, especially the spleen, liver and bone marrow [6, 7]. The Leishmania life cycle is shown in Figure II.1.

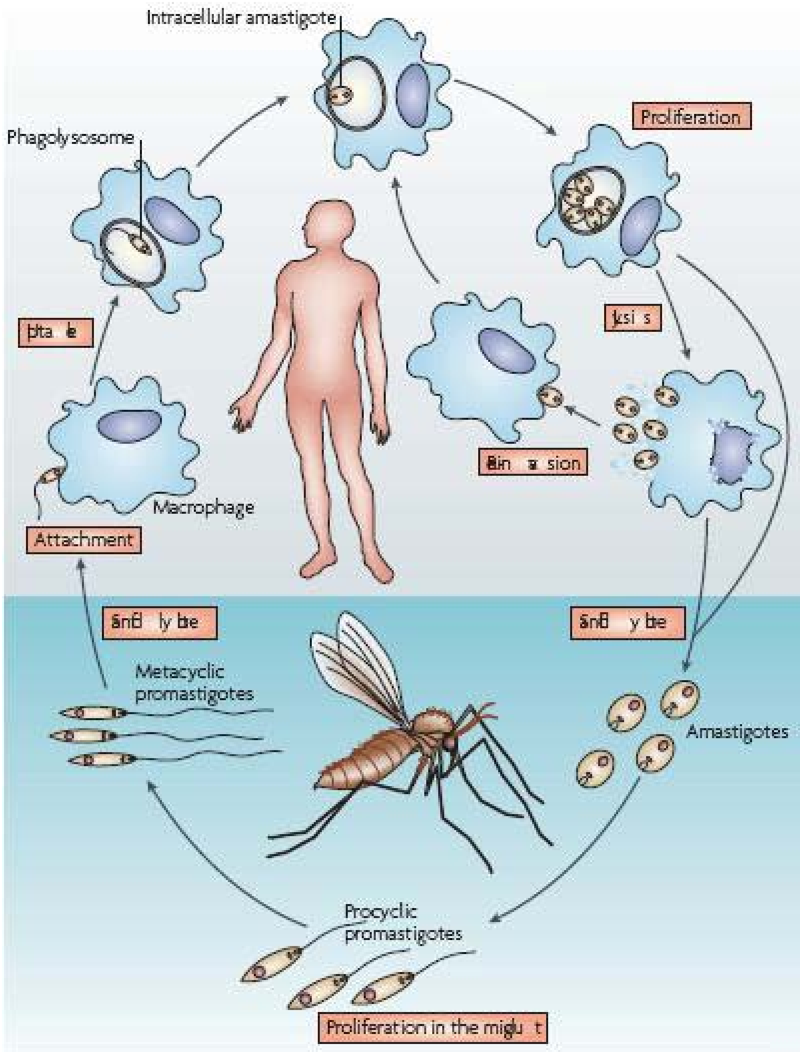


Figure II.1 - The *Leishmania* lifecycle. The promastigote form of Leishmania is transmitted into the skin by female sandflies. Then the parasites are internalized by macrophages and dendritic cells where they lose their flagella, transforming into the amastigote form. After multiplication, the amastigotes destroy the host cell and infect other phagocytic cells. The amastigotes disseminate through the lymphatic and vascular systems, eventually infiltrating the liver, spleen and the bone marrow. Adapted from Chappuis *et al.* [8].

Female sand flies of the genus *Phlebotomus sp* or *Lutzomyia sp* are responsible for the transmission to mammals, including humans [9]. The dog is considered the main peridomestic reservoir of the strains *Leishmania infantum / Leishmania chagasi* which are the causative agents of zoonotic visceral leishmaniasis in man [9, 10]. *Leishmania* infections can cause a broad spectrum of clinical outcomes, ranging from self-healing skin ulcers, to severe, life-threatening manifestations depending on the interactions between the species, the virulence of the infecting species and the host's immune response. According to its clinical manifestations leishmaniasis can be classified into: cutaneous leishmaniasis (CL); muco-cutaneous leishmaniasis (MCL) (also known as espundia) and visceral leishmaniasis (VL) (also known as kala-azar) [3, 8, 11, 12]. The CL is characterised by multiple ulcerative lesions that can result in disfiguring scars, creating a lifelong aesthetic stigma; MCL is a mutilating disease as it causes extensive destruction of the oral, nasal and pharyngeal cavities and VL is a severe, debilitating disease, characterized by intermittent fever, weight loss, massive hepatosplenomegaly, anaemia and progressive deterioration of the host; is usually fatal when untreated [9, 13].

Leishmaniasis was selected by the WHO for elimination by 2015, along with other neglected tropical diseases [14]. Since there is no approved anti-leishmanial vaccine in clinical use, control relies almost exclusively on chemotherapy. Several different classes of drugs have been used for the treatment of leishmaniasis. Pentavalent antimonials (Sb^V), administered intravenously in the form of either Sodium Stibogluconate or Meglumine Antimoniate, have been the first-line chemotherapy agents against leishmaniasis for more than 60 years and are currently still the mainstream treatment [5, 15, 16]. However, antimony treatments are accompanied by a combination of problems, including: variable efficacy, need of long course treatments and severe adverse reactions, such as cardiac and renal toxicity. These side

effects and the emergence of several antimony resistant strains are responsible for their progressive failure and discontinuation [3, 5, 11, 16, 17]. Second-line treatment includes amphotericin B deoxycholate (Fungizone®) and pentamidine, efficient drugs also limited by severe side effects and the need for parenteral administration. Originally developed as a systemic antifungal, Fungizone® is also an efficient anti-leishmanial drug, but has the major drawback of being acutely toxic and also requiring the need for prolonged hospitalization and close monitoring [5, 16, 18]. Pentamidine has been used as a second-line treatment for VL and CL for over 40 years [19]. In India it has shown promising activity in the treatment of relapsing VL after antimony treatment [17, 20]. Nevertheless the widespread use of this drug was always restricted due to low cure rates, considerable toxicity and the appearance of resistant strains. Miltefosine (hexadecylphosphocholine), a structural analogue of alkyl-lysophospholipids, originally developed as an anticancer drug, is the first efficient oral drug for the treatment of leishmaniasis. It was previously approved in India (2002) and recently in other countries [1, 11, 21, 22]. There is, however a general concern about miltefosine due to severe side effects, including teratogenicity and the easy appearance of resistant mutants [11, 17, 23, 24]. A number of other drugs, paromomycin, imiquimod and sitamaquine are currently at different stages of development as anti-leishmanial drugs [5, 16, 17]. With respect to paromomycin, recent Phase 3 clinical trials conducted in India proved an efficacy similar to amphotericin B, but higher occurrence of adverse reactions [16].

A strategy to improve the pharmaceutical efficacy and lessen the toxicity of some of the already existing anti-leishmanial drugs is the development of new, non-conventional formulations. These include the use of NanoDDS able to deliver the bioactive agents to the sites of infection and the use of nano-formulations as alternative to conventional ones.

Over the last decades, many attempts have been made to treat leishmaniasis with liposomal drugs [25, 26-30]. Despite the low encapsulation efficiencies that characterised some of the early liposomal formulations used for this purpose, they are claimed to be more efficient therapies than the conventional ones [26]. In general, liposomal formulations of anti-leishmanial agents have proved superiority, allowing the administration of considerably larger doses without revealing toxicity and reducing the dosing schedules [31, 32]. Among the drugs formulated in liposomes are amphotericin B; miltefosine and Sb^V compounds [29]. Of these, AmBisome®, a liposomal formulation of amphotericin B, is the safest and most successful formulation until now in curing human visceral leishmaniasis. It is considered the first choice treatment for patients who are unresponsive to antimonials [29]. AmBisome® can be administered at doses (7.5 mg/kg) much higher than amphotericin B free form (1.5 mg/kg) without signs of toxicity [33]. Nonetheless, the cost of such a treatment is currently too high hampering its use in the poor populations suffering from the disease [1, 5, 27, 34]. Antimonial compounds (meglumine antimoniate), efficiently encapsulated in liposomes, proved to be hundreds of times more effective than the free drug, while reducing its toxicity, in the treatment of canine leishmaniasis [28, 30, 35]. These formulations are, however, as the conventional drug formulations, limited by the existence of Sb^V-resistant strains [11, 36]. The latest therapeutic approaches in the treatment of leishmaniasis include the combination of Sb^V-drugs or amphotericin B with liposomes bearing stearylamine (SA), itself having leishmanicidal activity [32, 37, 38] or the evaluation of combination regimens of currently available drugs. A particular combination therapy regimen tested in India, consisting of a single-dose of liposomal AmphB (5 mg/kg) followed by short-course orally administered Miltefosine (50 mg capsules) presented cure rates > 90% [15, 39]. Although the results are very promising, the use of drugs with severe limitations will, again, confine the application of these therapies.

The aforementioned scenario points up that the current conventional chemotherapy for leishmaniasis, based on a handful of drugs with serious limitations such as toxicity and lack of efficacy in endemic areas, is far from satisfactory. A continuous search for new antileishmanial drugs and effective alternative approaches and administration strategies are thus urgently needed to reach the WHO goal for this disease.

Dinitroanilines, a known class of bioactive agents, are tubulin-binding agents that have been recognised and extensively used for their herbicidal properties [40]. More recently several dinitroanilines have also shown anti-leishmanial properties. Among those, trifluralin (TFL) and oryzalin (ORZ), are two well characterised and commercially available herbicides that bind to plant tubulins, have proved to be active against several protozoan parasites such as *Trypanosome*, *Toxoplasma* [41], *Cryptosporidium* [42] and *Leishmania* [43]. *Leishmania sp* tubulins have been shown to be very similar to plant tubulins. This similarity represents the basis of the anti-leishmanial activity of dinitroanilines, which have been shown to inhibit promastigote proliferation, reduce promastigote to amastigote transformation, interfere with amastigote replication and reduce amastigote infectivity [43]. An additional attractive feature of these agents is their lack of binding affinity to animal tubulins, meaning that they are not toxic to mammals [44]. *In vitro* studies confirmed TFL efficiency against several forms of leishmaniasis, while its *in vivo* topical administration, formulated as an ointment, was reported effective against *Leishmania major* and *Leishmania mexicana* in a murine model of cutaneous leishmaniasis [43]. However, after oral administration of 600 mg/kg TFL in mice, insignificant plasma concentrations were reported and this behaviour was related to the treatment failure against malaria [45]. Also the use of TFL by parenteral route has not been reported before probably due to its low water solubility (0.22 mg/L) and unusual low vapour pressure (0.61 mPa) [46]. With these features, and taking into account the *in vitro* (IC_{50} values on promastigotes

and amastigotes of *L. infantum* ranging from 20 to 34 µM) [47] and *in vivo* data (50-100 mg TFL/kg/day by oral route showed therapeutic activity against Chagas's disease) [48, 49], large volumes or solvents not compatible with parenteral administration would be needed to reach the therapeutic doses. Nonetheless, TFL and dinitroanilines in general seem good candidates for the treatment of leishmaniasis due to its specific mechanism of action and the consequently low toxicity in mammals [47, 49].

As referred above for conventional drugs, an alternative approach to circumvent the unfavourable physicochemical properties of these new anti-leishmanial agents is their optimization, through non-conventional approaches. These include their incorporation and stabilization in NanoDDS, such as liposomes, enabling the delivery of higher concentrations of the bioactive agent and providing sustained release and targeting to specific cells or organs [50].

The work presented in this Chapter is focused on two complementary strategies to overcome the difficulties in handling these agents, as well as to increase their therapeutic activity. The first strategy (included in Part A) consists in the association of TFL with liposomes and targeting them to the sites of infection. For this purpose several parameters that allow an efficient incorporation and stabilization of TFL in liposomes were assessed. Liposomal formulations of TFL were stabilized in several forms, such as in suspension, lyophilized and frozen form. TFL liposomes were reproducibly prepared in a larger scale that makes possible the preparation of the quantities required for tests in animal models. Furthermore, *in vivo* tests suggest that liposomal formulations of TFL have activity against *L. donovani* and *L. infantum* in mice and dogs models, respectively [51, 52].

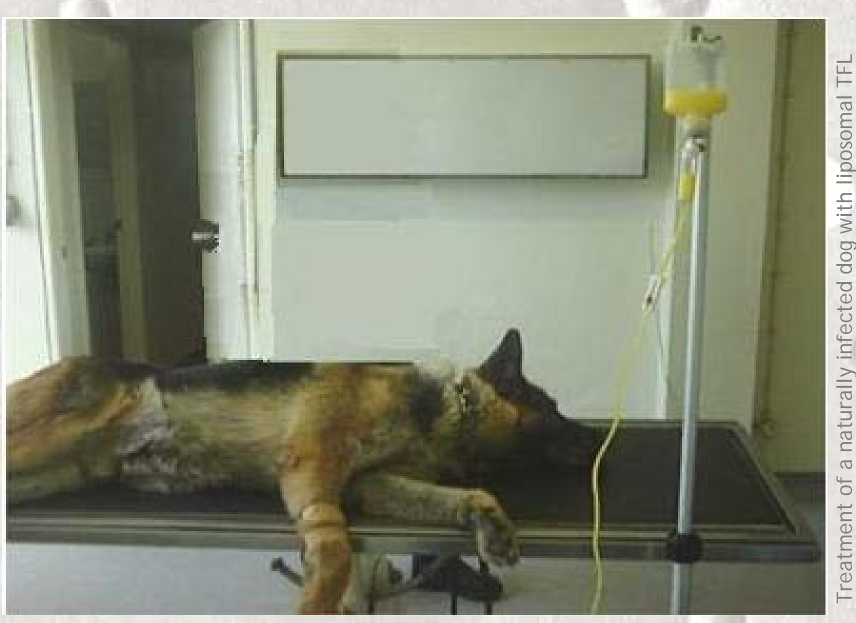
The second strategy (included in Part B) relies on the use of new chemical derivatives of TFL (TFL-D) with increased water or lipidic solubility as new improved

anti-leishmanial agents. Some synthesised TFL-D, in which the amine group was modified with different kind of substituents, showed enhanced *in vitro* anti-leishmanial activity either against the promastigote and/or the intracellular amastigote form [53]. Based on this screening two new compounds with potential anti-leishmanial activity were selected and incorporated in liposomes. Stable TFL-D liposomal formulations were obtained and evaluated *in vitro* and *in vivo* against *Leishmania* parasites. The results proved that not only liposomes can be appropriate solvents for TFL-D, but also that the correspondent liposomal formulations evidenced enhanced properties as antileishmanial drugs [54].

CHAPTER II

Part A

*Liposomal Formulations of Trifluralin active against Leishmania infections**



Treatment of a naturally infected dog with liposomal TFL

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Carvalheiro, M.C., Jorge, J., Eleutério, C., Pinhal, A.F., Sousa, A.C., Morais, J.G., Cruz, M.E.M. Trifluralin liposomal formulations active against *Leishmania donovani* infections. Eur. J. Pharm. Biopharm., 2009, 71, 292-296.

II-A.1 - Abstract

Leishmaniasis represents an immense public health problem affecting 12 million peoples. Trifluralin (TFL), a dinitroaniline, is an anti-microtubule herbicide that is commercially available and well characterized. It is also known to be active against several protozoan parasites such as *Leishmania*, *Trypanosoma*, *Toxoplasma* and *Cryptosporidium*. Its unfavourable physicochemical properties (low water solubility and sublimation) have prevented its systemic administration without the need of toxic solvents. In the studies presented here liposomes were developed for the incorporation of TFL allowing its application against *Leishmania* parasites. An efficient incorporation and stabilization of TFL in liposomal formulations was achieved after appropriate studies involving the lipid composition, presence of Chol, phase transition temperature of phospholipids, liposome surface charge and the presence of PEG-derivatized lipids. These lipid based formulations resulted in an appropriate solvent for TFL *in vivo* administration and together with a cryoprotectant they form a stabilized system for its maintenance/storage during a significant time after production. In addition, liposomal formulations containing TFL also acted as carrier systems able to transport the drug to the sites of infection. Using appropriate animal models we have demonstrated that all the tested liposomal TFL formulations are active against different strains of *Leishmania* in mice with significant reduction of the levels of visceral and cutaneous infections. Clinical tests with infected dogs also revealed promising results.

II-A.2 - Materials and Methods

II-A.2.1 - Lipids and Chemicals

Trifluralin was purchased from Riedel-de Haën (Germany). Egg phosphatidylcholine (PC), bovine liver phosphatidylinositol (PI), phosphatidylglycerol (PG), stearylamine (SA), dioleoylphosphatidylglycerol (DOPG), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Sigma (Spain). Hydrogenated phosphatidylcholine (HPC), dioleoylphosphatidylcholine (DOPC) and distearoylphosphatidylcholine (DSPC) were from Lipid Products (UK). Distearoylphosphatidylethanolamine-poly(ethyleneglycol) (2000) (DSPE-PEG) was purchased from Avanti Polar Lipids (USA). All lipids were used without further purification. Schneider's medium, penicillin-streptomycin and foetal calf serum (FCS) were from Sigma, (S. Louis, USA). Polycarbonate membranes were from Nuclepore (USA). Acetonitrile (HPLC grade) and trehalose di-hydrate were from Merck. All other reagents were analytical grade.

II-A.2.2 - *Leishmania* strains and animals

Three strains of *Leishmania* parasites were used in this study: *L. donovani* MHOM/ET/67/HU3 and *L. major* MHOM/SA/85/JISH118, both kindly provided by the London School of Hygiene & Tropical Medicine; and *L. infantum* MCAN/PT/03/IMT335 from Instituto de Higiene e Medicina Tropical (IHMT), Lisboa.

Amastigotes of *L. donovani* were continuously passed in golden hamsters (Mesocricetus auratus) obtained from Charles River-Criffa (Barcelona, Spain). 10^8 amastigotes were administered by intracardiac injection in golden hamsters under anaesthesia. Two to three months later, animal was sacrificed and the spleen removed. Smears were made for counting, the organ was homogenized and amastigotes isolated by centrifugation.

The cycle was repeated for maintenance of the infection or the amastigotes were used for therapeutic activity studies in animals.

L. major amastigotes were harvested from a nodular lesion removed from BALB/c female mice and allowed to transform to promastigotes in Schneider's medium with 10% heat-inactivated foetal calf serum (FCS) at 24 °C. They were grown in the same medium and subcutaneously injected in mice (10^7 promastigotes/mouse).

For therapeutic activity experiments BALB/c female mice, (6 to 8 weeks old; 20-25 g) were purchased from Gulbenkian Institute of Science (Oeiras, Portugal), and housed at INETI animal facilities. The animals were kept under standard hygiene conditions, fed with commercial chow and given acidified drinking water *ad libitum*. All *in vivo* experiments were conducted, under licence, according to the local laboratory animal committee guidelines and after approval from competent authorities (DGV).

Five healthy female beagle dogs, 3 years of age, were purchased from Estação Zootécnica Nacional, Santarém, (Portugal), housed at the kennels at the IHMT and maintained in compliance with European Union requirements (86/609/EEC), as recognised by Portuguese law (DR DL129/92 and Portaria 1005/92).

II-A.2.3 – Preparation of TFL liposomal formulations

Different phospholipid compositions were studied for the incorporation of TFL in liposomes. The formulations studied and respective molar ratios are summarized in Table II-A.3.1 and Table II-A.3.2. Liposomes were prepared by the thin lipid film-extrusion method. The lipidic components at 10 µmol/mL and TFL at 335 - 450 µg/mL corresponding to a TFL:lipid molar ratio of 1:10 or 1.3:10 respectively, were added to a round bottom flask of 25 mL, dissolved in a small volume of chloroform and dried under vacuum in a rotavapor, with controlled temperature above the lipids phase transition temperature (T_C). The temperature

used was 25 ºC for all formulations, except for those containing DPPC and DSPC where the temperature was 45 ºC and 58 ºC, respectively. Nitrogen was blown over the dried lipid film for about 5 min in order to remove traces of the organic solvents. The lipid film was subsequently hydrated in three steps with glass beads forming MLV. In a typical 2 mL formulation the first hydration step was done using 200 µL (1/10 of the final volume) of 154 mM NaCl or a sugar solution while keeping the osmolarity at 300 mOsm. The round bottom flask was subjected to mechanical and manual external stirring until all the film around the walls was hydrated. The suspension was allowed to stand for at least 15 min. This step was repeated using 200 µL of a 154 mM NaCl solution or of a sugar solution. The hydration was completed with 1600 µL (8/10 of the final volume) of 154 mM NaCl or a sugar solution. This liposomal suspension (MLV) was down-sized by successive extrusions through progressively smaller pore size (ranging from 1 µm down to 0.1 µm) polycarbonate membranes (∅ = 25 mm) in a 10 mL extrusion barrel (Lipex Biomembranes Extruder, Canada). Non-incorporated TFL and potential TFL/liposome aggregates or precipitates were removed from the liposome dispersion by centrifugation at 5000 x g for 10 min (Sigma 202MK laboratory centrifuge). Following centrifugation, the supernatant, containing TFL liposomes was assayed for TFL and lipid contents, vesicles size and zeta potential.

II-A.2.3.1 - For scale-up studies

For large laboratory scale preparations (LLS), the initial volume used was 600 mL. The lipidic components (6 mmol/600 mL total lipid, corresponding to 10µmol/mL) and TFL (1,05 mg/600 mL, corresponding to 335 µg/mL) in a molar ratio of 1:10 (TFL:lipid), were weighed and added to a round bottom flask of 1000 mL solubilised in a small volume of chloroform and dried under vacuum in a 1 L capacity rotavapor. The dry lipidic film containing TFL was kept overnight at 4º C, followed by the removal of the

residual organic solvent by a stream of N2. The lipidic film containing TFL was hydrated, to a final volume of 600 mL, using the same three steps procedure described. The liposomal suspension was down-sized by successive extrusions through progressively smaller pore size (ranging from 1 µm down to 0.1 µm) polycarbonate membranes (∅ = 47 mm) in a 100 mL extrusion barrel. The removal of non-incorporated material and liposome characterization was performed as above.

II-A.2.3.2 – For animal studies

For the liposomal formulations used in animal studies, the lipidic film was hydrated using a 300 mM trehalose solution, in the three steps of the procedure. The formulations used in the visceral animal model were extruded until 0.2 µm pore diameter filters while the formulations used in the cutaneous animal model were extruded until 0.05 µm pore diameter filters. After the removal of non-incorporated TFL, the obtained liposomal suspensions were concentrated by ultra-centrifugation at 180,000 x g for 1 h at 20 ºC (L8-60 M ultracentrifuge, Beckman Instruments, U.S.A.) and the pellet ressuspended in an appropriate volume of a 300 mM trehalose solution. The resulting suspension, containing TFL liposomes, was assayed as described above.

II-A.2.4 – Characterization of TFL liposomal formulations

Liposomal formulations were characterized for phospholipids and TFL concentration, mean particle size and zeta potential. The following incorporation parameters were determined: Trifluralin Incorporation Efficiency, loading capacity, Lipid and Trifluralin retention. Abbreviations and equations used to determine the incorporation parameters are as follows:

Table II-A.2.1 - Abbreviations and equations of TFL incorporation parameters

Trifluralin	TFL
Total Lipid	Lip
Initial TFL to Lip ratio (g/mol)	$[TFL/Lip]_i$
Loading capacity (L.C.) (g/mol)	$[TFL/Lip]_f$
TFL retention (%)	$([TFL]_f/[TFL]_i) \times 100$
Lipid retention (%)	$([Lip]_f/[Lip]_i) \times 100$
Incorporation Efficiency (I.E.) (%)	$([TFL/Lip]_f)/([TFL/Lip]_i) \times 100$

TFL retention after centrifugation reflects the yield of the drug in liposomes during the process (incorporation and stabilization). I.E. is a measure of the efficiency of the initial system (TFL + lipid) to incorporate the TFL in the final liposomal form.

II-A.2.4.1 - TFL quantification

TFL quantification was performed by HPLC. The HPLC system used consisted of a System Gold Nouveau, Beckman with a 126 Pump Direct Control and an auto sampler Midas, type 830 with a 20 µL sample loop. An analytical column, Bio-Sil C18 HL-90-5 (150 x 4.6 mm) from Bio-Rad was used. The instrumental settings were: flow rate 1 mL / min; column temperature 25º C and detection at 220 nm in a diode-array Detector Module model 168. The mobile phase consisted of 0.02 M sodium acetate (pH 6.55): acetonitrile gradient from 46 to 50 % of acetonitrile in 5 min and 50 to 80 % of acetonitrile in 15 min.

II-A.2.4.2 - Total Lipid quantification

Phospholipid determinations were performed using the colorimetric method described by Rouser and co-workers based on quantification of inorganic phosphorous [55]. The inorganic phosphorous is first released from phospholipids by hydrolysis with perchloric acid (in a dry bath at 180 ºC). Then, in the presence of ammonium molybdate, the inorganic phosphorous is converted into phosphomolybdic acid and reduced to a blue complex with ascorbic acid (in a water bath at 100 ºC). The blue colour was quantified by spectroscopy at 797 nm.

II-A.2.4.3 - Mean particle size and zeta potential determinations

Mean particle size (∅) was determined by photon correlation spectroscopy (PCS) at 25 ºC with a Malvern Zetasizer 3 (Malvern Instruments, Malvern, UK) using a 25 mW He-Ne laser under an angle of 90°. As a measure of particle size distribution of the dispersion, the system reports the polidispersity index (P.I.) ranging from 0.0 for an entirely monodisperse sample up to 1.0 for a polydisperse suspension. All samples were diluted to an adequate scattering intensity prior to measurement.

The liposome surface charge properties were evaluated by measuring the zeta potential by Laser Doppler Anemometry, using a Malvern Zetasizer 3 (Malvern Instruments, Malvern, UK). The scattering angle was 12º and the electric field intensity ranged from 18.5 to 19.6 V/cm. The zeta potential is measured by applying an electric field across the dispersion. Particles within the dispersion will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. This velocity is measured using the technique of laser Doppler anemometry. The frequency shift or phase shift of an incident laser beam caused by these moving particles is measured as the particle mobility, and this mobility is

converted to the zeta potential by inputting the dispersant viscosity, and the application of the Smoluchowski theories.

II-A.2.5 – Stability evaluation of TFL liposomal formulations

The stability experiments for TFL formulations were performed in different conditions: freshly made at 4º C in saline, freeze-dried and frozen at low temperatures. The stability of the formulations was characterized by evaluating TFL and total lipid retention after separation of destabilised material by centrifugation, as described above, and also by variations in mean particle size.

II-A.2.5.1 – Stability of TFL liposomes in suspension

For stability studies at 4º C, freshly made liposomes were prepared as described in section II-A.2.3 using 154 mM NaCl as final hydration medium. At selected time points samples were taken and centrifuged at 5000 x g for 10 min (Sigma 202MK laboratory centrifuge) to remove lipid aggregates and non-incorporated TFL that crystallizes and precipitates. The supernatant containing TFL liposomes was analysed.

II-A.2.5.2 – Stability of TFL liposomes as freeze-dried cakes

The stability of freeze-dried liposomes was done using different hydration media, with or without the use of cryoprotectants/lyoprotectants. The lipidic film was hydrated as described in liposome preparation (II-A.2.3). Ultra-centrifuged liposomal pellets were suspended either in 154 mM NaCl or in various 300 mM sugar solutions (trehalose, glucose, sucrose). The liposomal suspension was divided in 1 mL aliquots in 10 mL lyophilisation glass vials and frozen at -70 ºC for, at least, 30 min. The freeze-drying process took place overnight (12 – 18 h) at a pressure of about 10 Pa and at an initial

temperature of -40 ºC. Vials were sealed under vacuum and stored at room temperature. At selected time points, vials were opened and reconstituted with water. After rejection of non-incorporated material, as described above, TFL and phospholipid recovery and variations in mean particle size were determined to evaluate stability. After reconstitution of lyophilized TFL liposomes the resulting suspensions were then used to assess the stability of reconstituted freeze-dried formulations kept at 4 ºC for a short period. These samples were treated as described above for freshly made formulations.

II-A.2.5.3 – Stability of frozen TFL liposomes

Stability studies at low temperatures were performed with formulations kept at -70 ºC for at least 24 h. The ultra-centrifuged liposomal pellets were suspended in 154 mM NaCl, divided in 1 mL aliquots in 10 mL glass vials and frozen. At selected time points, samples were thawed to room temperature and homogenized. After removal of the non-incorporated material, the supernatant was analyzed for TFL and lipid contents and particle size was also measured.

II-A.2.6 – *In vivo* studies in animal models of infection

II-A.2.6.1- Visceral murine model of infection

A total of $2x10^7$ amastigotes were used to infect (i.v.) female BALB/c mice via the tail vein. At one week post-infection (day 7), animals were randomly sorted into groups of five and submitted to dosing.

In a first series of experiments, groups of mice received either free TFL or three different formulations of liposomal TFL at 15 mg TFL/kg body weight/day by i.v. route for 5 consecutive days (at days 7, 8, 9, 10, 11). Another group of mice received 1 dose

of meglumine antimoniate (Glucantime®) (s.c.) at 15 mg Sb^{V}/kg at day 8, as a positive control. The negative control group received 5 doses of 300 mM trehalose by i.v. route.

In a second set of experiments, each group of infected mice received liposomal TFL at 2, 5 or 10 mg TFL/kg/dose i.v single dose (day 7), 3 doses (at day 7, 9 and 11) or 5 doses (days 7, 8, 9, 10 and 11) regimens. Liposomal TFL was concentrated up to 10 times, in such a way that the required dose could be administered in 200 μL.

For all the experiments, at day 15 post-infection, animals were sacrificed, the liver removed and smears were made. Smears were fixed with methanol and stained with Giemsa. Parasite numbers were determined by counting the number of amastigotes per 500 nuclei on each smear. The parasite load was calculated according to Stauber's formula: number of amastigotes per 500 nucleated liver cells times the organ weight (in milligrams) [56]. The suppression of parasite growth was calculated as the percentage inhibition relative to parasite load of negative control animals (animals injected with 300 mM trehalose).

II-A.2.6.2- Cutaneous murine model of infection

Female BALB/c mice (6 to 8 week-old) were infected with 10^7 *L. major* (MHOM/SA/85/JISH118) first-passage, stationary-phase promastigotes, subcutaneously at the base of the tail. At 7 days post-infection, lesions were measured in two dimensions and animals were randomly sorted into groups of five and submitted to dosing.

In the first series of experiments, 7 days post-infection, mice with established lesions were treated with two different TFL liposomal formulations, at a dose of 6 mg TFL/kg of body weight, administered once a day for 6 days either by i.v., i.p. or s.c. (close to the lesion) routes of administration. Positive control groups received the same 6 doses

of Glucantime® (400 mg Sb^V/kg) either by i.v., i.p. or s.c. route and the negative control mice received 6 doses of 300 mM trehalose by i.v. route (100 µL/dose).

In a second series of experiments, 7 days post-infection, groups of mice received 10 doses of TFL liposomes at a concentration of 10 mg TFL/kg of body weight, once a day for 10 days, either by i.v., i.p. or s.c. (close to the lesion) routes of administration. Mice of the negative control group received 300 mM trehalose every day for 10 days (100 µL/dose).

The evaluation of the antiparasitic effect of the formulations was assessed at days 14 and 21 post-infection, by changes in the lesion size. The lesions were measured in two dimensions and the percentage increase/decrease of lesion size where calculated according to the following formula:

$$\left(\frac{D_f - D_i}{D_i}\right) \times 100 \ (\%)$$

where D_i and D_f are the measurements of the mean lesion diameters immediately before the first treatment (D_i), and 7 or 14 days (D_f) after the beginning of treatment, respectively.

II-A.2.6.3- Experimental canine model of infection

The canine model used was described elsewhere [52]. Briefly, dogs were infected (i.v.) with 1×10^6 amastigotes/kg (*L. infantum*: MCAN/PT/03/IMT335). Six months later animals were treated with a daily dose of 10 mg/kg of liposomal TFL during ten days. Five months after infection and 1 and 3 months after treatment, bone marrow and popliteal lymph node aspirates were collected and used for quantification of viable parasites using the limiting dilution assay [57].

II-A.2.7 – Statistical Analysis

Data presented is expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), as mentioned in the legends of figures and tables. Statistical analysis was performed using ANOVA single factor. The acceptable probability for a significant difference between mean values was $p<0.05$.

II-A.3 - Results

The incorporation of TFL in liposomes is proposed not only as the inclusion of a hydrophobic bioactive agent in a lipid matrix to increase its pharmacological properties, but also as a solvent stabilizing system. The TFL liposomal drug delivery system is expected to deliver TFL to the targets of *Leishmania* infection.

II-A.3.1 – Development of trifluralin liposomal formulations.

II-A.3.1.1 – Effect of lipid composition on the incorporation of TFL in liposomes

The incorporation of TFL in liposomes was studied as a function of lipid composition. As a first approach the properties of the liposomes were modulated by the inclusion of positively and negatively charged lipids, by varying the Chol content and by shifting the membrane fluidity using phospholipids with different phase transition temperatures (T_c). The incorporation parameters of the resulting TFL liposomal formulations were systematically determined and are presented in Table II-A.3.1.

Table II-A.3.1 - Incorporation parameters for TFL liposomal formulations.

	Phospholipid Composition (molar ratio)	$^*T_c$ (ºC)	$[TFL]_i/[Lip]_i$ (g/mol)	L.C. (g/mol)	TFL retention (%)	LIP retention (%)	I.E. (%)	Zeta Potential (mV)
F_1	PC	<0	30 ± 4	2 ± 1	5 ± 1	68 ± 5	8 ± 1	-10 ± 2
F_2	PC:Chol (4:1)	<0	30 ± 1	19 ± 1	37 ± 1	59 ± 1	62 ± 3	-12 ± 4
F_3	PC:Chol (2:1)	<0	29 ± 6	9 ± 3	23 ± 16	62 ± 17	35 ± 17	-10 ± 6
F_4	DMPC	23	36 ± 5	4 ± 1	9 ± 4	81 ± 9	11 ± 1	-7 ± 3
F_5	DMPC:Chol (4:1)	23	32 ± 2	7 ± 1	13 ± 6	58± 14	22 ± 5	-9 ± 2
F_6	DMPC:Chol (2:1)	23	30 ± 6	7 ± 4	13 ± 6	64 ± 3	21 ± 9	-6 ± 4
F_7	DPPC:Chol (4:1)	42	35 ± 2	9 ± 5	17 ± 10	69 ± 14	25 ± 14	n.d.
F_8	DPPC:Chol (2:1)	42	35 ± 2	0	0	70 ± 12	0	n.d.
F_9	DSPC:Chol (4:1)	55	30 ± 5	11 ± 3	26 ± 6	70 ± 9	38 ± 9	n.d.
F_{10}	DSPC:Chol (2:1)	55	29 ± 5	12 ± 4	24 ± 5	61 ± 15	44 ± 22	n.d.
F_{11}	PC:Chol:SA (10:5:1)	<0	28 ± 2	8 ± 7	14 ± 8	58 ± 27	27 ± 20	20 ± 2
F_{12}	PC:Chol:PI (10:5:1)	<0	28 ± 3	13 ± 3	32 ± 17	63 ± 13	49 ± 17	-19± 5
F_{13}	PC:Chol:PG (10:5:1)	<0	26 ± 3	12 ± 5	33 ± 11	73 ± 12	47 ± 21	-22 ± 4

Liposomes were extruded until a mean diameter (mean vesicle size) of 100 to 120 nm was achieved with a P.I. < 0.20.
TFL concentration before extrusion [TFL]i = 331 – 398 µg/mL (0.98 – 1.19 µmol/mL);
Lip concentration before extrusion [Lip]i = 9,03 - 13,5 µmol/mL (6.8 - 10.1 mg/mL).
*Tc = Phase Transition Temperature of the major phospholipid in the mixture according to Uster et al. [58]
The data shown is an average ± SD from, at least, three independent experiments.

The incorporation parameters (L.C. and I.E.) were found to be highly dependent on the lipid composition used. The average L.C. values diverge between 0 and 13 g/mol, corresponding to I.E. values below 50 % in 12 out of the 13 formulations studied. The liposomal formulations made from fluid lipids and Chol (F_2, F_{12} and F_{13}) were found to have the highest incorporation parameters. In contrast, low TFL incorporation parameters were obtained for liposomes composed of pure lipids, PC or DMPC, (F_1 and F_4). However, acceptable Lip retention values, 68 and 81%, have been observed for both formulations made from PC and DMPC, respectively.

By varying the fluidity of the liposomal membrane either by the inclusion of Chol in the lipidic bilayer or by using phospholipids with different T_c, it was observed that both the T_c of the phospholipids and the Chol content affects the incorporation of TFL into liposomes. For formulations F_1 to F_{10} the L.C. was significantly higher ($p<0.05$) in liposomes with a fluid membrane (T_c of the major phospholipid below room temperature, ± 20º C) and a low Chol content ($F_{2,}$ corresponding to 20 mol %) than in liposomes with a fluid membrane at a higher Chol content ($F_{3,}$ corresponding to 33 mol%) or in liposomes with a rigid membrane at both Chol contents (F_5 to F_{10}).

Lipidic mixtures containing PC, Chol and either positively or negatively charged lipids or molecules (PI, PG and SA) were used to study the effect of liposomes surface charge on the incorporation of TFL. No significant differences in incorporation parameters were observed when comparing neutral and charged formulations with similar Chol content (F_3 versus F_{11} to F_{12}).

Lipid mixtures containing PG were evaluated taking two facts into consideration: the results showing that PG promotes acceptable incorporation (F_{13}) and the fact that this negatively charged lipid has been described to target organs and cells of the MPS where *Leishmania* parasites reside [59]. In addition, liposomes prepared with mixtures containing PEG-derivatized lipids (DSPE-PEG) were also evaluated. These

PEG-formulations were tested to determine the influence of prolonged circulation of liposomes in the blood stream on the delivery of the bioactive agent to other reservoirs of the parasites, such as bone marrow and skin [60]. Data is shown in Table II-A.3.2.

Liposomes with a fluid membrane containing 30 % (mol/mol) of PG (F_{14}, F_{15}) showed acceptable TFL incorporation parameters: L.C. higher than 20 g/mol and I.E. around 85%. These values were consistently reduced when the incorporation was performed in liposomes with rigid membrane and prepared with the same PC and PG molar ratios (F_{16} versus F_{14} and F_{15}) ($p<0.01$). When DSPE-PEG was added to lipid mixtures based on PC with or without PG (F_{17}, F_{19}) the incorporation parameters of the resulting liposomes were comparable with those of liposomes with fluid membranes without PEG (F_{14}) ($p>0.05$). Although evidencing similar L.C., the formulation composed of PG and DSPE-PEG (F_{18}) presented smaller TFL and lipid retentions. For similar $[TFL/Lip]_i$ (26-34 g/mol), formulations with up to 27 µg/µmol were obtained (F_{17} - F_{19}), corresponding to a typical preparation of 280 µg TFL/mL. The increase on $[TFL/Lip]_i$ to around 52 µg/µmol (F_{20} to F_{22}), significantly increased the L.C. in the PG based formulations (F_{21} *versus* F_{17} and F_{22} *versus* F_{18}). A concomitant reduction was observed in the TFL and Lip retentions and in the I.E. parameter, indicating that saturation was not yet reached. The inclusion of Chol in one of these mixtures reduced by 50% the incorporation parameters including the L.C. (F_{23} *versus* F_{22}). The zeta potential of TFL formulations containing PG was about -40 mV. The zeta potential of the formulations containing DSPE-PEG was around neutrality due to the shielding effect of PEG, meaning that TFL does not interfere with the liposomal charge.

Table II-A.3.2 - Incorporation parameters of various TFL formulations composed of PG and/or PC with or without DSPE-PEG$_{2000}$.

	Phospholipid Composition (molar ratio)	$[TFL]_i/[Lip]_i$ (g/mol)	L.C. (g/mol)	TFL retention (%)	LIP retention (%)	I.E. (%)	Zeta Potential (mV)
F_{14}	PC:PG (7:3)	34 ± 2	27 ± 1	66 ± 8	81 ± 4	80 ± 6	-38 ± 2
F_{15}	DOPC:DOPG (7:3)	31 ± 3	24 ± 4	71 ± 12	52 ± 18	81 ± 12	-35 ± 2
F_{16}	DPPC:DPPG (7:3)	35 ± 2	9 ± 1	11 ± 4	44 ± 20	25 ± 3	-35 ± 2
F_{17}	PC:PG:DSPE-PEG (12:3:1)	28 ± 5	23 ± 3	71 ± 14	85 ± 8	84 ± 17	-5 ± 3
F_{18}	PG:DSPE-PEG (15:1)	27 ± 1	23 ± 1	56 ± 1	65 ± 1	86 ± 2	-5 ± 1
F_{19}	PC:DSPE-PEG (15:1)	26 ± 6	23 ± 6	74 ± 12	84 ± 8	88 ± 9	-5 ± 2
F_{20}	PC:PG:DSPE-PEG (12:3:1)	53 ± 2*	28 ± 1	41 ± 1	77 ±2	53 ± 1	-5 ± 3
F_{21}	PG:DSPE-PEG (15:1)	51 ± 4*	39 ± 8	43 ± 6	56 ± 1	76 ± 7	-6 ± 2
F_{22}	PC:DSPE-PEG (15:1)	53 ± 1*	27 ± 1	40 ± 1	78 ± 2	50 ± 2	-5 ± 1
F_{23}	PC:Chol:DSPE-PEG (12:3:1)	54 ± 2*	15 ± 1	19 ± 1	68 ± 1	27 ± 1	-4 ± 1

Liposomes were extruded until a mean diameter (mean vesicle size) of 100 to 120 nm was achieved with a P.I. < 0.20.
[TFL]i = (335 µg/mL) or *(450 µg/mL); [Lip]i = 9.03 - 13.5 µmol/mL (6.8 - 10.1 mg/mL)
The data shown is an average ± SD from, at least, three independent experiments.

The purpose of the lipid composition studies was to obtain one or more formulations with high intraliposomal TFL concentration and high TFL retention. Based on that, the first selection of TFL liposomal formulations for further studies was F_2, F_7, F_9, F_{14}, F_{15} and F_{19}. All the liposomal formulations used hereinafter were prepared with an $[TFL/Lip]_i$ of 1:10 mol:mol (33.5 g/mol). Under these conditions the liposomal membrane is not yet saturated nevertheless this is the best ratio as it maximizes the incorporation parameters I.E. and the L.C.

II-A.3.1.2 – Stability of TFL liposomal formulations under different storage conditions

The physical stability of TFL liposomal formulations was studied under different conditions: as a liposomal suspension, in the presence of a saline solution; as a freeze-dried powder in the presence of sugars; and as a frozen suspension, at low temperature. To evaluate the stability of liposomes, the L.C., TFL retention in liposomal form and mean vesicle size variation were studied for all storage conditions.

II-A.3.1.2.1 - In suspension

The short-term stability of several TFL liposomal suspensions was assessed. The formulations chosen for this study were: one formulation made with fluid lipid and one formulation made with rigid lipid both with Chol (F_2 and F_9 respectively); two fluid formulations containing PG (F_{14} and F_{15}) and one with PEG (F_{19}). The assayed formulations were freshly made, stored in saline at 4° C during 11 days and analysed for stability. Data is shown in Figure II-A.3.1.

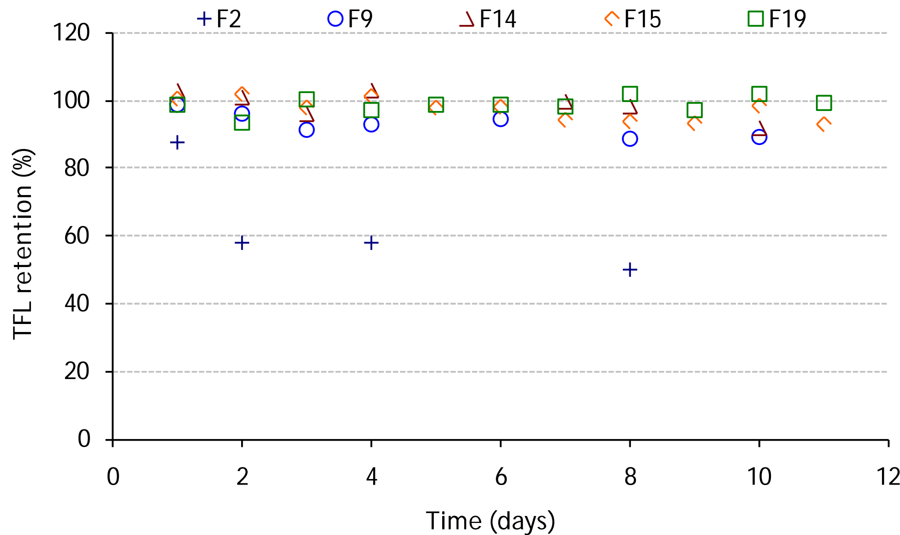

Figure II-A.3.1 - Stability of TFL liposomal formulations as suspensions. TFL retention for conventional and long circulating liposomes maintained at 4 ºC during 11 days. The composition of the formulations and respective denomination is as in Table II-A.3.1 and Table II-A.3.2. $[TFL/Lip]_i$ ranges between 26 and 34 µg/µmol. The lipidic film hydrations were done with 154 mM NaCl. The diameter of these formulations was 150 ± 15 nm. The data points represent the average ± S.D. of three independent experiments.

The most stable formulations, for which a 11 days storage at 4 ºC resulted in a minimal loss in TFL after centrifugation are conventional liposomes composed of fluid lipids without Chol (F_{14} and F_{15}) and long-circulating liposomes without Chol (F_{19}). These formulations presented less than 5% TFL loss while formulations prepared with rigid lipids and Chol (F_9) showed around 13% TFL loss. Liposomes made with fluid lipids and Chol (F_2) were much less stable, presenting a substantially greater loss with only 58 % of TFL retention after only 2 days in suspension.

II-A.3.1.2.2 - Freeze-dried

Due to its high hydrophobicity and low water solubility, TFL is expected to distribute in the lipid bilayer of the liposomes and to remain there during the process of freeze-drying, as long as appropriate conditions are used [61]. Furthermore, TFL tendency to

sublimate [46] makes the stabilization of this drug in the lipid bilayer critical and probably difficult to achieve without cryoprotection.

The protective effect of two different saccharides (a mono and a disaccharide) was investigated during freeze-drying procedure of TFL liposomes. After re-hydration of the freeze-dried formulation with water, the impact caused by the presence of sugars was evaluated through TFL retention, as well as liposome mean vesicle size and zeta potential variations as shown in Figure II-A.3.2. The TFL retention in reconstituted liposomes was only possible if this process was done in the presence of sugars, with no significant differences between the use of glucose or trehalose, in any of the studied parameters. No such sugar dependency was found for the lipid content as for all the conditions used more than 92% of total lipid was present after liposomes reconstitution. However, when liposomes were freeze-dried in water only, TFL retention was null and the mean vesicle size of the vesicles changed dramatically with increases of almost 120% in diameter. In the presence of sugars, a small reduction in size (15 - 18%) was observed after reconstitution, with no significant differences between trehalose and glucose. Changes in zeta potential of 15 to 25% were also observed. These changes, in mean vesicle size and in zeta potential, are not expected to affect the *in vivo* behaviour of liposomes.

Trehalose was selected as the cryoprotectant agent for TFL liposomes. Although no significant differences were found between this sugar and glucose, the lyophilisation cake obtained was more homogeneous and easier to hydrate.

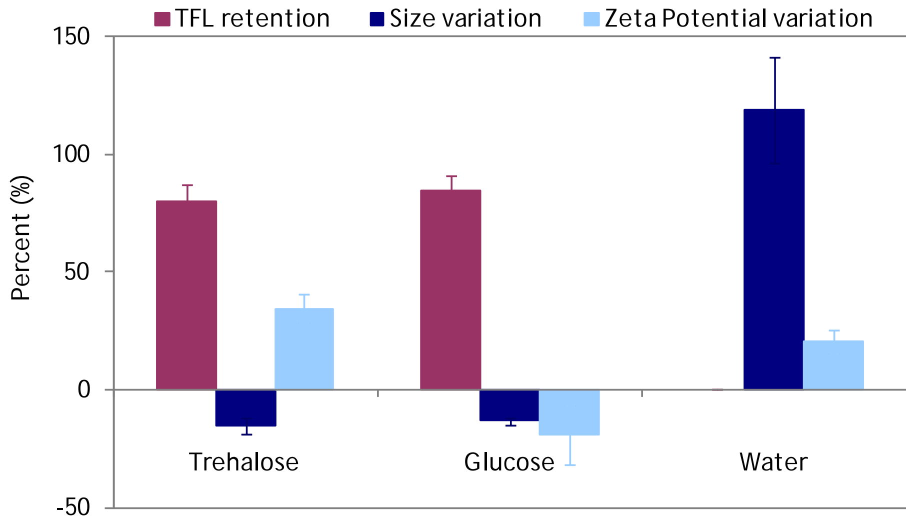

Figure II-A.3.2 - Effect of sugars on TFL retention, mean vesicle size and zeta potential variation in freeze-dried TFL liposomes. Lipidic films composed of DOPC:DOPG (7:3) and containing TFL were hydrated with a 300 mM solution of trehalose or glucose or with deionised water in order to afford a suspension containing 10 µmol/mL total lipid and 1 µmol/mL of TFL. TFL retention (%) is the ratio between TFL before and after lyophilisation. Mean vesicle size and zeta potential variation is the increase and/or decrease of mean vesicle size and zeta potential before and after lyophilisation as percentage of the respective values before lyophilisation. Initial mean vesicle size were 253 ± 5 nm for trehalose, 216 ± 7 nm for glucose and 227 ± 8 nm for water (P.I. < 0.1). Initial zeta potential values were -42 ± 4 mV for trehalose, -50 ± 3 mV for both glucose and water. The data represents the average ± S.D. of three independent experiments.

Another set of experiments intended to clarify if the localization of trehalose either in the inner or the outer medium of liposomes affects TFL retention and other characteristics of liposomes such as size. Two of the selected formulations (A.3.1.1) were prepared in such a way that the lipid film containing TFL was hydrated either with NaCl (154 mM) or with 5% (w/v) or 10% (w/v) trehalose solutions, thus becoming the inner medium (internal aqueous space) of liposomes. The liposomal pellet was suspended either in 154 mM NaCl or in a 10% (w/v) trehalose solution, thus being this the composition of the outer medium. All liposomal formulations were freeze-dried in 1 mL aliquots and reconstituted with 1 mL of water. The osmolarity was always kept constant at 300 mOsm, either with trehalose or NaCl or both. Table II-A.3.3 shows

that, TFL recovery in freeze-dried liposomes was not substantially affected by the presence of trehalose in the inner space (hydration medium). However its presence on the outer space (suspension medium) is crucial for TFL retention and for the preservation of liposome size and surface properties. When TFL liposomes were freeze-dried in the absence of trehalose there was no TFL recovered after rehydration, which could be explained by drug sublimation and/or spun-down drug aggregates or precipitates. When trehalose was present only in the inner space, TFL recovery was null or very small (up to 8%). However, if trehalose was present in the outer space before freeze-drying, TFL recovery in lyophilized/rehydrated liposomes varied from 80% to 96%. This finding was independent from either the presence or the concentration of trehalose in the inner space and from the lipid compositions studied (F_{14} or F_{15}). The size of liposomes was also preserved by the presence of trehalose before freeze-drying. In the absence of trehalose in the outer medium, liposomes increased their sizes up to 155% of the initial value. Small reductions (up to 14%) were observed when liposomes were freeze-dried in the presence of trehalose in the outer medium.

In this case, sizes were reduced by an average of 18 to 20 nm corresponding to variations smaller than 14% irrespectively of the lipid composition and of the presence of trehalose in the inner space of liposomes.

To better understand this behaviour, TFL recovery was also analysed after the ultra-centrifugation used to change the suspension medium before lyophilisation. At this step TFL recovery was not affected by the presence or the concentration of trehalose (data not shown).

Table II-A.3.3 - Evaluation of the protective effect of trehalose on TFL recovery and size variation in freeze-dried liposomes.

Formulation	Hydration Medium	TFL retention (%)		Mean vesicle size variation (%)	
		Suspension medium		Suspension medium	
		NaCl	Trehalose	NaCl	Trehalose
F_{14}	0%	0	96±4	86±8	-3±10
	5%	0	95±3	155±63	-10±1
	10%	8±1	89±10	123±65	-13±1
F_{15}	0%	0	90±7	50±13	-12±4
	5%	0	94±1	38±45	-14±3
	10%	0	80±15	76±35	-9±1

(0%) = 154 mM NaCl; (5%) = 154 mM NaCl and 300 mM trehalose; (10%) = 300 mM trehalose. Mean vesicle size variation is the increase and/or decrease of mean vesicle size before and after freeze-drying as percentage of the respective value before freeze-drying. The mean vesicle size before freeze-drying were 192 ± 7 for F_{14} and 187 ± 17 nm for F_{15}, and zeta potential values before freeze-drying were 40 ± 5 mV and 38 ± 4 mV respectively. The data shown are the average ± S.D. from three to nine independent experiments.

The stability of lyophilized liposomes prepared in the presence of trehalose, as a function of time, was evaluated in two sets of experiments: one experiment with a total time span of two weeks and another with a total duration of 12 months. Sampling was performed at three month time intervals.

Three selected TFL liposomal formulations (F_{14}, F_{15} and F_{19}) were freeze-dried and at different times after lyophilisation they were reconstituted with water and centrifuged at low speed to reject the potential TFL that leaked out. Figure II-A.3.3 shows the results obtained for the three TFL liposomal formulations, studied over a period of two weeks.

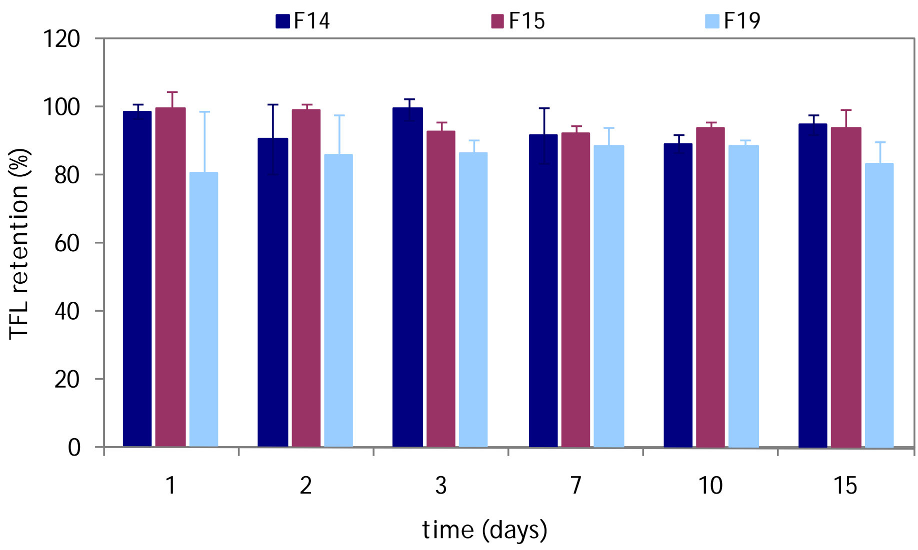

Figure II-A.3.3 – Short term stability of freeze-dried TFL liposomal formulations. TFL values before freeze-drying corresponding to 100% retention are 266 µg/mL for F_{14}, 275 µg/mL for F_{15}, and 252 µg/mL for F_{19}. [Lip] = 10 µmol/mL for all formulations. The data points represent the average ± S.D. of at least three independent experiments.

The most stable formulations were those composed of PC:PG (F_{14}) and DOPC:DOPG (F_{15}) showing more than 95 % of TFL still present two weeks after lyophilisation and subsequent reconstitution. Lipid retention was also higher than 95% for these lipid compositions. Small mean vesicle size changes were observed for both formulations. F_{14} increased their sizes between around 7% of the initial value (97 ± 2 nm) at day one to 14% of the initial value at day 15 after lyophilisation. On the contrary, the mean vesicle size of F_{15} decreases up to 24% in diameter compared to the values before freeze-drying (144 ± 4 nm). A P.I. smaller than 0.2 was observed for both formulations. The third formulation studied, F_{19} showed an overall lower TFL retention and a very irregular behaviour with respect to vesicle mean vesicle size, with variations up to 55 % of the initial value (137 ± 5 nm) and with a P.I. higher than 0.2.

The stability of freeze-dried liposomes over a longer period of time was studied for the most stable TFL formulations. Figure II-A.3.4 shows the results obtained for F_{14} and F_{15} kept lyophilized up to 12 months and reconstituted at various times.

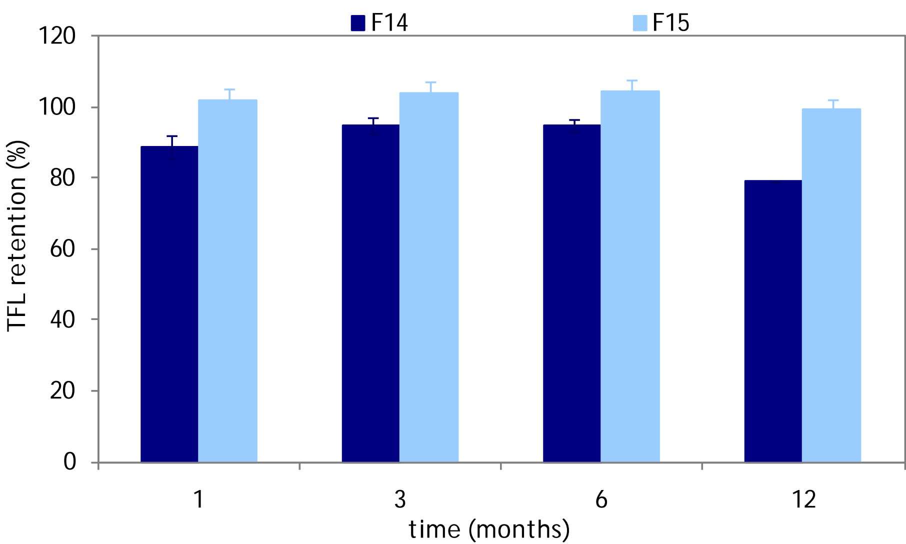

Figure II-A.3.4 - Long-term storage stability of freeze-dried TFL liposomes. Liposomal formulations composed of PC:PG (F_{14}) and DOPC:DOPG (F_{15}) (1 µmol TFL:10 µmol Lip) were suspended in 300 mM trehalose (after ultra-centrifugation), freeze-dried in 1 mL aliquots and kept at room temperature. TFL/Lipid ratio before lyophilisation is 31 and 32 µg/µmol respectively for F_{14} and F_{15}. Lipid recoveries were 92 % for F_{14} and 95 % for F_{15}. Initial mean vesicle sizes are 123 ± 4 nm and 140 ± 6 nm respectively for the two formulations. The data points represent the average ± S.D. of, at least, three independent experiments.

The F_{15} formulation retained more than 95% of TFL 12 months after lyophilisation. The F_{14} formulation presents a lower amount of TFL after the same period, although more than 85% was still incorporated during the first 6 months, decreasing by 10% in the following 6. Variations in the mean particle size smaller than 20% for both formulations were observed. One year after the mean diameters were below 120 nm for both formulations.

The stability of the above reconstituted formulations kept for 72 h at 4 °C was studied. Table II-A.3.4 shows the results obtained of TFL retention and size variation as a function of time for the two TFL liposomal formulations studied.

Table II-A.3.4 - TFL retention and mean vesicle size stability of reconstituted freeze-dried TFL liposomal cakes.

Formulation	24 hours		48 hours		72 hours	
	TFL retention (%)	Mean vesicle size variation (%)	TFL retention (%)	Mean vesicle size variation (%)	TFL retention (%)	Mean vesicle size variation (%)
F_{14}	98 ± 10	-4	95 ± 7	-2	90 ± 8	-3
F_{15}	92 ± 5	+6	87 ± 14	+8	84 ± 5	+8

At the time of reconstitution (t = 0 h) TFL contents varied between 204 and 300 µg/mL for DOPC:DOPG and 237 to 323 µg/mL for PC:PG. Liposomal suspensions were kept at 4 °C. Final TFL content was determined at t = 24, 48 and 72 h after rejection of the potential leaked out material by low speed centrifugation.
Mean vesicle size variation is the increase and/or decrease of size after respectively 24, 48 and 72 h, as percentage of the value at the time of reconstitution. These values were 106 ± 2 nm for PC:PG and 117 ± 8 nm for DOPC:DOPG with a P.I. < 0.2.
The data shown is an average ± SD from, at least, three independent experiments.

After 24 h both formulations kept more than 99% of incorporated TFL and after 72 h still maintain 87 to 90% of the drug, with no significant different values for both lipid compositions. Size is maintained unchanged during this period of time.

The maintenance of the liposome properties 3 days after reconstitution will allow their safe usage in a number of other experiments, e.g. *in vivo* studies.

II-A.3.1.2.3 - Frozen at low temperature

The stability of frozen TFL liposomes, at low temperature, over more than 80 days, was studied for F_{15} and F_{19}. Figure II-A.3.5 shows TFL retention and mean vesicle size

variation after thawing and low speed centrifugation of frozen TFL liposomes. No significant variation in TFL retention compared to the values before freezing ($p>0.05$), was observed. The mean vesicles size experienced an average increase of 6 and 11% of the original values, respectively for DOPC:DOPG (F_{15}) and PC:Chol:DSPE-PEG (F_{19}). For the latter lipid composition, the standard deviations were higher and the P.I. varied between 0.1 and 0.3. The zeta potential of the vesicles before (-42 mV) and after freezing was not statistically different ($p>0.05$).

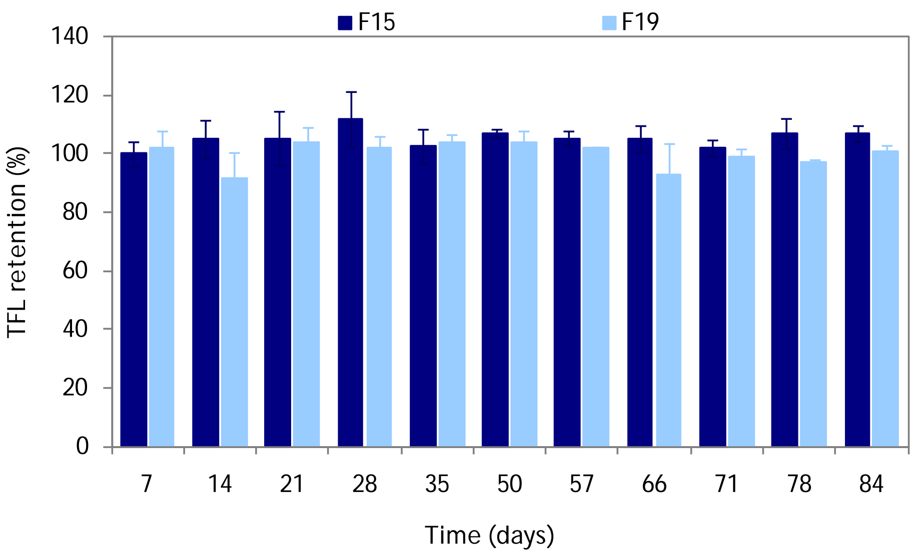

Figure II-A.3.5 - Stability of TFL liposomes stored at low temperature. Liposomes were frozen at -70 ºC and at various times were thawed; allowed to reach room temperature and centrifuged at low speed (5000 x g for 10 min) to remove the potential leaked TFL. $[TFL]_i$ = 190 ± 4 μg/mL for F_{15} and 236 ± 4 μg/mL for F_{19}. $[Lip]_i$ = 10 ± 1 μmol/mL. The data shown is an average ± SD from, at least, three independent experiments.

The finding that it is possible to keep certain TFL formulations frozen with no further treatment after preparation is important due to the convenience and simplicity of this procedure.

II-A.3.1.3 - Laboratory Large-Scale preparation of TFL liposomal formulations

The previous incorporation studies were done using formulations with small laboratory volumes, on average no more than 5 mL per preparation. However, some of the therapeutic activity studies used to evaluate the biological effect or *in vivo* activity of any drug incorporated in liposomes usually requires the preparation of high quantities of the liposomal drug. The scale-up involved in planning these experiments is, sometimes, difficult to overcome. The feasibility to prepare liposomes at a laboratory scale and at the same time compatible with experiments involving either mice or dogs was assessed.

The formulation with the best incorporation parameters and stability characteristics (F_{15}), was prepared in a volume of 2 to 3 mL herein referred as Small Laboratory Scale (SLS) and in a volume 200 to 300 times higher (600 mL) herein referred as Large Laboratory Scale (LLS). The incorporation parameters and characteristics of these formulations were compared. Both scales were prepared using the same method with some adjustments for LLS regarding the size of glassware, in particular a round bottom flask for solvent evaporation of acceptable capacity, longer hydration times and an extruder device with higher volume capacity.

The LLS liposomes were prepared in daily batches of 600 mL until the required final formulation volume was reached. All batches were individually characterised as well as the final formulation resulting from the assemblage.

According to the results presented in Figure II-A.3.3, no significant differences were observed for the I.E. and L.C. parameters and characteristics of liposomes prepared by the SLS or by the LLS. The mean diameters of liposomes prepared in both scales showed no significant differences ranging from 201 to 236 nm respectively with an

average zeta potential of -40.3 ± 2.2 mV. The TFL retention of both scales (55 to 66%) was also not statistically different.

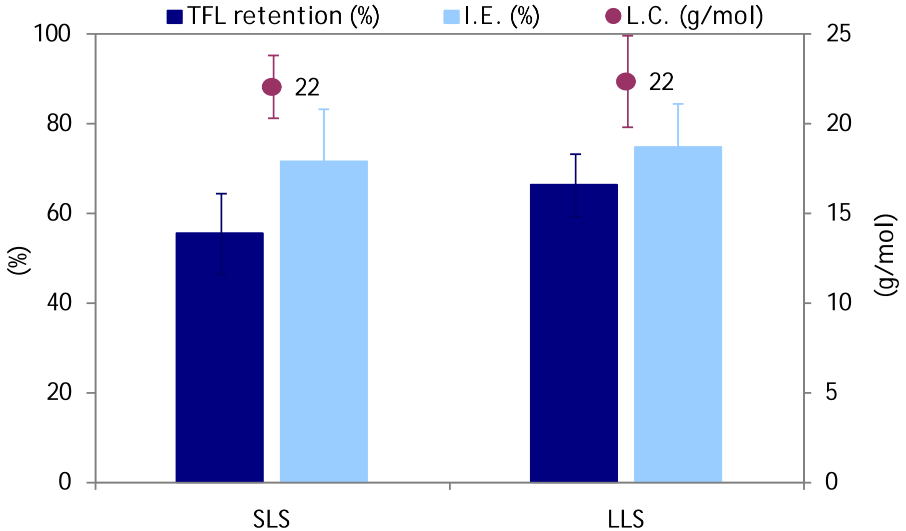

Figure II-A.3.6 - Incorporation parameters obtained for TFL liposomes, prepared as a SLS or as a LLS. The parameters studied for the F_{15} formulation include TFL retention (dark blue bars), I.E. (light blue bars) and L.C.(dark red dots). $[TFL]_i$ and $[Lip]_i$ for SLS are 323 ± 30 µg/mL and 10 ± 1 µmol/mL respectively. For LLS, $[TFL]_i$ and $[Lip]_i$ are respectively 320 ± 29 µg/mL and 11 ± 1 µmol/mL. The final size (obtained for LLS preparations was 201 ± 39 while for SLS preparations was 236 ± 4 nm. The SLS scale corresponds to 2-3 mL preparations; the LLS correspond to 600 mL preparations. The data points represent the average ± S.D. of, at least, ten independent experiments.

For LLS it was also demonstrated a high reproducibility of the properties from batch to batch among ten independent experiments. Some these batches were further stored frozen at -70 ºC in order to maintain their characteristics.

When required, several batches were thawed and vigorously mixed before final characterisation. The macroscopic and microscopic observations revealed the absence of precipitated TFL indicating good stability. The final homogenised formulation presented 239 ± 5 µg TFL and 8 ± 3 µmol total lipid per mL.

The TFL formulation prepared in the LLS has all the appropriated characteristics in terms of intraliposomal TFL concentration and vesicle size to be compatible with the

requirements of the *in vivo* experiments. That is, the administration of a therapeutically effective dose in a volume no greater than 200 µL per mice, by intravenous route. In addition, this formulation can still be concentrated, at least 10-fold, without signs of precipitation.

Selection of TFL liposomal formulations

According to the incorporation, stability and scale-up studies, the following formulations were selected for *in vivo* studies: F_9 (DSPC:Chol); F_{14} (PC:PG); F_{15} (DOPC:DOPG) and F_{19} (PC:PEG-DSPE).

II-A.3.2 – Evaluation of the anti-leishmanial activity of TFL liposomal formulations in animal models of *Leishmania sp* infections

The antiparasitic activities of the selected TFL liposomal formulations were evaluated in experimental mouse models of visceral (*L. donovani*) and cutaneous (*L. major*) leishmaniasis and also in a canine model (*L. infantum*).

The influence of the lipid composition, the TFL dose effect, the treatment schedule and the route of administration, on the reduction of parasite burden, was assessed.

II-A.3.2.1 – Therapeutic activity in a murine visceral model

The therapeutic activity of TFL liposomes was evaluated in a murine visceral model of infection against *L. donovani*.

Effect of the lipid composition of TFL liposome

TFL incorporated in three different liposomal formulations (F_9; F_{14} and F_{15}) were compared to free TFL (in 10% Tween 80) and to Glucantime® (positive control). Infected, untreated, animals were used as a negative control. The TFL liposomal formulations prepared as described in section II-A.3.1.3 (LLS), were concentrated in such a way that the used dose (15 mg TFL/kg body weight) could be administered in an appropriated volume (up to 200 µL/mouse). The parasite load in the liver of treated and untreated mice was calculated as described in section II-A.2.6.1. Data is shown in Figure II-A.3.7.

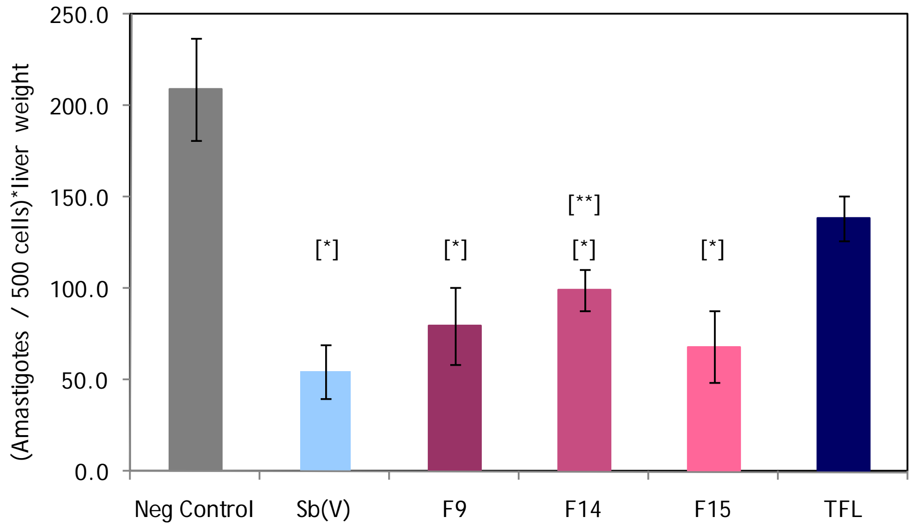

Figure II-A.3.7 - *In vivo* activity of TFL liposomal formulations, free TFL and Glucantime® (Sb^V) against *L. donovani* (MHOM/ET/67/L82) infections in the liver of BALB/c mice. TFL liposomes: F_9 = DSPC:Chol; F_{14} = PC:PG and F_{15} = DOPC:DOPG. Mice were treated during days 7 to 11 post-infection and the parasite load in the liver was determined at day 14 post-infection. All treatment groups (5 animals) received a dose of 15 mg TFL/Kg body weight/day for 5 days. The negative control group (dark blue dots) received 5 doses of 300 mM trehalose. The positive control group (Sb^V) received one dose of 15 mg Sb^V/kg body weight at day 7. Black bars represent the mean value. Results are expressed as the number of parasites in the liver ± SEM, from, at least, three independent experiments
[*]-Significantly different from negative control and from free TFL.
[**]-Significantly different from Sb^V.

Free TFL induced a therapeutic effect reflected by a 33% inhibition of parasite growth as compared to untreated mice. A significant superior antiparasitic effect was revealed by all three TFL liposomal formulations as well as by Glucantime®, compared to free TFL ($p<0.05$). The inhibition of parasite growth observed for the three TFL liposomal formulations ranged from 55 to 68%, depending on the lipid composition. However, the therapeutic activity of the formulations is not statistically dependent on the lipid composition; nevertheless, F_{15} formulation is 2-fold more active than free TFL and displayed a similar activity as Glucantime® at similar dose (1 administration). On the contrary the PC:PG liposomes (F_{14}) are significantly lower than Glucantime® ($p<0.05$).

Effect of liposomal TFL dose and number of treatments

The therapeutic effect of the formulation displaying higher activity, F_{15}, prepared in three different doses (2, 5 and 10 mg TFL/kg body weight) and administered in 1, 3 or 5 treatments was explored. The evaluation of the activity of liposomal TFL for all the doses and treatments of F_{15} is shown in Figure II-A.3.8.

Free TFL administered in Tween 80 at a dose of 10 mg/kg induced a small reduction of parasite growth, ranging from 18 to 26% inhibition, compared to the negative control. No effect was observed with the increase of the number of treatments. Free TFL was significantly less active than the respective liposomal form ($p<0.01$).

For mice treated with TFL liposomes, a significant parasite inhibition was observed in the liver compared to the untreated control group ($p<0.05$), irrespectively of the treatment regimens and doses used. When increasing the number of treatments and the dose, it was observed a tendency to increase the parasite inhibition, ranging from 36% (single administration of 2 mg TFL/kg) to a maximum of 70% (five administrations, 10 mg/kg). However, significant differences were only observed between the animal

groups treated with a single administration of any of the tested doses of liposomal TFL and the group treated with 5 doses at 10 mg TFL/Kg/day ($p<0.05$). This latter group presented a similar antiparasitic activity (70% parasite inhibition) as the group treated with Glucantime® (5 mg/kg/day, 5 days).

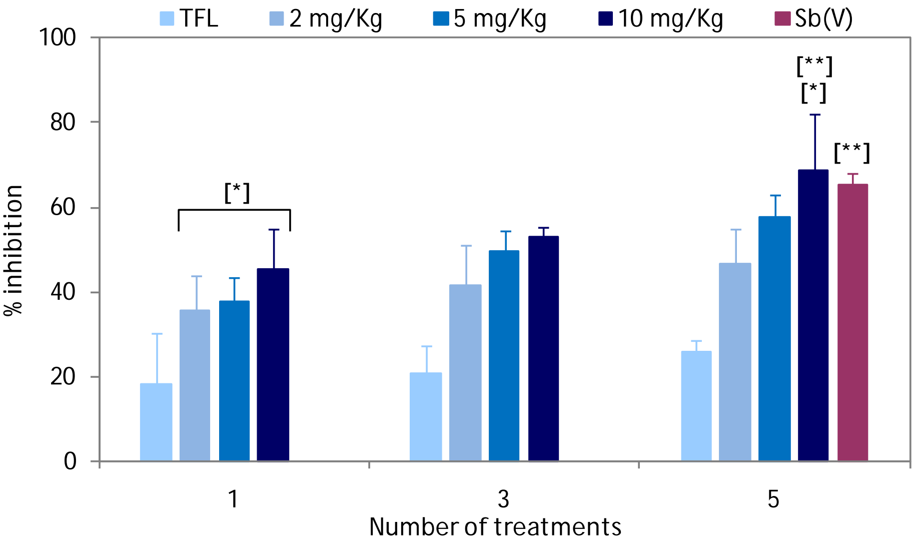

Figure II-A.3.8 – Dose and treatment-response activity of free and liposomal TFL against *L. donovani* infections in the liver of BALB/c mice. Mice (5 animals per group) were treated during days 7 to 11 of infection and the inhibition of liver amastigotes was determined at day 14 post-infection. The TFL doses incorporated in DOPC:DOPG liposomes were 2 (light blue bars), 5 (blue bars) and 10 (dark blue bars) mg/kg/day and the number of treatments were one (single dose), three (in alternate days) and five (five consecutive days). Free TFL (grey bars) was given at a dose of 10 mg/kg/day. The positive control group ($Sb^{(V)}$) (dark red bars) received 5 doses of 5 mg/kg. Results are expressed as percent parasite inhibition ± SEM.
[*]-significant difference between the values
[**]-Not significant difference between the values

II-A.3.2.2 - Therapeutic activity in a murine cutaneous model

The therapeutic effect of TFL incorporated into two liposomal formulations was also evaluated against *L. major* in a cutaneous animal model of *Leishmania* infection. One of the selected formulations was the one used before for the visceral model with the best results (F_{15}). A second formulation with long circulation characteristics, F_{19}

(PC:PEG-DSPE) was also included with the intention of reaching the skin where the target cells in this model are located. These two formulations were prepared with a final mean particle size of around 80 nm. The therapeutic effect was analysed by measuring the size of the skin lesions produced by the *L. major* infection.

In one set of *in vivo* studies the anti-parasitic activities of F_{15}, F_{19} TFL liposomal formulations and Glucantime® administered i.v., i.p. and s.c., against *L. major* were compared. Data is shown in Figure II-A.3.9. In comparison to untreated mice that presented an average increase in the lesion size of 40%, both TFL formulations significantly reduced lesions size ($p<0.001$). Glucantime® also had a significant effect on lesion size ($p<0.001$). An interesting result was the observation that the treatment with TFL liposomes at 6 mg/kg dose, was able to slow the lesion progression at a similarly rate than the same 10 administrations of a 66-fold higher dose of the Glucantime® for all the administration routes.

Regarding the lesion reductions it was observed that they were irrespective of the administration route for all treated groups. However the s.c. route (close to the lesion) offered the most promising results for all formulations. This route of administration presented the most consistent results for the three formulations. On the other hand some irregularity of results (high SD values) was observed for the i.p. and i.v. routes in F_{15} and for the i.v. route in F_{19}.

The therapeutic effect of free TFL was also assessed in 2 independent experiments, however results were inconsistent and several animals had to be euthanized before the end of the experiment to avoid unnecessary animal suffering (data not shown).

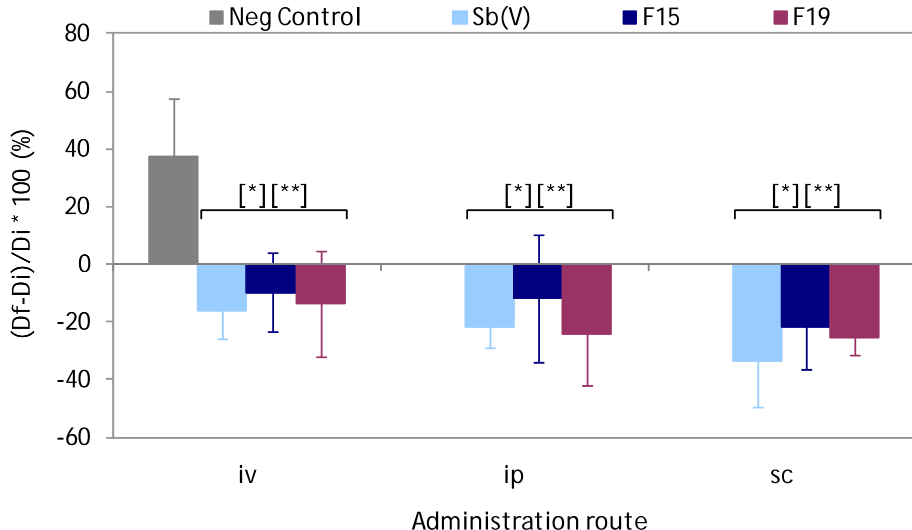

Figure II-A.3.9 - Antiparasitic activity of TFL liposomes against *L. major* (MHOM/ET/67/L82) infections in the skin of BALB/c mice. Mice (5 animals per group) were treated every day, between days 7 and 12 post-infection with Glucantime® (400 mg Sb^V/kg/day) and TFL incorporated in DOPC:DOPG(F_{15}) and PC:PEG-DSPE (F_{19}) liposomes (6 mg/kg/day) either by i.v., i.p. or s.c. (close to the lesion) route. Untreated mice (Neg. Control) were given 300 mM trehalose. The lesion sizes were measured 14 days post-infection. Results are expressed as the percent increase/decrease in lesion size ± SD of two representative experiments.
[*]- Statistically different from negative control ($p < 0.001$)
[**]- Not statistically different between values ($p > 0.05$)

In a second set of *in vivo* experiments and based on the above results, mice with a higher *L. major* infection level were treated with the long circulation TFL formulation F_{19}. In this study the activity of 10 doses of F_{19} (10 mg TFL/kg/day) was compared to Glucantime® (400 mg Sb(V)/kg/day), both administered i.v., i.p. and s.c. Data is shown in Figure II-A.3.10. The lesion sizes of untreated mice observed 21 days after infection increased about 90%. In this study no therapeutic effect was achieved for the treated mice groups with the exception of the s.c administrations of F_{19} liposomes with a significant reduction in the rate of lesion development ($p < 0.01$). These results demonstrate the superiority of the s.c. administration route close to the lesion for the treatment of this model of cutaneous leishmaniasis with TFL liposomes.

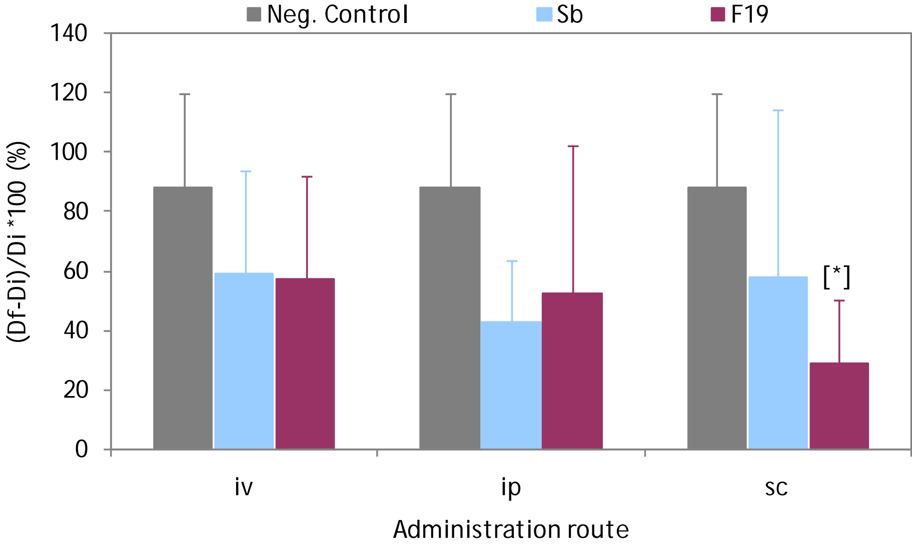

Figure II-A.3.10 – *In vivo* activity of TFL liposomes against *L. major* (MHOM/ET/67/L82) infections in the skin of BALB/c mice. Mice (5 animals per group) were treated between days 7 and 17 post-infection with TFL incorporated in PC:PEG-DSPE (F_{19}) liposomes (10 mg/kg/day) by i.v., i.p. and s.c. routes. Untreated mice (Neg. Control) were given 300 mM trehalose. The lesion sizes were measured 21 days post-infection. Results are expressed as the percent increase in lesion size ± SD.
[*]- Statistically different from negative control.

The lesions size of mice with the lower level of *L. major* infection (from Figure II-A.3.9) were monitored for 7 weeks after the beginning of treatment with the intention to evaluate the progression of the disease after the end of treatment. During this time animals that presented signs of distress were removed from the experiment. Figure II-A.3.11 shows the weekly evolution of the lesion size in all the mice groups. Considering that treatment was completed at day 10, it is interesting to notice that a negative mean value (-5%) for the lesion size increase was still obtained 21 days from the start of the treatment for the TFL liposomes administered s.c. After 28 days the lesion size for these animals increases to positive values (25%). However, this value is significantly different from the control that is already 70% higher than the initial value. The standard drug Glucantime® had a more marked effect in the evolution of

this model. In particular, when administered s.c. it was responsible for a negative progression of the lesion size up to 1 month from the completion of the treatment.

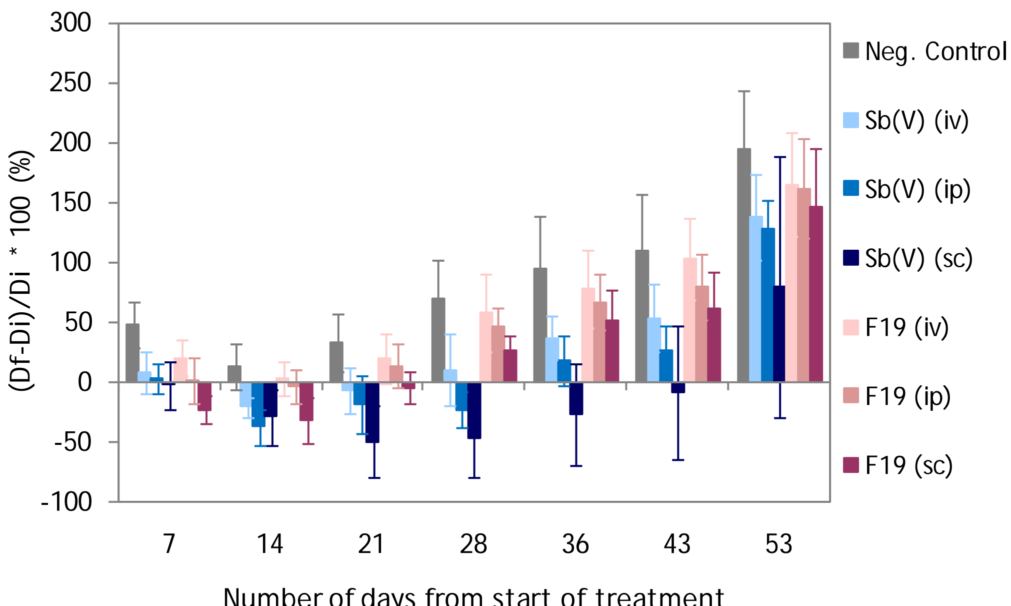

Figure II-A.3.11 – *In vivo* activity of TFL liposomes against *L. major* (MHOM/ET/67/L82). Mice (5 animals per group) received 10 doses of either TFL incorporated in PC:PEG-DSPE (F_{19}) liposomes (10 mg TFL/kg/day) or Glucantime® (Sb(V)) (400 mg/kg/day) by i.v., i.p. and s.c. routes. Untreated mice (Neg. Control) were given 300 mM trehalose x 10 doses. Treatment was completed at day 10. The lesion sizes were measured once a week. Results are expressed as the increase/decrease in lesion size ± SD of two representative experiments.

II-A.3.2.3 - Therapeutic activity in a canine model of L. infantum

The therapeutic activity of liposomal TFL (F_{15}) was also studied in the treatment of dogs experimentally infected with *L. infantum*. Infected female Beagles were treated for 10 days with a daily dose of 10 mg TFL/Kg (i.v.). The therapeutic effect was analysed by quantification of viable parasites in the bone marrow and lymph nodes and in the evaluation of the clinical condition of the dogs. Figure II-A.3.12 shows some of the external clinical signs observed in the dogs five month after infection (a to d) and at 1 (e and f) and 3 (g, h and i) months after treatment. It was observed a general clinical recovery with remission of symptoms after treatment, namely: conjunctivitis and nose dry dermatitis (e and h *vs.* a); ulcerative lesions of the paws (f and i *vs.* b) and hair loss and desquamation (g *vs.* c)

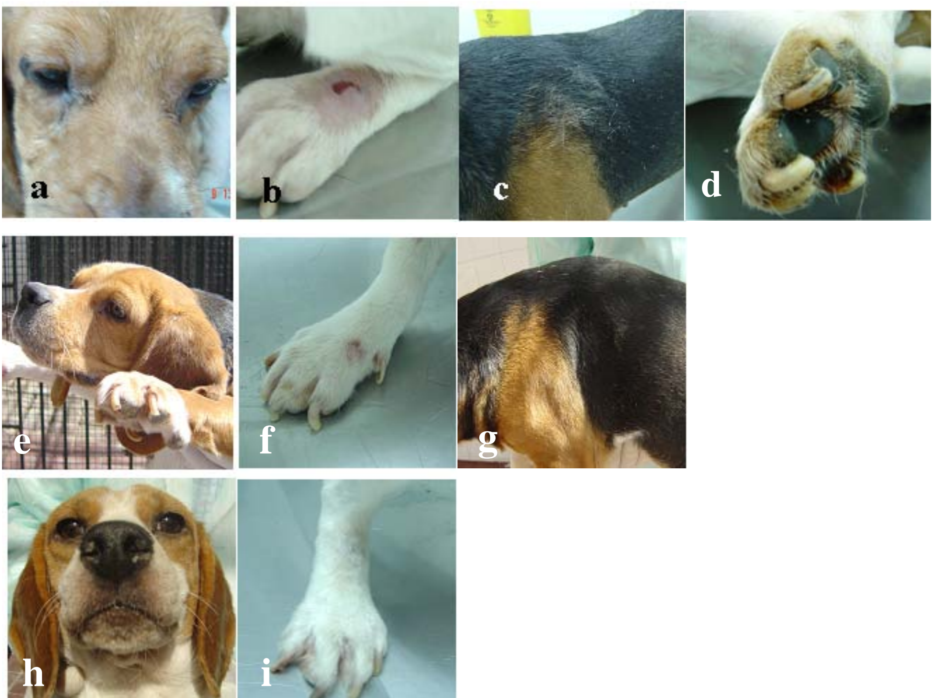

Figure II-A.3.12 – External clinical signs of leishmaniasis shown by dogs after infection (a to d) and general clinical recovery with remission of symptoms observed after treatment (e to i). (a) mild conjunctivitis; (b) ulcerative lesions of the paws; (c) hair loss and desquamation; (d) onycogryphosis (nail bed changes); (e and h) remission of conjunctivitis and nose dry dermatitis; (f and i) nearly and completely healed ulcerations of the paws, (g) absence of hair loss and desquamation. Adapted from [52].

Table II-A.3.5 shows the evolution of the number of viable parasites detected in one of the dogs before and after treatment with liposomal TFL.

Table II-A.3.5 – Viable *L. infantum* parasites detected in one of the dogs before and after treatment with liposomal TFL (F_{15}).

Time Line	Parasites/g of tissue	
	Bone Marrow	Lymph Nodes
5 months after infection (*)	1000	6400
1 month after treatment	negative	1600
3 months after treatment	4000	1600

(*) before treatment

Results show that there was either elimination or a significant reduction in the parasite burden at 1 month after treatment, respectively in the bone marrow and in the lymph nodes. Nonetheless, 3 months after treatment parasites numbers increased in the bone marrow whereas in lymph nodes remained constant and 4-fold lower than before treatment.

The induction of a potential protective immune response due to the increments of protective cytokines (IFN-γ and IL-2) in the parasite target organs was detected in the dogs following treatment (data not shown). In addition, no variation on haematological and biochemical blood parameters (haemogram and leukocytic formula) was observed along the course of the experiment (measured at 0, 6 and 10 months after the start of the study) [52].

II-A.4. Discussion

Dinitroanilines, in particular TFL, have shown attractive features as potential drugs for the treatment of parasite infections since the pioneer work of Chan and co-workers [44]. The recent increasing interest in dinitroanilines as selective chemotherapeutic agents is documented by chemical modification studies aiming at increasing the activity against the tubulins from parasites [62-66].

Besides chemical modification, incorporation in liposomes represents another strategy to overcome the difficulties of handling and to correctly formulate TFL for *in vivo* administration. The work presented here represents the first attempt to incorporate TFL in liposomes, simultaneously providing a solvent, and a stabilizing system for TFL and a NanoDDS for its systemic administration targeted towards the *Leishmania* infected organs.

To achieve an efficient incorporation and stabilization of TFL in liposomal formulations, studies involving the effect of lipid composition, presence of Chol, T_c of lipids, lipids surface charge and the presence of PEG-derivatized lipids, were performed. The reduction in TFL incorporation observed in conventional fluid liposomes and in PEG containing liposomes, resulting from the inclusion of Chol in the lipid mixture (Table II-A.3.1), can be explained by TFL hydrophobicity and by the competition of both molecules for the same domain in the phospholipid bilayer. These results are in accordance with previous observations involving other anti-parasitic drugs [67, 68]. The low incorporation obtained for formulations composed of lipids with higher T_c without Chol, as compared to fluid lipids (Table II-A.3.2), can be explained by the difficulty of insertion and stabilization of TFL in ordered bilayers, as already observed for other drugs with a high hydrophobicity [67, 69]. A cumulative inhibitory effect of high T_c and Chol on drug incorporation seems to be responsible for low or no TFL incorporation in rigid lipidic bilayers combined with 30% Chol content

(F_6, F_8 and F_{10}). The presence of a negative surface charge in the liposomal bilayer is favourable for the incorporation of TFL in liposomes. As such the best compositions for the incorporation of TFL in liposomes are mixtures of phospholipids with low T_c, without Chol and with charged polar head groups, in particular PG, with or without PEG-derivatized lipids.

In addition to a successful incorporation, the maintenance of the drug in the liposomes is mandatory to allow the performance of long term studies and *in vivo* experiments. This is particularly critical in the case of unstable hydrophobic drugs such as TFL that can affect liposomes stability by interacting with the lipid bilayers, as opposed to water-soluble drugs [70, 71]. We have reached this goal as the TFL formulations that presented the best incorporation parameters can be used safely 10 days after preparation as they were also the most stable in saline at 4 ºC.

When higher amounts of liposomes are needed, their efficient stabilization in lyophilized form has been widely used [72]. In addition, freeze-drying is a well-accepted technique in the pharmaceutical industry. Due to the tendency of TFL to be incorporated in the lipid matrix, it would be possible to stabilise this drug during freeze-drying without the need for lyoprotection [61]. However, due to the drug's labile nature, protection may be required. Therefore, we have investigated the effect of sugars on the stabilization of freeze-dried TFL liposomes. Although it is sustained that sugars are required, both in the inner aqueous space and in the medium outside the vesicles, to reach optimal lyoprotective effect of either hydrophobic or hydrophilic drugs [61, 72, 73], our results did not show such a sugar dependency. Regardless of the presence or absence of trehalose inside liposomes, its presence in the outside space was crucial for the retention of TFL and for the preservation of liposome size and surface properties. When lyophilisation took place in the absence of lyoprotectant, changes occurring in the vesicles structure due to the formation of ice

crystals [72], and in lipids distribution in the liposomal outer layer may be partially responsible for the leakage of TFL.

When a loss of drug occurred during the lyophilisation process, a discoloration from the typical yellow colour of TFL to a white colour of the freeze-dried cake was observed. The possible sublimation of TFL during the freeze-drying process may account for these observations. In the absence of a lyoprotectant, it is described an increase in competing interactions between acyl chains resulting from the drying of the bilayers [72]. Under these conditions the association of TFL to the acyl chains of the phospholipids is not strong enough to avoid leakage of the drug and sublimation.

The presence of trehalose, replacing water in the interaction with the phospholipids head groups [74], allows the maintenance of the original TFL-acyl chains interaction, thus protecting TFL in liposomes by acting as an anti-sublimating agent [75]. In the experiments with TFL liposomes made from fluid lipids (F_{14} and F_{15}), the presence of trehalose was crucial to keep liposomes properties during lyophilisation and during storage for periods of time of up to one year without significant loss of TFL. Furthermore, the presence of trehalose during the preparation of TFL liposomes also allowed a better manipulation of the liposomal suspension. In fact, an easier extrusion was observed and it prevented the shrinkage of the freeze dried cake assuring a good and instantaneous rehydration and homogenization after the addition of water. This represents a common feature previously reported for other drugs and other types of lyophilised liposomes [76, 77]. These formulations also evidenced other interesting pharmaceutical aspects like the conservation of their properties, particularly the loading capacity and vesicle size during, at least, 3 days after reconstitution. This behaviour will allow a safe usage of reconstituted liposomes in other experiments, *e. g. in vivo* studies. The finding that it is possible to keep certain TFL liposomal formulations frozen in the presence of trehalose, for at least 3 months, with no

further treatment after preparation, is important due to the convenience of this procedure. The slow freezing process used in the present work was already described for cationic liposomes, as allowing to maintain smaller size variations than fast freezing [61, 78].

Another important finding of this work was the possibility to increase 300-fold the scale of liposomes preparation, without affecting the incorporation parameters and the characteristics of the final TFL liposomes. This indicates that all the critical steps for efficient drug incorporation like the formation of the lipid film, its hydration, separation of non-incorporated material and sizing [72], defined before, were not affected by the modifications in the preparation method and experimental conditions attempted. Batch-to-batch reproducibility of liposomes properties was found in a significant number of individual 600 mL preparations. Although the scale increase tested with TFL liposomes cannot be extrapolated for the large production of formulations, it is worthwhile at laboratory scale, allowing the production of the needed liposomal volume for the treatment of leishmaniasis in infected mice and dogs.

As a result of the above described systematic evaluation of parameters affecting TFL incorporation and stabilization in liposomes, some TFL formulations were selected for biological evaluation. These liposomes can act as a stable solvent system for TFL, increasing its water concentration at least 10.000 times.

The second goal of this chapter was to demonstrate if liposomes were appropriate NanoDDS to carry TFL to the sites of *Leishmania* infection. While TFL has been described in the literature as an anti-leishmanial agent [79], its biological behaviour was characterized mostly based on *in vitro* tests. The only known results on the use of TFL *in vivo* concern topical application, as an ointment, in a cutaneous animal model [43], which is not an appropriate formulation to treat the visceral form of the disease.

In addition, TFL is not suitable for oral or systemic administration due to very low water solubility and easy sublimation [46] and the large volumes needed to reach the therapeutic dose [48, 49]. Through the evaluation of the therapeutic activities of TFL liposomal formulations in murine and canine models of *Leishmania* infections, it was evidenced the superiority of the liposomal formulations over the free TFL. In addition, all the liposomal formulations have the advantage of not requiring organic solvents to be administered as is the case of the free drug. Despite the fact that the TFL liposomal formulations assayed in a murine visceral model of *L. donovani* infection have different properties like membrane fluidity and surface charge, no significant differences were evidenced in their antileishmanial activity. However, one of the formulations made with fluid lipid (DOPC:DOPG), can be pointed out as the best formulation developed in this work, as it inhibited up to 67% parasite growth in the liver, being two fold more active than the free TFL and presented a similar inhibition pattern as Glucantime®. This formulation was used to test different doses and treatment schedules with the aim to further improve the parasite growth inhibition in mice and dogs. A total dose of 50 mg/kg administered as 5 treatments of 10 mg/kg/day was responsible for a substantial reduction of the parasite growth (around 70%), presenting simultaneously a pattern similar to Glucantime® (5 treatments of 5 mg $Sb^{(V)}$/kg/day) while displaying significantly higher therapeutic effect from lower doses (2 and 5 mg/kg/day) at different administrations. This finding indicates that to reach the goal of 100% parasite inhibition a further increase on the dose and/or number of administration should be the correct direction to follow. The dose increase could be safely done, as no signs of toxicity were reported in mice for TFL liposomes of the same lipid composition at a dose of at least 60 mg TFL/kg body weight (by i.v. or i.p. route) [80].

In the cutaneous model, we had to face a more difficult task for drug delivery, as the cellular target for TFL are *L. major* amastigotes-infected macrophages located in the dermis of the skin [81]. For this reason the type of chosen liposomes to deliver the drug were long circulating ones and conventional liposomes of smaller mean particle size (below 100 nm). Liposomes with these characteristics have been described as appropriate to gain access to the dermis where they are able to extravasate through the leaky blood vessels caused by local inflammatory responses [81, 82]. The therapeutic activity studies proved a significant reduction in the lesion size of treated animals compared to control following administration of both TFL formulations (Figure II-A.3.9). This observation was not found for free TFL, demonstrating that a proper delivery was achieved. The small mean particle size of the administered liposomes (around 80 nm) was most probably the cause for the similar activity observed for both the conventional and long circulating formulations. Interestingly the route of administration seems to have a more relevant influence than that of liposomes composition. A superiority of TFL liposomes was found for the s.c. route as compared to i.p or i.v. administration in experiments with higher infection levels (Figure II-A.3.11). Such a dependence of the effectiveness of liposomal formulations from the administration route was observed by other authors [83]. However in studies with liposomal amphotericin B the i.v. route was superior for the treatment of cutaneous leishmaniasis [81, 82]. The different chemical properties of both amphotericin B and TFL molecules and their specific interaction and diffusion in the tissues after local administration may explain these discrepancies. In addition, and according to biodistribution studies of small liposomes, a fraction remains at the injection site following s.c. administration, the percentage depending from the anatomical site of injection [84, 85]. The subsequent formation of a s.c. liposomal depot at the site of injection and, in this case, close to the lesion may provide a

sustained release of TFL increasing the drug concentration and maintaining a higher drug level for a longer time in its site of action. This process is not only dependent from the ability of small liposomes to migrate through the interstial spaces between cells [83], but also from the ability of free molecule to diffuse through lipophilic media from the site of injection to the lesion. The contribution of this latter mechanism may be crucial for the overall result, closely dependent from the type of molecule under study.

The overall results obtained have demonstrated that not only TFL liposomes are active in the treatment of cutaneous leishmaniasis, they also presented a similar therapeutic activity to Glucantime® at a high dose (400 mg of $Sb^{(V)}$/kg). In particular the results for the s.c. route, a simpler route for patient self-administration, opens the way for new studies where different treatment regimens including higher TFL doses may be expected to have a curative effect.

Our most ambitious aim was to test, at least, one TFL liposomal formulation in dogs experimentally and naturally infected (data not shown) with *Leishmania* parasites. This was carried out with formulation F_{15}. The activity of TFL liposomes (DOPC:DOPG) and the absence of signs of toxicity were demonstrated in both types of infected dogs. Improvement in physical signs including the disappearance of nose dry dermatitis, articulary skin oedemas and mild conjunctivitis were observed in a naturally infected dog treated with F_{15} [86]. The therapeutic effects exhibited by experimentally infected dogs, after treatment with liposomal TFL included the improvement of the animal's clinical condition, the reduction of parasite load, and the induction of protective immune response with the increments of protective cytokines [52]. In addition, no variation on haematological and biochemical blood parameters were registered [52, 86].

Taking into account the results obtained in the mouse and dog infection models, we can conclude that the formulations developed in this study are promising TFL delivery systems for the treatment of leishmaniasis. However, further systematic studies are needed for a complete knowledge of the usefulness of TFL liposomes to fight this infection.

CHAPTER II

Part B

*Liposomal Formulations of TFL synthetic derivatives active against Leishmania infections**

TFL-A6

TFL-A3

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II-B.1 - Abstract

Leishmaniasis treatment failures are becoming a common problem in endemic areas with the emergence of resistance towards the traditional first line drugs. Second-line drugs such as pentamidine and amphotericin B, which are more toxic and difficult to administer and the recent oral agent miltefosine, have failed in providing a clear alternative for the treatment of visceral leishmaniasis. The continuous investigation for a thriving vaccine together with the search for potentially new bioactive agents and new strategies are needed to overcome the increasing incidence of the disease and fight the emergence of resistant strains.

In Part B of Chapter II, new dinitroaniline derivatives prepared by hemi-synthesis methods (TFL-D) were incorporated in lipid-based NanoDDS and studies were performed to evaluate their anti-leishmanial activity. With the intent of target macrophages, the host cells of *Leishmania* parasites, conventional DMPC:DMPG liposomes were prepared and optimised. The TFL-D compounds included in these studies (TFL-A6 and TFL-A3) were selected based on their *in vitro* anti-leishmanial activity. To provide evidence on the effectiveness of the liposomal TFL-Ds, the *in vitro* and *in vivo* anti-leishmanial activities were evaluated using appropriated assays and animal models. The *in vitro* biological evaluation of the free and liposomal TFL-D has demonstrated that they are active against *Leishmania* and more potent than miltefosine. The absence of adverse toxic effects *in vitro* was also established. Extensive parasite load inhibition was observed after treatment with liposomal TFL-A3 in a murine model of zoonotic visceral leishmaniasis.

Overall, the chemical synthesis of potentially effective new bioactive agents together with the use of NanoDDS that naturally target the diseased organs can be considered a promising new combined strategy to the discovery of novel non-conventional anti-leishmanial systems.

II-B.2 - Materials and Methods

II-B.2.1 – Lipids and Chemicals

Trifluralin derivative molecules (TFL-A6 and TFL-A3) were synthesized and were a generous gift from the Fuel Cells and Hydrogen Unit of LNEG. The phospholipids dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were purchased from Avanti Polar Lipids (USA) and were used without further purification. Polycarbonate membranes were from Nuclepore (USA). PD-10 columns were purchased from Bio-Rad. Acetonitrile (HPLC grade) was from Merck. RPMI 1640 media (20 mM HEPES), Schneider's Drosophila medium, penicillin-streptomycin, and foetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). Foetal calf serum (FCS) and Histopaque 1077 were purchased from Invitrogen (USA). LIVE/DEAD viability kit was obtained from Molecular Probes (UK). The BCA™ Protein Assay Kit is from Pierce (USA). All other reagents were analytical grade.

Synthesis of TFL Derivatives

The TFL derivatives (TFL-D) were synthesised by Dr. A. Esteves (Fuel Cells and Hydrogen Unit, LNEG). The general procedure was described elsewhere [42, 53]. The method consisted of reacting chloralin, with primary or secondary amines in the presence of triethylamine using ethanol as solvent. Substituent in the amines was aryl and alkyl groups. The following TFL-Ds were kindly supplied:

TFL-A6: 4-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol

TFL-A3: 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-butylamino)-ethanol

II-B.2.2 - Cell lines, *Leishmania* strains and mice

The human monocytic cell line THP-1 was maintained in culture in RPMI 1640 medium, supplemented with 10% heat inactivated FBS, L-glutamine, Penicillin 100 U/mL and Streptomycin 100 μg/mL, pH 7.4 at 37 ºC, 5% CO_2.

Three different *Leishmania* strains were used in these studies. For *in vitro* assays *Leishmania infantum* promastigotes (MHOM/TN/80/IPT1/LEM 235) and *Leishmania donovani* promastigotes (MHOM/IN/80/DD8/LEM 703) were cultured in RPMI 1640 medium, supplemented with 10% heat inactivated FCS, L-glutamine, and Penicillin 100 U/mL plus Streptomycin 100 μg/mL, pH 7,4 at 26 ºC. For *in vivo* studies *L. infantum* MON-1 (MHOM/PT/89/IMT 151) was maintained by passage in Syrian golden hamsters and amastigotes were isolated from infected spleens. After *in vitro* transformation, virulent promastigotes collected from the stationary phase of a subculture with less than five passages was used for mice inoculation.

BALB/c mice 6 to 8 weeks old (weight 25-30 g) were purchased from Gulbenkian Institute of Science, Portugal, and housed at the INETI animal facilities, fulfilling the European Union Council Directive (86/209/CEE), recognised by Portuguese law (DR DL129/92 and Portaria 1005/92). The experiments were repeated at least three times.

II-B.2.3 – Preparation of TFL-D liposomes

The incorporation of TFL-D in liposomes was done by the thin film-extrusion method described for TFL liposomes in section II-A.2.3. Typically, mixtures of either DMPC and DMPG or DOPC and DOPG (16 μmol/mL for TFL-A6 and 20 μmol/mL for TFL-A3, total lipid), at different molar ratios, and TFL-D at various initial concentrations were dissolved in an organic solution and dried under a nitrogen stream. The hydration of the film was performed in two steps; first, was added two-tenth of the total volume of

a trehalose-citrate buffer (10 mM Sodium Citrate, 135 mM NaCl, 29 mM Trehalose, pH 5.5) and the film was allowed to disperse by stirring. The hydration was completed with the addition of the appropriated volume (eight tenth) of citrate buffer (10 mM Sodium Citrate, 145 mM NaCl, pH 5.5), under stirring. Liposomes containing TFL-D were down-sized by extrusion until a final pore size of 200 nm and a polydispersity index (P.I.) below 0.2. The non-incorporated TFL-D was removed by gel filtration in PD-10 columns. Final liposomal suspensions were obtained after ultra-centrifugation of the eluted liposomes at 180,000 x g for 2 h at 20 ºC in a Beckman L8-60M ultracentrifuge (Beckman Instruments, Inc., USA). The pellets were suspended in the same buffer to their volume before the gel filtration. Liposomes were assayed for TFL-D and lipid contents, vesicles size and zeta potential.

II-B.2.3.1 – Freeze-dried TFL-D liposomes

The ultra-centrifuged TFL-D liposomal pellets prepared above were suspended with the trehalose-citrate buffer, divided into 1 mL aliquots and freeze-dried overnight. The lyophilized cakes were reconstituted with water to the same volume and the potential released TFL-D was removed by gel filtration.

For the cellular association studies, 1 mol% of Rhodamine-DOPE (Rh-PE) was included in the lipid composition. It was added to the lipid mixture in the organic solvent. The remaining preparation process was the same as above.

For the remaining *in vitro* biological evaluation assays, liposomal TFL-D formulations were concentrated 4-fold, after ultra-centrifugation, by suspending the liposomal pellets in a smaller volume of the trehalose-citrate buffer. The resulting suspension was then freeze-dried overnight in 2 to 3 mL aliquots. Each lyophilized cake was reconstituted with 2 to 3 mL of water in order to reach the concentration required in each particular assay.

The liposomal TFL-D formulations used in animal studies were concentrated around 6-fold, by suspending the ultra-centrifuged liposomal pellet in a smaller volume of the trehalose-citrate buffer. The resulting concentrated suspension was divided into 10 vials with equal volume (± 1.5 mL) and freeze-dried overnight. A freeze-dried sample was reconstituted with water to the same volume and assayed to determine TFL-D concentration. The remaining vials were reconstituted with water to ±1.5 mL in order to afford 3.7 mg TFL-D/mL. The freeze-dried liposomes were reconstituted daily, a few hours before their administration to infected mice.

II-B.2.4 - Characterization of TFL-D liposomal formulations

All liposomal formulations and processes for their preparation were characterized throughout the various steps (from initial bulk solution to final liposomal suspension). The incorporation parameters used for the characterization of the final formulations, their abbreviations and equations are described in Table II-B.2.1.

An HPLC method was set-up for the quantification of free and liposomal TFL-D. The HPLC system used consisted of a Beckman System Gold Nouveau (Beckman Instruments, USA) with a 126 Pump Direct Control and a Midas type 830 auto-sampler with a 20 µL sample loop. This system is connected to specific computer software (32 Karat, version 7.0) for the integration of chromatograms. A Nucleosil C18, 5 µm (150 x 4.6 mm) analytical column (Supelco, USA) was used. The mobile phase consisted of 0.02 M sodium acetate (pH 6.55): acetonitrile (40:60). Samples were extemporaneously prepared by dilution in acetonitrile, mixed by vortex and then the appropriate volume of 0.02 M sodium acetate (pH 6.55) was added in order to have an acetonitrile:buffer ratio of 60:40. Samples were filtered into appropriated HPLC vials. The chromatographic run started with a gradient from 60 – 80 % of acetonitrile in 8 min and kept at 80 % of

acetonitrile for 5 min. At min 13 a gradient from 80 – 60 % of acetonitrile in 2 min was started. The instrumental settings were: flow rate 1 mL/min; column temperature 25° C and detection at 280 nm for TFL-A6 and at 360 nm for TFL-A3 in a diode-array Detector Module model 168 (Beckman Instruments, USA).

Phospholipid concentration was determined according to the method by Rouser, *et al* [55] and liposomes mean particle size and surface charge properties were evaluated as described for TFL liposomes in section II-A.2.4.

Table II-B.2.1 - Abbreviations and equations of TFL-D incorporation parameters

Trifluralin synthetic Derivatives	TFL-D (D = A6 or A3)	[TFL-A6 or TFL-A3]
Total Lipid	Lip	
Initial TFL-D to Lip ratio (g/mol)	$[\text{TFL-D/Lip}]_i$	
Loading Capacity (L.C.) (g/mol)	$[\text{TFL-D/Lip}]_f$	
TFL-D retention (%)	$([\text{TFL-D}]_f/[\text{TFL-D}]_i) \times 100$	
Lipid retention (%)	$([\text{Lip}]_f/[\text{Lip}]_i) \times 100$	
Incorporation Efficiency (I.E.) (%)	$([\text{TFL-D/Lip}]_f)/([\text{TFL-D/Lip}]_i) \times 100$	

II-B.2.5 - Evaluation of the *in vitro* biological behaviour of Lip-TFL-D

II-B.2.5.1 - Cellular association studies

The cellular association studies required the use of Rhodamine-DOPE (Rh-PE) labelled liposomes. Empty DMPC:DMPG liposomes or TFL-D containing DMPC:DMPG liposomes were prepared with 1 mol% of Rh-PE and incubated in concentrations 0.1, 0.4 and 0.8 mM phospholipid per well (PL/well) with one million differentiated THP-1 cells. The studies were done either at 4 or 37 ºC to distinguish between cell binding and cell

internalization. To assess the effect of the incubation time on the degree of cellular association, this assay was carried out for 0.5, 1 and 4 h. After the incubation, the cells were washed three times with cold phosphate buffered saline (PBS) (1.4 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 137 mM NaCl, pH 7.4) and lysated with 0.1% Triton X-100 in PBS (pH 8.0). The Rh-PE fluorescence was measured in the supernatant using excitation and emission wavelengths of 560 nm and 590 nm respectively, in a SpectraMax Gemini EM plate reader fluorimeter (Molecular Devices, Canada). The cellular association of TFL-D liposomes was calculated based on an Rh-PE standard curve and normalized to the amount of protein in the lysate determined by the BCA^{TM} Protein Assay Kit. Data was expressed as nmol of phospholipid per mg of protein (nmol PL/mg protein).

II-B.2.5.2 - Anti-leishmanial activity against intracellular L. infantum

The THP-1 cell line differentiated with 1 mM retinoic acid (3 days at 37 ºC, 5% CO_2), was used as the host cell line of the parasite. Differentiated THP-1 cells were infected with *L. infantum* (LEM 235) promastigotes by mixing cells and parasites, in cell culture flasks, at a ratio 1:4 followed by 24 h incubation at 37 ºC and 5% CO_2. The cellular suspension was centrifuged at 400 *x* g for 10 min and the pellet ressuspended in RPMI medium. The suspension volume was covered with an equal volume of Histopaque 1077 and centrifuged at 1000 *x* g for 20 min to remove the free promastigotes. The infected cell layer was washed with PBS (pH 7.4) twice and resuspended in RPMI at 4×10^5 cells/mL.

Free and liposomal TFL-Ds, in concentrations ranging from 50 to 0.4 µM, were mixed with 200 µL of infected THP-1 cells in a 24 well tissue culture plate (Cellstar, Greiner). Incubation proceeded for 48 h at 37 ºC and 5% CO_2. After incubation cells were smeared onto glass slides in a cytocentrifuge (Cytospin, Japan) at 45 x *g* for

1 min, air-dried, fixed with methanol and stained in Giemsa. The percentage of infected THP-1 cells was observed microscopically at 1000 x magnification. The IC_{50} values were calculated using sigmoidal regression analysis from the data of three independent experiments [87].

II-B.2.5.3 – Anti-leishmanial activity against promastigote cultures

For this assay free TFL-D were dissolved in DMSO:ethanol, 50:50 (v:v), and free or liposomal TFL-D solutions were prepared to a final concentration of 65 mM TFL. Linear 5-fold dilutions were prepared in RPMI culture medium ranging from 100 to 0.8 µM. A volume of 200 µL of *L. donovani* promastigote culture was seeded at 2×10^6 cells/mL in a 24-well tissue culture plate. An equal volume of the appropriate compound concentration was added to the wells and promastigotes were allowed to grow for 72 h at 26 ºC. After incubation, cultures were washed in PBS, resuspended in 300 µL of HEPES buffered solution, and stained with propidium iodide (PI) and SYBR-14 using the LIVE/DEAD viability kit according to the manufacturer recommendations. Cell samples were analyzed by flow cytometry on an Epics Elite model flow cytometer (Coulter, USA) equipped with a 488 nm argon laser. Differential monitoring of the dyes was achieved by reading the green fluorescence of SYBR-14 at 545 nm and the red fluorescence of PI at 645 nm. At least 10000 cells were analyzed per sample and data analysis was performed on fluorescence intensities that excluded cell auto fluorescence and cell debris. Results were obtained as percentage of live or dead promastigotes in each sample. SYBR-14, a fluorescent nucleic acid stain, binds to the DNA of living cells while PI intercalates into double-stranded nucleic acids of dead cells only since it cannot penetrate intact membranes of live cells. Calculations of IC_{50} values were done as described in section II-B.2.5.2.

II-B.2.5.4 - Cytotoxicity on THP1 cell line

The THP-1 cell line was used to quantitatively estimate the degree of cytotoxicity of free and liposomal TFL-D in a macrophage-like cell line. In this assay, THP-1 cell cultures were incubated at 1×10^6 cells/mL during 72 h at 37 ºC with free and liposomal TFL-D, in concentrations ranging from 50 to 6.25 µM. After incubation, the cells were suspended in labelling buffer (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4) containing 12 µM of PI and 100 nM of SYBR-14. The cytotoxicity was analysed by flow cytometry as described in section II-B.2.5.3.

II-B.2.5.5 - Haemolytic activity on human whole blood

The haemolytic properties of free and liposomal TFL-D were assessed since it is intended to use these formulations as injectable pharmaceuticals. This assay is used as a biomarker of a general toxic effect to the cell membrane. Free and liposomal TFL-D were diluted with PBS and distributed in a 96 well plate (100 µL/well) in concentrations ranging from 250 to 6.25 µM TFL. EDTA-preserved peripheral blood of healthy volunteers was centrifuged to remove the serum. The red blood cells (RBCs) were resuspended in PBS (pH 7.4) and washed a total of three times. An equal volume of the RBCs suspension was added to the 96 well plates where the formulations were previously placed. After 1 h incubation at 37 ºC, RBCs were centrifuged at 800 *x*g for 10 min. The absorbance of the supernatant was measured at 540 nm with the reference filter at 620 nm. The percentage haemolytic activity of each formulation at different concentrations was estimated as $(A-A_0/A_{max}-A_0)x100$ where A_0 is the background haemolysis obtained by the incubation of RBCs with PBS and A_{max} is the 100% haemolysis achieved upon incubation of RBCs in distilled water. The HC_{50}, concentration that exhibited 50% haemolysis was also determined.

II-B.2.6 – *In vivo* therapeutic activity studies

Therapeutic efficacy of free and liposomal TFL-D was assessed in a mouse model for zoonotic visceral leishmaniasis (*L. infantum*). The therapeutic efficacy was examined in BALB/c mice (6 to 8 weeks old), each infected intraperitoneally (i.p.) with 1 x 10^7 virulent promastigotes freshly transformed. After 45 days of infection, the mice were randomly assigned to three groups. The first group was injected i.p. (150 µL) with trehalose/citrate buffer and used as a control group (not-treated). The second group was treated i.p. (150 µL) with free TFL-D (25 mg TFL-D/kg of body weight) dissolved in trehalose/citrate buffer containing 5% DMSO. The third group was similarly treated with TFL-D liposomes (25 mg TFL-D/kg of body weight) freshly reconstituted from freeze-dried cakes. To establish the best experimental conditions, mice from the three groups were daily treated for 5 consecutive days.

In all subsequent studies the treatment followed was a multiple-dose therapy schedule where mice received 10 doses for two consecutive weeks with two days interval in-between. In these studies the free TFL-D were dissolved in trehalose/citrate buffer containing 5% Tween 80. Additional control groups were included in these studies, sodium stibogluconate (Glucantime®), used as a positive control was administered subcutaneously (s.c.) in a dose of 15 mg/kg for 5 consecutive days and empty liposomes (20 µmol Lip/mouse/day), also used as control of the lipid vesicle, were administered i.p. following the same treatment schedule as the free and liposomal TFL-D. In all regimens described mice were sacrificed 3 days post-treatment and spleens were aseptically collected and weighted. Viable parasite loads in infected (control) and treated animals were estimated by limiting dilution assay [88]. The spleen from each mouse was homogenised individually in 1.5 mL Schneider's Drosophila medium supplemented with 10% heat-inactivated FCS. The homogenized spleen tissue suspensions were diluted to a total volume of 3 mL with the same

medium. An additional 1:2 dilution was made for the spleen suspensions. A volume of 200 µl of the tissue suspensions was placed into the first well and four-fold serial dilutions were distributed in 96 well plates and incubated at 24 ºC. Two weeks after incubation a sample of each well was examined under the microscope and labelled as positive or negative depending on the presence or absence of promastigotes. The final titre was set as the highest dilution for which the well contained at least one parasite and the number of parasites per gram of tissue was calculated as follows:

*[(Reciprocal titre of the last positive well * total volume of homogenised tissue) / (volume of first well * Dilution Factor)] / Weight (g) of homogenised tissue*

The viable parasitic load was expressed as the number of *Leishmania* per gram of homogenized organ.

II-B.2.7 - Statistical analysis

Data from *in vitro* studies are expressed as mean values (±) standard deviation (SD) or as mean values (±) standard error (SEM) according to samples in each study. Statistical analysis was performed using ANOVA Single Factor. The acceptable probability for a significant difference between mean values was $p<0.05$ or as stated.

The non-parametric Mann-Whitney U test was used to compare parasite load from treated and non-treated infected mice. Differences were considered significant with a 5% significance level ($p<0.05$). Statistical analysis was performed with the SPSS 13.0 for Windows software (SPSS Inc., USA) using values from at least three independent experiments.

II-B.3 - Results

Several synthetic TFL-D were prepared starting from a commercially available chlorinated precursor, chloralin [53]. Two of these TFL-D, designated TFL-A6 and TFL-A3 were selected to be incorporated in liposomes based on their *in vitro* biological evaluation studies [53]. The compound names and chemical structures are presented below in Table II-B.3.1.

Table II-B.3.1 - Chemical structures of TFL and of the two new synthetic derivatives and their solubility in water [(a)]

Compound Name **(abbreviation)**	Chemical Structure	Water Solubility (ppm)
2,6-Dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine **(TFL)**	NO_2, N, F_3C, NO_2	0.5
4-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol **(TFL-A6)**	NO_2, NH, OH, F_3C, NO_2	11.4
2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-butylamino)-ethanol **(TFL-A3)**	NO_2, N, OH, F_3C, NO_2	22.6

[(a)] Data is from Esteves *et al.* [53]

II-B.3.1 - Incorporation of TFL-D in conventional liposomes

II-B.3.1.1 - Preparation of TFL-A6 liposomes with different lipid composition

The part A of this chapter reported a study for the incorporation of TFL in liposomes. The effect of lipidic composition, membrane fluidity, presence of cholesterol and charged lipids, on the incorporation parameters was assessed. The liposomal formulation selected for TFL was DOPC:DOPG (7:3 molar ratio).

In part B of this chapter two lipid compositions were selected to study the incorporation of the TFL-D. Based on the results obtained for TFL, both formulations contain phosphatidylcholine (PC) and phosphatidylglycerol (PG), but differ in lipid properties such as membrane fluidity. As discussed in Part A of this Chapter, the rational for using PG is the fact that this lipid has been described to target macrophages of liver and spleen, organs were *Leishmania* parasites reside [89].

TFL-A6 was incorporated in two different liposomal formulations. Liposomes were prepared as described in section II-B.2.3 and used to comparatively study the effect of lipid composition on TFL-A6 incorporation.

As shown in Table II-B.3.2, the compared phospholipids compositions were DOPC:DOPG, a mixture with T_c below 0 ºC and DMPC:DMPG with a T_c of about 23 ºC. Both liposomal formulations exhibited relevant incorporation parameters, with L.C. values of 71 and 56 g/mol for DOPC:DOPG and DMPC:DMPG, respectively, and I.E. of 93% and 74% respectively for DOPC:DOPG and DMPC:DMPG. However, the more fluid at room temperature, DOPC:DOPG formulation allowed a higher TFL-A6 retention (82%). The average sizes obtained were as expected for the preparation method used, which includes extrusion through pore size membranes of 200 nm pore diameter. In addition the final polydispersity index of all the formulations was below 0.2. The zeta

potential values are negative as the phospholipid composition includes negatively charged lipids (PG).

Table II-B.3.2 – Characteristics of two liposomal formulations containing TFL-A6.

Phospholipid Composition	TFL-D Retention (%)	L.C. (g/mol)	I.E. (%)	Mean vesicle size (nm)	Zeta potential (mV)
DOPC:DOPG 7:3	82 ± 3	71 ± 3	93 ± 5	188 ± 12	-41 ± 2
DMPC:DMPG 7:3	63 ± 9	56 ± 5	74 ± 11	178 ± 9	-43 ± 4

$[\text{TFL-A6}]_i$ = 3 µmol/mL (approx.1 mg/mL); $[\text{Lip}]_i$ = 16 µmol/mL
The data shown is an average ± SD from, at least, three independent experiments.
Vesicles were sized to a mean diameter below 200 nm with a P.I. < 0.2.

These liposomal formulations were prepared mainly with the purpose of assessing the parasitic activity of TFL-D liposomes against leishmanial infections *in vivo*. The experimental design for these studies often requires the preparation of large batches of a concentrated liposomal formulation so as to have the TFL-A6 amount and/or concentration required for the relevant study. One approach to concentrate the formulation is to ressuspend the liposomal pellet (after ultra-centrifugation) in a smaller volume of buffer. Freeze-drying the liposomal formulations is also one of the techniques used to concentrate the preparation apart from being a form to store batches of liposomes during long course animal studies. To evaluate if the combination of these two processes affects the properties and stability of TFL-A6 liposomes, both formulations were concentrated by ultra-centrifugation and then freeze-dried in the presence of trehalose, used as a lyoprotectant. The resulting lyophilised cake was reconstituted with water to the initial volume. TFL-A6 content and liposomes properties, before freeze-drying and after the reconstitution of the lyophilised cake, were compared. Results are displayed in Table II B.3.3.

***Table II-B.3.3* -** Evaluation of the freeze-drying process of different TFL-A6 liposomal formulations.

Phospholipid Composition	[TFL-A6] (mg/mL)		Mean vesicle size (nm)		Zeta potential (mV)	
	Before	After	(*)Before	After	Before	After
DOPC:DOPG 7:3	1.3 ± 0.1	1.1 ± 0.1	195 ± 3	> 2000	-41 ± 2	-40 ± 1
DMPC:DMPG 7:3	1.5 ± 0.2	1.4 ± 0.3	130 ± 12	193 ± 19	-43 ± 4	-42 ± 6

Liposomes containing TFL-A6 were ultra-centrifuged, the pellets ressuspended in citrate buffer containing 29 mM of trehalose, freeze-dried overnight and reconstituted with water to the initial volume.
(*)Vesicles were sized to a mean diameter below 200 nm with a P.I. < 0.2.

The mean diameters of the DOPC:DOPG liposomal formulation was the only parameter that presents a significant variation. The mean diameter not only increased to values a thousand times higher, they also correspond to a non-homogenous population (P.I. = 1.0), comparable to the size distribution of a non-extruded formulation. The freeze-drying process has clearly caused a massive fusion of the sized homogeneous liposomal population. As for the DMPC:DMPG formulation the mean diameter also suffered an increase. However the values obtained after the freeze-drying process were still acceptable to target MPS cells *in vivo*.

Despite the fact that the incorporation parameters were lower for the rigid formulation, DMPC:DMPG was chosen for subsequent studies as the parameters are acceptable and liposomes prepared with this lipidic mixture can be easily stored.

II-B.3.1.2 - Optimization of TFL-D liposomal formulations

Liposomal formulations of the selected TFL-D (TFL-A6 and TFL-A3) were prepared as described previously in section II-B.2.3 using DMPC:DMPG lipid mixtures. The effect of different TFL-D:Lip molar ratios and surface charge characteristics of the vesicles on the incorporation parameters and liposome properties were evaluated. Theoretical

molar ratios of lipidic components (DMPC:DMPG) of 7:3 and 9:1 as well as TFL-D:Lip of 1:4; 1:5 and 1:10, were assayed. The results for all the formulations prepared for each derivative are shown below in Table II-B.3.4.

TFL-A6 liposomes present, in general, acceptable incorporation parameters for all the experimental conditions used. The L.C. values range between 30 - 77 g/mol and 35 - 75 g/mol for DMPC:DMPG 7:3 and 9:1, respectively. These variations were for both cases closely related with TFL-D:Lip molar ratio and are similar for constant ratios. The TFL-A6 retention values were analogous for all studied formulations, regardless of TFL-D:Lip or lipidic components molar ratio (determinant of surface charge). The corresponding I.E. values were between 68 and 84%, indicating that DMPC:DMPG are appropriate lipid mixtures for incorporation of this derivative either in the 7:3 or 9:1 molar ratio. The lipid retentions obtained for this derivative range between 80 to 95% irrespective of TFL-D:Lip molar ratio or liposomal surface charge. The overall results point out that the conditions that allowed higher incorporation of TFL-A6 correspond to 1:4 TFL-D:Lip molar ratios regardless of DMPC:DMPG ratios.

For the TFL-A3 derivative, the incorporation parameters were more dependent from the experimental conditions. The liposomal formulation composed of DMPC:DMPG at 9:1 with a TFL-D:Lip molar ratio of 1:5 was found to have maximized L.C. and I.E. In general all DMPC:DMPG mixtures with the same 9:1 molar ratio showed higher parameters than the ones observed for the 7:3 ratio.

Table II-B.3.4 – Incorporation parameters of TFL-D containing liposomes as a function of lipid composition and $[TFL\text{-}D/Lip]_i$.

TFL-D	DMPC:DMPG (molar ratio)	TFL-D:Lip (molar ratio)	$[TFL\text{-}D/Lip]_i$ (g/mol)	L.C. (g/mol)	TFL-D retention (%)	Lip retention (%)	I.E. (%)	Zeta potential (mV)
TFL-A6	7:3	1:10	42 ± 1	30 ± 2	62 ± 4	90 ± 8	70 ± 4	-42 ± 7
		1:5	77 ± 13	56 ± 5	63 ± 9	85 ± 9	74 ± 11	-42 ± 6
		1:4	105 ± 3	77 ± 3	69 ± 4	95 ± 1	73 ± 3	-45 ± 5
	9:1	1:10	42 ± 2	35 ± 5	67 ± 2	80 ± 4	84 ± 6	-34 ± 7
		1:5	90 ± 2	61 ± 3	63 ± 4	93 ± 1	68 ± 3	-33 ± 6
		1:4	104 ± 2	75 ± 8	66 ± 2	92 ± 7	72 ± 8	-30 ± 5
TFL-A3	7:3	1:10	62 ± 8	43 ± 4	57 ± 6	81 ± 12	71 ± 9	-47 ± 2
		1:5	99 ± 3	49 ± 7	32 ± 5	65 ± 2	49 ± 6	-45 ± 7
		1:4	121 ± 5	55 ± 5	35 ± 9	77 ± 4	45 ± 8	-46 ± 4
	9:1	1:10	68 ± 3	58 ± 2	64 ± 8	75 ± 6	86 ± 4	-36 ± 2
		1:5	83 ± 7	75 ± 9	63 ± 4	69 ± 4	91 ± 4	-31 ± 2
		1:4	151 ± 4	75 ± 2	32 ± 9	64 ± 2	50 ± 4	-34 ± 5

Vesicles were sized to a mean diameter between 170 and 200 nm in all formulations, with a polydispersivity index (P.I.) < 0.2. Values are mean ± S.D. of at least three independent experiments. The zeta potential measurements were determined in citrate buffer, pH 5.5, 300 mOsm.
TFL-A6 $[Lip]_i$ = 16 µmol/mL; TFL-A3 $[Lip]_i$ = 20 µmol/mL

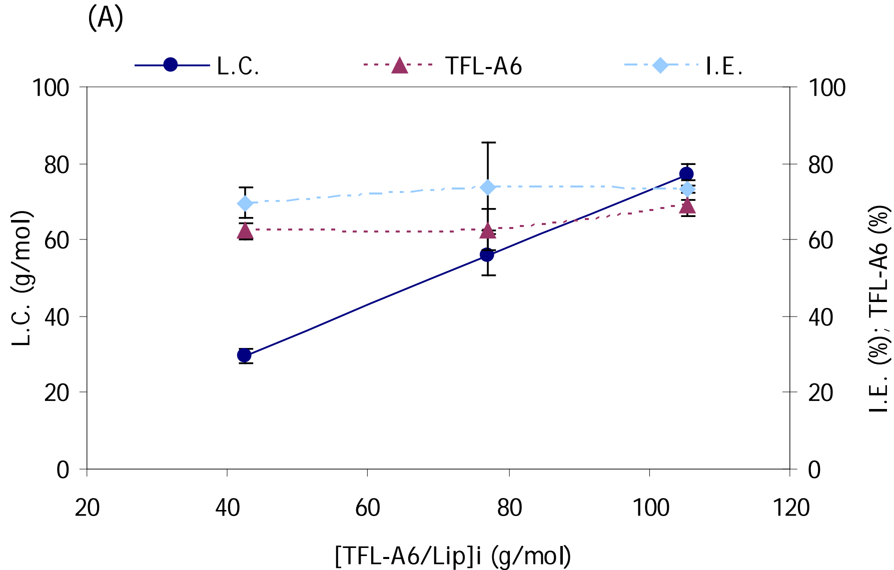
The zeta potential of the DMPC:DMPG liposomes incorporating either TFL-A6 or TFL-A3 were not significantly affected by the amount of incorporated derivatives (L.C.). This parameter is closely related to the percentage of PG in the formulation. For DMPC:DMPG 7:3 liposomes, the average zeta potential values were around -44 mV while for the 9:1 liposomes this parameter exhibits values of about -33 mV, when incorporating either TFL-A6 or TFL-A3. Nevertheless, TFL-A3 shows higher incorporation parameters for the less negatively charged formulations containing 10% DMPG (DMPC:DMPG 9:1). Conversely, the TFL-A6 shows a preference for more negative bilayer structures containing 30% DMPG (DMPC:DMPG 7:3).

II-B.3.1.3 - Effect of initial TFL-D to lipid ratio on incorporation parameters

To determine the possible saturation of the liposomal membrane with the derivatives, several experiments were carried out in the presence of increasing amounts of initial TFL-D. The dependence of $[\text{TFL-D/Lip}]_f$, I.E. and TFL-D retention as a function of the initial experimental conditions ($[\text{TFL-D/Lip}]_i$) was studied for a selected liposomal formulation from each derivative. The resulting saturation profiles are plotted in Figure II-B.3.1.

For TFL-A6 (Figure II-B.3.1, panel A) the selected liposomal formulation was DMPC:DMPG with a 7:3 lipid ratio. The L.C. parameter is strongly dependent on the $[\text{TFL-A6/Lip}]_i$, increasing from 30 to about 80 g/mol with the increase in $[\text{TFL-A6/Lip}]_i$ between 42 and 105 g/mol. The other two incorporation parameters remain constant through the range of $[\text{TFL-A6/Lip}]_i$ used (corresponding to an I.E. of about 72% and a TFL-A6 retention around 66%). These results indicate that the lipid membrane, for this derivative, is not yet saturated. Further studies, in particular animal experiments,

were performed with an [TFL-A6/Lip]$_i$ of 105 g/mol as it presents high values for I.E and drug retention.



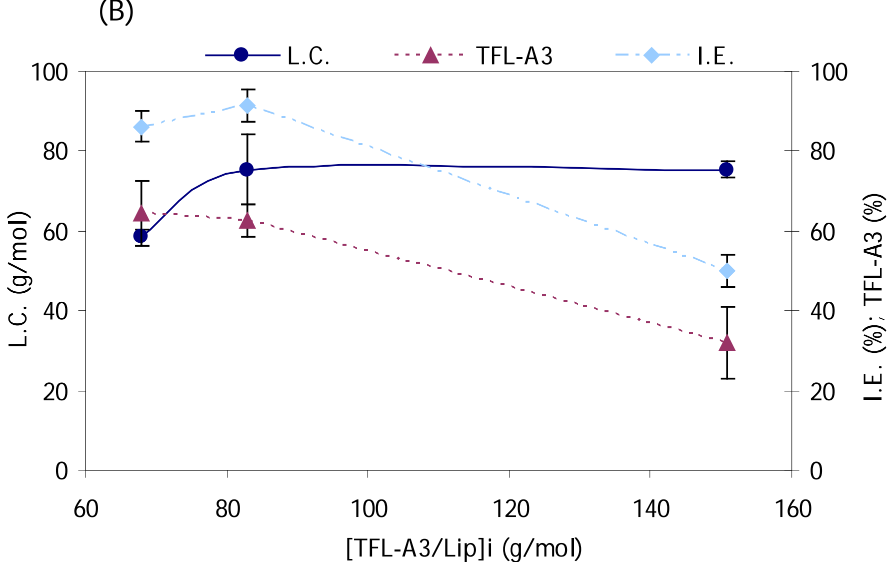


Figure II-B.3.1 – Saturation profiles for DMPC:DMPG liposomal formulations containing TFL-D. Influence of [TFL-D/Lip]$_i$ on the incorporation parameters. ***Panel A***: TFL-A6 containing DMPC:DMPG (7:3) liposomes. Lipid films (16 μmol/mL total lipid) and TFL-A6 (1.6; 3.2 and 4 μmol/mL), corresponding to [TFL-A6/Lip]$_i$ of 42, 77 and 105 g/mol, were prepared as described in section II-B.2.3. ***Panel B***: TFL-A3 containing DMPC:DMPG (9:1) liposomes. Lipid films (20 μmol/mL total lipid) and TFL-A3 (2; 4 and 5 μmol/mL), corresponding to [TFL-A3/Lip]$_i$ of 68, 83 and 151 g/mol, were prepared as described in section II-B.2.3. [TFL-D/Lip]$_i$ represents the ratio after the hydration of the lipidic film and [TFL-D/Lip]f is the same ratio after ultra-centrifugation.

The liposomal formulation selected for TFL-A3 was DMPC:DMPG (9:1) (Figure II-B.3.1, panel B) and the saturation of the lipid membrane was reached at an $[TFL\text{-}A3/Lip]_i$ of around 80 g/mol corresponding to a L.C. of about 75 g of TFL-A3 per mol of lipid. For higher initial ratios the L.C. remains constant. The I.E. is dependent on the $[TFL\text{-}A3/Lip]_i$, increasing from 86 to 91% with the increase of $[TFL\text{-}A3/Lip]_i$ from 68 and 83 g/mol and then decreasing to 50%. The TFL-A3 retention decreases from 64 to 32% over the range of $[TFL\text{-}A3/Lip]_i$ assayed. The results obtained for the later two parameters confirm that saturation of the lipidic bilayer was reached for $[TFL\text{-}A3/Lip]_i$ conditions above 83 g/mol.

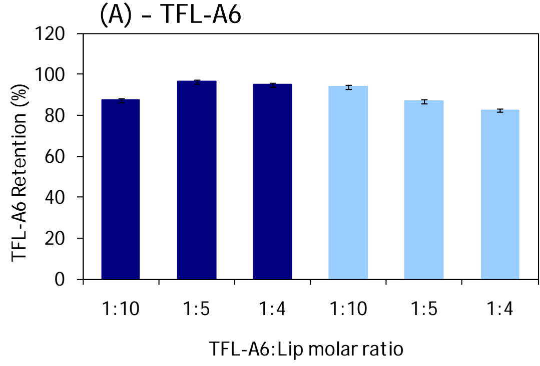
The size of the vesicles was found to be independent from the amount of TFL-D incorporated in the range studied, showing mean average values of about 185 nm for both derivatives.

II-B.3.1.4 - Stability of freeze-dried TFL-D formulations

The stability of freeze-dried TFL-D liposomes of the DMPC:DMPG lipid composition (see Table II-B.3.4 formulation details) was studied. By freeze-drying and storing aliquots of the TFL-D liposomal formulations, it was possible to prepare the total required amount of liposomes with one large batch. These large laboratory scale preparations were made for the *in vitro* and *in vivo* biological evaluation assays. The results on the effect of freeze-drying in TFL-D retention, for all formulations prepared are displayed in Figure II B.3.2.

The TFL-A6 formulations presented similar results, independent of the lipid molar ratios (7:3 and 9:1). TFL-A6 retentions were higher than 80% for all preparations which is representative of stable liposomes. The best result was observed for the

DMPC:DMPG 7:3 formulation with a TFL-A6:Lipid molar ratio of 1:5. This preparation lost less than 4% of the incorporated TFL-A6 during freeze-drying.



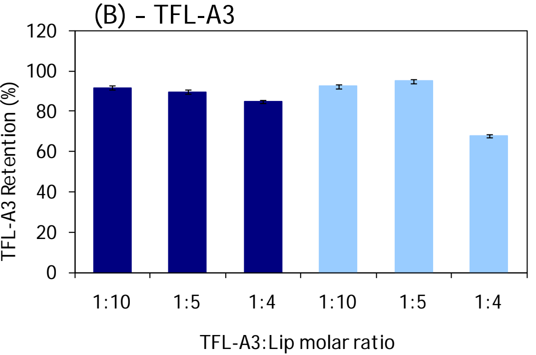


Figure II-B.3.2 – Stability of the TFL-A6 and TFL-A3 liposomal formulations in the freeze-drying process. Influence of TFL-D:Lip (1:10; 1:5 and 1:4) and DMPC:DMPG molar ratios (7:3 – dark blue bars; 9:1 – light blue bars). TFL-D retention (%) is the ratio between the concentration immediately before freeze-drying and after the procedure. The samples were reconstituted with water and filtered through a PD 10 column to remove any non-incorporated/released TFL-D. The mean particle sizes of the reconstituted liposomes range between 225 and 250 nm for both derivatives.

The results obtained for the TFL-A3 derivative are similar to those observed for TFL-A6, with an overall TFL-A3 retention over 80%, with one exception. The formulation prepared in a DMPC:DMPG molar ratio of 9:1 and with a TFL-A3:Lip ratio of 1:4 presents a lower TFL-A3 retention (68%). Interestingly, this result is in accordance with the low TFL-A3 retention (32%), obtained for DMPC:DMPG (9:1) liposomes in suspension in the incorporation studies presented in Table-II-B.3.4. This suggests that this formulation not only incorporates less TFL-A3, but after the incorporation is not stable in freeze-dried form.

Selection of TFL-D liposomal formulations

The studies presented in the previous paragraphs have demonstrated that it was possible to incorporate both TFL-D in liposomes using the same experimental

conditions and lipid composition (DMPC:DMPG) but changing the phospholipid and TFL-D/Lip molar ratios. These incorporations resulted in 77 g of TFL-A6 and 75 g of TFL-A3 per mol of total lipid. According to the results, future assays were performed with the following initial conditions:

* For TFL-A6 the liposomes were composed of DMPC:DMPG in a 7:3 molar ratio and with a TFL-A6:Lip 1:4 molar ratio.
* For TFL-A3 the liposomes were composed of DMPC:DMPG in a 9:1 molar ratio and with a TFL-A3:Lip 1:5 molar ratio.

II-B.3.2 - *In vitro* biological evaluation of TFL-D liposomal formulations

The *in vitro* biological behaviour of the new synthetic dinitroanilines either free or incorporated in liposomal formulations was estimated by several studies:

* Cellular association/uptake studies in human monocytic THP-1 cells.
* Potential adverse effects of the derivatives and their formulations evaluated by two models. In one model their effect on apoptosis of THP-1 host cell line was assessed by cytotoxicity assays; and in the other model their haemolytic activity on human red blood cells was determined.
* Anti-leishmanial activity assessed against promastigote cultures of *L donovani* and intracellular amastigote form of *L. infantum* in infected human monocytic THP-1 cells.

II-B.3.2.1 - Cellular association studies

For the cellular association studies Rh-PE was used as a fluorescent probe. In order to estimate if its inclusion in the lipid composition interfered with TFL-D incorporation,

liposomal formulations containing TFL-A6 or TFL-A3 with or without 1 mol% Rh-PE were prepared. The effect of the presence of the fluorescent probe on the incorporation parameters of both TFL-D and on the liposome properties were assessed, as shown in Table II-B.3.5. These data refers to reconstituted freeze-dried formulations made from DMPC:DMPG liposomes.

Table II-B.3.5 – Effect of the presence of Rh-PE in the incorporation of TFL-A6 and TFL-A3 in liposomes.

	$[TFL\text{-}D/Lip]_i$ (g/mol)		L.C. (g/mol)		I.E. (%)		Mean vesicle size (nm)		Zeta Potential (mV)	
Rh-PE	TFL-A6	TFL-A3	TFL-A6	TFL-A3	TFL-A6	TFL-A3	TFL-A6	TFL-A3	TFL-A6	TFL-A3
Without	105±3	83±7	77±3	75±9	73±3	91±4	177±10	190±12	-45±5	-31±2
With	67±5	70±7	54±5	51±6	81±2	73±1	192±9	203±7	-49±4	-35±6

TFL-A6:Lip molar ratio: 1:4, DMPC:DMPG (7:3). TFL-A3:lip molar ratio: 1:5; DMPC:DMPG (9:1).
Vesicles were sized to a mean diameter around 200 nm with a P.I. < 0.2.
Results are mean ± S.D. of three independent experiments

Results show comparable incorporation efficiency in the presence or absence of Rh-PE in the bilayer for both TFL-D. The reduction of L.C. (around 30% for both TFL-D) is not crucial considering the study for which they were prepared and might be due to the lower $[TFL\text{-}D/Lip]_i$ exhibited by the formulation containing Rh-PE for both TFL-D. However, the parameter that better describes the influence of a new molecule in the system is the I.E. This parameter reflects how the initial experimental conditions affect the final outcome of the formulation. Mean vesicle sizes does not show a significant increase by the presence of Rh-PE. The zeta potential of both formulations presents no changes in the presence of Rh-PE.

In general, the presence of Rh-PE does not prevent the incorporation of TFL-D and does not change in great extent the properties of the liposomes. So the Rh-PE labelled formulations can be used for future tests and stored in lyophilized form.

The influence of incubation time, temperature and lipid concentration on cellular association of TFL-A6 incorporated in DMPC:DMPG liposomes previously selected (section II-B.3.1) was assessed in differentiated THP-1 cells. To determine the potential interference of TFL-A6 on liposomal cellular association, DMPC:DMPG empty liposomes were also evaluated. The cellular association levels were determined by measuring the Rh-PE fluorescence in the cell lysate. The values were expressed in terms of total liposomal lipid per cell content protein.

Figure II-B.3.3 presents the cellular association of empty DMPC:DMPG liposomes with THP-1 cells at 4 ºC, a temperature that is non-permissive for endocytosis, and 37 ºC where endocytosis-mediated internalization can occur. The level of cellular association of these liposomes increases, for both temperatures, with the initial concentration of lipid and also with the incubation time. It has to be noted that no saturation is reached for any of the lipid concentrations used (0.1 mM to 0.8 mM) in all the incubation times assayed.

At 4 ºC, the maximum association level observed was around 80 nmol Lip/mg protein, obtained for 0.8 mM lipid. Increasing the temperature to 37 ºC produced a significant increase in the association levels. After 0.5 h incubation the association levels ranged from 32 to 163 nmol Lip/mg protein for 0.1 and 0.8 mM Lip, respectively. However, the maximum level of association, 290 ± 70 nmol Lip/mg protein, was obtained after 4 h incubation (0.8 mM). Therefore, the increase in the levels of association as the temperature is raised from 4 ºC to 37 ºC suggests that liposomes are being internalized. Nevertheless, to have a more clear understanding of internalization, the

difference between the association levels at 37 °C and 4 °C, after 1 h incubation, were calculated as an example, and results range from 41 ± 2 and 144 ± 32 nmol Lip/mg protein respectively for 0.1 mM and 0.8 mM. It is clear that the internalization increases with initial lipid concentration.

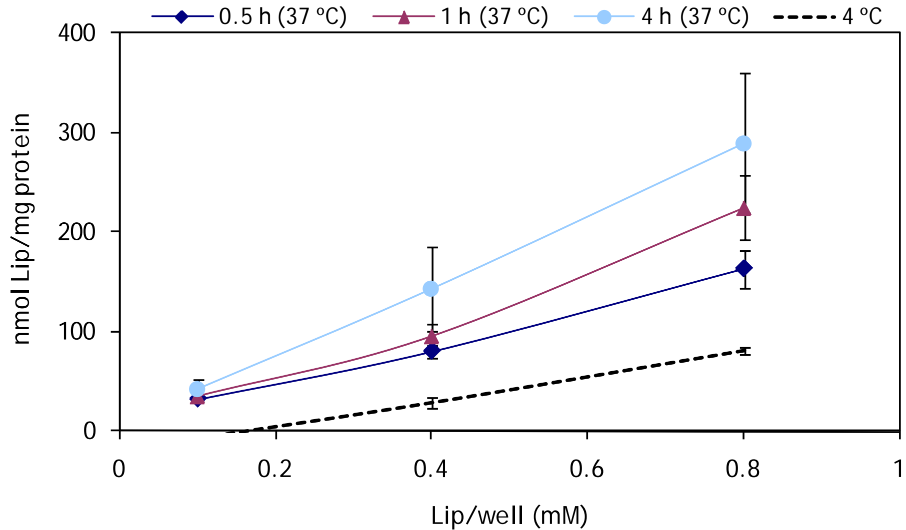


Figure II-B.3.3 - Cellular association of empty DMPC:DMPG (7:3) liposomes with THP-1 cells as a function of lipid concentration and incubation time. Empty Rh-PE-labelled liposomes (0.1, 0.4 and 0.8 mM Lip/well), were incubated with one million differentiated THP-1 cells at 37 °C for various incubation periods (0.5; 1 and 4 h) and at 4 °C for 1 h. After incubation cells were washed, lysed, and assayed to determine the Rh-PE levels of fluorescence. Data is expressed as nmol of lipid (Lip)/mg protein, and are the mean ± S.D. of six values in two independent experiments, each done in triplicate.

In Figure II-B.3.4 it is shown the cellular association profiles for TFL-A6 containing DMPC:DMPG (7:3) liposomes, in the same conditions as described before, for one hour incubation time. The level of cellular association observed for TFL-A6 liposomes in THP-1 cells increases as the lipid concentration raises from 0.1 to 0.4 mM where a level of 157 ± 37 nmol Lip/mg protein is reached. For the highest concentration tested the level of association is maintained at 158 ± 21 nmol Lip/mg protein, suggesting

saturation. It as to be noted that, at 4 °C, the levels of cellular association were in the same range as the values observed for the empty liposomes (Figure II-B.3.3.).

When temperature was raised from 4 °C to 37 °C a 3.5-fold increase in the levels of association (corresponding to around 115 nmol Lip/mg protein) was observed for the highest concentrations used, suggesting that the TFL-A6 containing liposomes were being internalized in these particular experimental conditions.

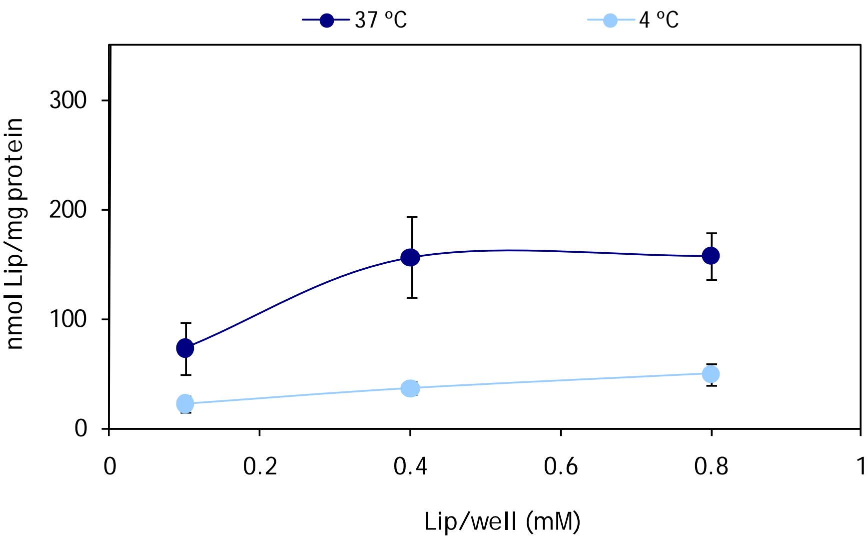


Figure II-B.3.4 - Cellular association of TFL-A6 containing DMPC:DMPG (7:3) liposomal formulations by THP-1 cells as a function of temperature and lipid concentration. Rh-PE-labelled TFL-A6 liposomes were incubated with one million differentiated THP-1 cells at 4 °C and at 37 °C for 1 h incubation period. After the incubation, Rh-PE fluorescence levels were assayed by spectrofluorometry. Data is expressed as nmol of lipid (Lip)/mg protein, and are the mean ± S.D. of six values in two independent experiments, each done in triplicate.

II-B.3.2.2 - Cytotoxicity assays of TFL-D liposomes

The assessment of the *in vitro* cytotoxicity was performed after incubation of increasing concentrations of the TFL-D liposomal formulations and free derivatives with human monocytic THP-1 cells given that these cells were used to evaluate the anti-leishmania potency of the formulations against the intracellular stages of the

parasite. Results plotted in Figure II-B.3.5 show that both TFL-A6 and TFL-A3 liposomal formulations as well as free TFL-A3 are non-cytotoxic in the range of tested concentrations, exhibiting IC_{50} values higher than 50 µM.

On the opposite free TFL-A6 presents a cytotoxic potential, with an IC_{50} value of 40 µM (Table II-B.3.6).

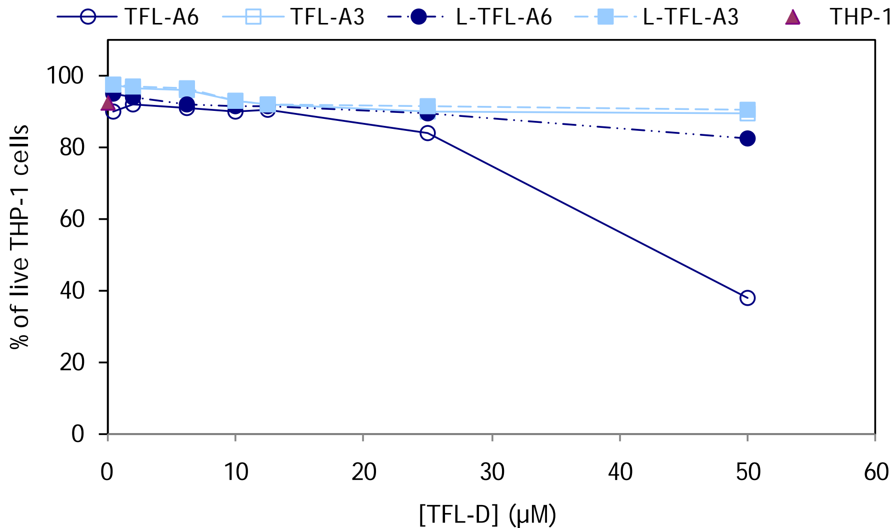


Figure II-B.3.5 - Cytotoxic activity of free and liposomal TFL-D. Percentage of live THP-1 cells in the presence of different concentrations of both free and liposomal TFL-A6 and TFL-A3 formulations. THP-1 cells were double-stained with PI and SYBR-14, and dead/alive cells were sorted by flow cytometry. The control represents non-treated THP-1 cells.

II-B.3.2.3 - Haemolytic activity of TFL-D liposomes

The haemolytic activity of the free and liposomal TFL-D was estimated against human RBCs. As Figure II-B.3.6 shows, for concentrations up to 250 µM, both TFL-D liposomal formulations and the free derivatives, did not exhibit any relevant haemolytic activity as the obtained HC_{50} values were higher than 250 µM.

These results show that for TFL-D concentrations lower than 100 µM the haemolysis is lower than 4% and the values are similar for both the free derivative and the liposomal TFL-D. For higher drug concentrations (125 - 250 µM) the haemolysis values increase.

For liposomal TFL-D, the high drug concentrations correspond to high lipid concentrations which may also contribute to the haemolysis of RBCs.

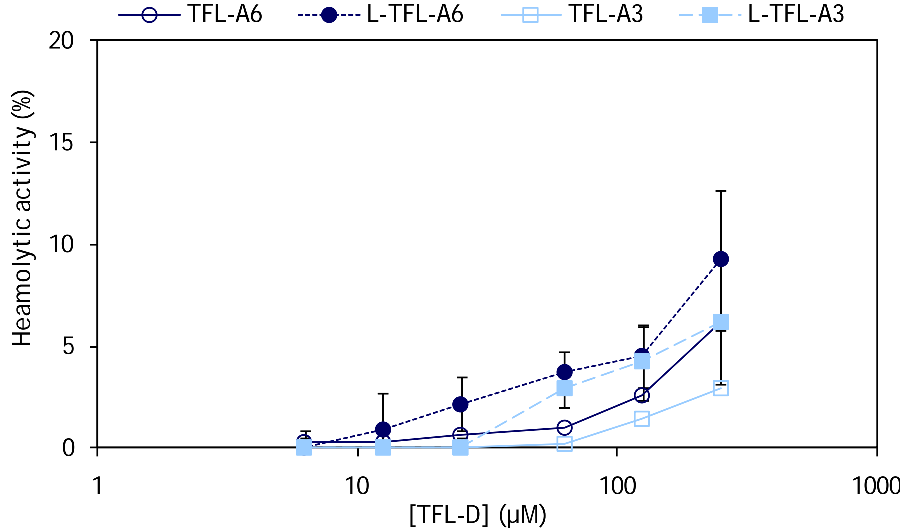


Figure II-B.3.6 – Haemolytic activity induced by free and liposomal TFL-D in RBCs. Different concentrations of free and liposomal TFL-A6 and TFL-A3 were incubated with RBCs for 1 h at 37 °C. The haemolysis was calculated by measuring the absorbance at 540 nm. The 100% haemolysis was induced upon incubation of the RBCs with water.

II-B.3.2.4 – Activity of TFL-D liposomes in vitro *against the intracellular amastigote form of* L. infantum

To study the anti-leishmanial potency of the free and liposomal TFL-D against the intracellular form of *Leishmania* parasites, the THP-1 cell line was infected with *L. infantum* LEM 235 strain and incubated with the derivatives. Results were plotted as concentration-effect curves (Figure II-B.3.7) and show that no significant differences ($p<0.05$) were obtained when comparing all the formulations and derivatives involved in the assay. In addition, the intracellular activity of both TFL-A6 and TFL-A3 liposomal formulations presents an analogous profile. None of the derivatives achieved a complete clearance of the parasite.

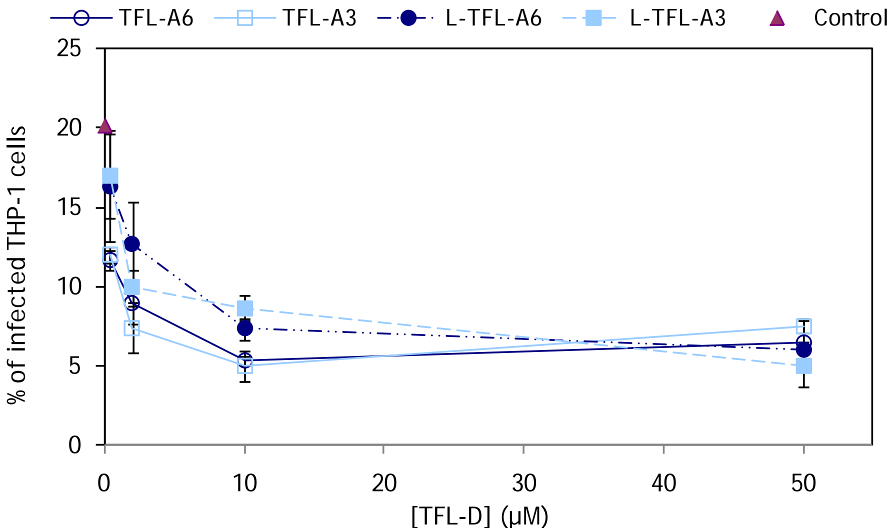


Figure II-B.3.7 – Anti-leishmanial activity of free and liposomal TFL-D against intracellular *L. infantum* amastigotes in THP-1 infected cells. Percentage of infected THP-1 cells in the presence of different concentrations of free and liposomal TFL-A6 and TFL-A3. The control represents non-treated THP-1 infected cells.

Table II-B.3.6 summarizes the results of all the assays performed for the evaluation of the *in vitro* behaviour of the TFL-D and their formulations providing a general analysis and also a comparative evaluation with the non-modified TFL and a standard drug Miltefosine. It is also presented the results of a preliminary screening of the anti-leishmanial activity of free and liposomal TFL-A6 against the free-living promastigote form of *L. donovani*. We observed that, in contrast to TFL, both the free and liposomal TFL-A6 are active against free-living promastigote, while significantly lower than that of miltefosine.

Table II-B.3.6 *- In vitro* cytotoxicity, haemolytic activity and anti-leishmanial activity *of free and liposomal TFL-D.*

Formulation	Cytotoxicity IC_{50} (µM)	Haemolysis HC_{50} (µM)	Intracellular amastigotes *L. infantum* IC_{50} (µM)	Promastigotes *L. donovani* IC_{50} (µM)
Miltefosine[(a)]	28.6±2.5	38.3±2.8	2.7	8.7±0.7
TFL[(a)]	>50	>100	>50	>100
TFL-A6	40	>500	1.8±1.3	22.4
L-TFL-A6	>50	>500	3.2±0.9	77.8
TFL-A3	>50	>500	1.2±0.4	ND
L-TFL-A3	>50	>500	1.4±0.2	ND

The cytotoxicity was evaluated in the THP-1 cell line and the haemolytic activity in human RBCs. The anti-parasitic activity was evaluated against intracellular amastigotes of *L. infantum*. The promastigotes viability was measured (by flow cytometry) after incubation with different concentrations of the formulations.
TFL-A6 and TFL-A3 = free derivatives; L-TFL-A6 = DMPC:DMPG (7:3), TFL-A6:Lip (1:4).
L-TFL-A3 = DMPC:DMPG (9:1), TFL-A3:Lip (1:5). ND = non determined.
The data are means ± SEM of three separate experiments.
[(a)] Data for Miltefosine and TFL is from Esteves *et al.* [53]

Overall, results show that both free and liposomal formulations of TFL-A6 and TFL-A3 are active against the intracellular form of the parasite with IC_{50} values ranging between 1.2 ± 0.4 and 3.2 ± 0.9 µM. In addition, all are more active than TFL (IC_{50} >50) and miltefosine (IC_{50} of 2.7 µM) with the exception of liposomal TFL-A6.

The compounds and formulations under study together with TFL displayed irrelevant haemolytic activity as compared to miltefosine.

II-B.3.3 – Evaluation of the therapeutic effect of free and liposomal TFL-D in a model of visceral leishmaniasis

The therapeutic efficacy of free and liposomal TFL-D was evaluated in a rodent model for zoonotic visceral leishmaniasis (*L. infantum*) through the determination of the parasite load reduction in the spleen. These activities were compared to that of the first line drug for treatment of leishmaniasis, Glucantime® (meglumine antimoniate) and, in order to establish the possible effect of the lipid carrier, the activity of empty liposomes was also assessed.

The first objective was to establish the best experimental conditions (*e. g.* treatment schedule) to evaluate the therapeutic activity of our formulations. For that purpose 3 groups of infected mice were treated daily for 5 consecutive days. Group 1 was treated with 25 mg/kg of liposomal TFL-A6, Group 2 with the same dose of free TFL-A6 diluted in a solution containing 5% (v/v) DMSO and Group 3 was used as control (untreated). The mice in Group 2 died during treatment probably caused by the DMSO. In addition, no reduction of the parasite load in the spleen of mice treated with liposomal TLF-A6 (Group 1) was observed when compared to the control. Thus changes were made to the experimental design. The treatment schedule was increased to 10 doses in 10 consecutive days, while the TFL-D dose administered per mouse was maintained at 25 mg/kg as well as the administration route.

The *in vivo* i.p. administration of 25 mg of TFL-D/kg for 10 days proved to inhibit parasite growth in the spleen of mice as is displayed in Figure II-B.3.8. In particular, liposomal TFL-A3 was significantly ($p < 0.01$) more active than the free drug. An almost complete removal of the parasites from the spleen with a 93% inhibition of parasite load was achieved as compared to the 48% inhibition obtained for the free drug.

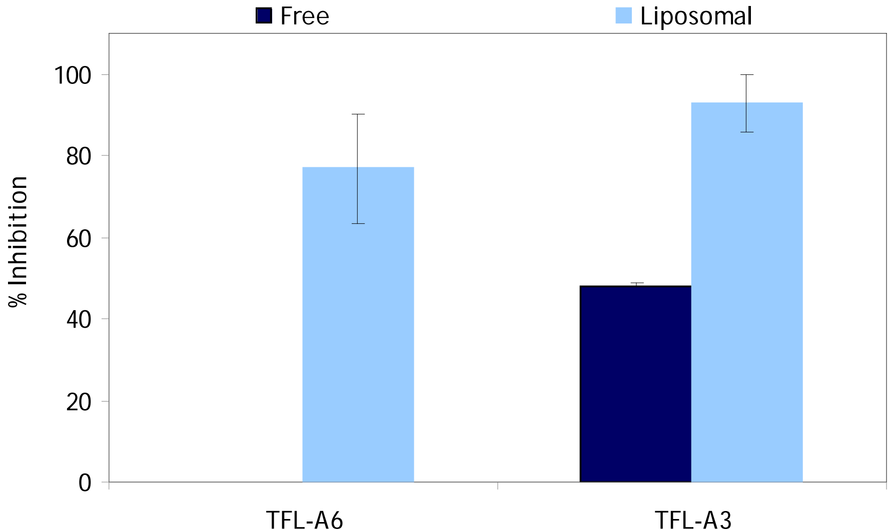


Figure II-B.3.8 - *In vivo* anti-leishmanial activity of free and DMPC:DMPG liposomal TFL-D preparations against *L. infantum* MON-1 (MHOM/PT/89/IMT151) amastigotes in the spleen of BALB/c mice. Mice were treated during days 45 to 56 post-infection with 25 mg/kg/day of the respective free or liposomal derivative (TFL-A3, L-TFL-A3 and L-TFL-A6), by i.p. route. The control group received the same 10 daily doses of only the vehicle (Citrate/trehalose buffer). The parasite load in the spleen was determined at day 60 post-infection by the limiting dilution assay. Results are expressed as the inhibition (%) of spleen amastigotes growth relative to numbers in untreated controls. Each point is the mean ± SD of three to four independent experiments.

The reduction of spleen amastigotes obtained for liposomal TFL-A6 (77% inhibition) was not significantly different ($p<0.05$) from that obtained for liposomal TFL-A3. The treatment with free TFL-A6 displayed very irregular results, inducing either no reduction in the number of viable parasite or an accentuated inhibition in *Leishmania* growth (91%) (data not shown). In an attempt to improve these results, the activity of TFL-A6 incorporated in fluid liposomes (DOPC:DOPG 7:3 molar ratio) was also evaluated using the same experimental conditions as in the above studies. However, no reduction in the number of viable parasites was observed for this formulation when compared with non-treated animals, indicating that the liposomes prepared with more rigid lipids were, nonetheless, more appropriate (data not shown).

In order to evaluate the effect of the liposomal carrier on the therapeutic activity of the TFL-A3 liposomal formulation, empty liposomes with the same lipid composition were assayed. In these studies Glucantime® was also included as an anti-leishmanial standard drug. The therapeutic activity of these formulations was estimated by comparing the parasite loads in infected spleens of treated animals with those of untreated mice. As shown in Figure II-B.3.9, treatment with Glucantime caused a reduction of parasite load about 2.5 fold lower compared to the inhibition induced by liposomal TFL-A3 (96%). In contrast, treatment with empty liposomes had little effect on *Leishmania* growth, generating only 15% of parasite load inhibition. These results confirm the therapeutic activity of the liposomal TLF-A3 formulations and the absence of effect of empty liposome in zoonotic visceral leishmaniasis.

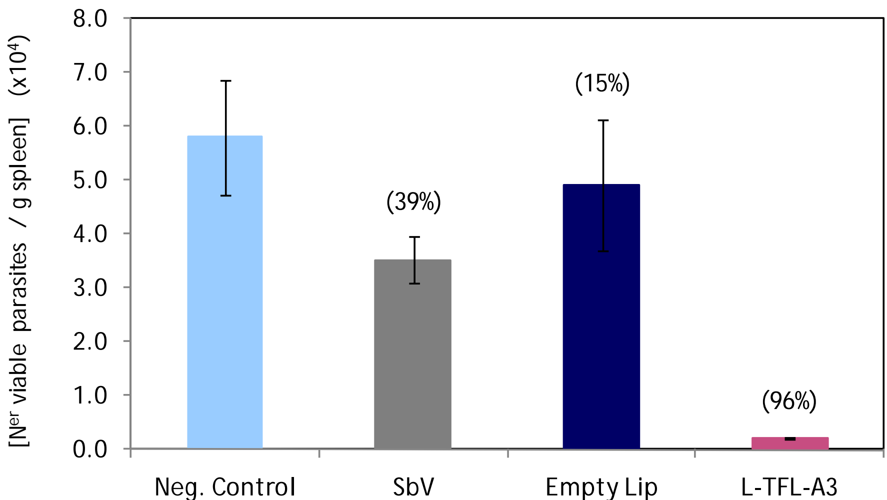


Figure II-B.3.9 - *In vivo* therapeutic effect of glucantime (Sb^V), empty and TFL-A3 DMPC:DMPG liposomes against *L. infantum* in comparison to untreated mice in the spleen of BALB/c mice. Mice infected with *L. infantum* MON-1 (MHOM/PT/89/IMT 151) received 10 doses (i.p.) of 25 mg TFL/kg of L-TFL-A3 or 20 µmol Lip/mouse of empty liposomes. Glucantime (Sb^V) was administered s.c., 15 mg Sb^V/kg, 5 doses. Negative control group received 10 doses of the vehicle (Citrate/trehalose buffer). The parasite load in the spleen was determined by the limiting dilution assay. Numbers in brackets correspond to the % of parasite load inhibition. Results are expressed as the number of viable parasites per gram (g) of homogenized spleen. Data represent mean values ± SEM derived from four mice per group in one representative experiment.

II-B.4 – Discussion

In Part A of this chapter it was described the incorporation of one dinitroaniline, TFL, in conventional and long-circulating liposomes, their *in vitro* and *in vivo* behaviour. Moreover, therapeutic activity assays in animal models have demonstrated that liposomal TFL is active against different strains of *Leishmania* infecting both mice and dogs. Despite the good therapeutic results, no complete elimination of parasites was reached [51, 52].

The search for new effective dinitroaniline compounds, driven by both the need to improve the properties of TFL formulations and anti-leishmanial activity, has led to the synthesis of new TFL derivatives (TFL-D) [53]. There was also the need to construct tailor made liposomes with properties that enable the delivery of the new active TFL-D to the sites of *Leishmania* infection. The work performed in Part B, represents a step further in the search for alternative strategies for the treatment of leishmaniasis, by using liposomal formulations of novel dinitroanilines.

The pharmaceutical optimisation of liposomal TFL-D through the study of critical incorporation parameters was one of the objectives of this chapter. The study of the *in vitro* and *in vivo* behaviour of the optimised formulations was another objective. Thus, the first approach was to incorporate, in these liposomes, two chemically synthesized TFL-D molecules, with proven *in vitro* anti-leishmanial activity [53]. To fulfil this objective, the effect of the lipid composition, surface charge properties, TFL-D to lipid ratios and lyophilisation on TFL-D incorporation in macrophage directed conventional liposomes was studied [90, 91]. Taking advantage from the successful incorporation of TFL (Part A), a similar lipid composition was chosen for the incorporation of TFL-D in liposomes. The selection of a second lipid composition, presenting higher T_c was based on previous work by Gaspar *et al.* [92]. They reported using rigid lipids containing the negatively charged PG moiety with the aim to achieve a more stable formulation for

in vivo administration of rifabutin. With similar objectives, the use of rigid lipids was also adopted by others [93], namely to immobilise the antifungal hydrophobic drug Amphotericin B, also active against *Leishmania* [94, 95].

Accordingly, we have compared the formulation properties of one of the derivatives incorporated in fluid (DOPC:DOPG) and rigid (DMPC:DMPG) liposomes and have observed that, in general, both formulations present good incorporation parameters. The hydrophobic nature of TFL-A6 anticipated a high interaction between this molecule and the hydrophobic domain of the liposomal bilayer. The greater the order of the lipid acyl chains, the lower L.C. and TFL-A6 retention was observed. These results are in agreement with the incorporation of hydrophobic drugs like rifampicin [96], rifabutin [67] and paclitaxel [69]. However, the exclusion of any of the formulations from further *in vitro* and *in vivo* studies can not be based only on the lower incorporation parameters. It is well known that the *in vivo* behaviour of liposomal formulations is significantly influenced by the mean diameter, the fluidity and the charge of the membrane, among other factors [67]. These characteristics are also responsible for the release rate of the incorporated substance from liposomes [97]. The maintenance of the liposome properties after freeze-drying and reconstitution of the formulations was an additional and very important factor to select the lipid composition of the formulations. This procedure allowed us to prepare and store the necessary amount of liposomes either for the *in vitro* or *in vivo* studies. The freeze-drying process of the TFL-A6 formulations was done in the presence of trehalose acting as a lyoprotectant [51, 61]. These studies allowed to select the DMPC:DMPG lipid composition over the DOPC:DOPG as this later formulation, although retaining TFL-A6 after the freeze-drying/rehydration cycle, suffered a drastic increase in the vesicle mean size suggesting massive liposome fusion and/or aggregation. This different behaviour might be due to significant interactions between trehalose and saturated lipids, like DMPC,

that are markedly reduced in the DOPC based liposomes [98]. In the present studies, 1% (w/v) trehalose was adequate to lyoprotect the DMPC:DMPG formulation incorporating TFL-D. This represents a technological advantage as it allows for an easier and straightforward manipulation of the formulations. Opposed to the need for 10% trehalose for DOPC:DOPG liposomes incorporating TFL, which were more viscous and difficult to manipulate [51]. Apart from the different percentage of trehalose used for both lipids, these results suggest that the type of derivative incorporated seems to play a role in the stabilisation of the formulation during freeze-drying and so for each molecule different conditions are needed.

The DMPC:DMPG lipid composition was selected for the remaining incorporation studies with TFL-D, that is the optimisation of the molar ratios of the phospholipids and the incorporation studies. These studies suggested that each molecule had a preferred TFL-D:Lip molar ratio and liposome surface charge properties to achieve maximal incorporation: TFL-A6 (containing a phenol group as the substituent) required a lower TFL-D:Lip molar ratio and a more negative liposomal surface charge, while TFL-A3 (containing an alcohol group as the substituent) preferred a less negative liposomal charge but needed a higher TFL-A3 to lipid molar ratio.

The incorporation profiles of both TFL-D in liposomes as a function of the $[TFL\text{-}D/lip]_i$ (Figure II-B.3.1), followed the common behaviour consistently observed either for hydrophilic [99] or hydrophobic molecules [100]. However, the experimental conditions required to reach saturation were also dependent from each TFL-D, indicating a different interaction of the molecules with the phospholipid matrix. Another important fact was the observed independency of the zeta potential from the amount of both TFL-D incorporated in liposomes, indicating that these molecules are inserted in the lipidic matrix and do not expose any charged portion to the external medium.

These observations allow concluding that there is no 'uniform rule' for the efficient incorporation of a bioactive agent in a liposomal formulation which might be experimentally established. It relies on two main features: the parameters that influence liposome properties, (*e. g.* preparation method, liposome size, surface charge and bilayer rigidity) and the characteristics of the molecule being incorporated [101]. The overall studies have established that the use of DMPC:DMPG lipid mixture for the incorporation of both TFL-D molecules resulted in liposomes that accommodate the desired pharmacological characteristics, including high TFL-D loadings and acceptable homogeneous sizes (below 200 nm) required for their *in vitro* and *in vivo* use. The option of freeze-dry these liposomes adds yet another advantage to this formulation as this technique is well accepted for long-term storage of liposomes [61].

The *in vitro* biological evaluation of the selected TFL-D liposomal formulations was focused on cellular association, potential toxic effects and evaluation of anti-leishmanial activity. To study the cellular association of the liposomal formulations, assays, well accepted in the literature, were performed [102, 103]. In our studies, based on the differences obtained between the non-specific cell binding and the cellular internalization of liposomes it was concluded that empty DMPC:DMPG liposomes were internalised by THP-1 cells in culture in an incubation time and phospholipid concentration dependent manner (Figure II-B.3.3). However, the association profile for this formulation containing TFL-A6 did not follow the exact same behaviour. Although a reasonable level of cellular association was observed, the absolute values were lower and saturation was reached. These observations may indicate that TFL-A6 interferes with the internalization of DMPC:DMPG liposomes by THP-1 cells. This interference might be due to modifications of the fluidity and/or the liposomes surface properties related to the intercalation of TFL-A6 within the

phospholipids acyl chains. There are no data in literature on similar effects on liposomes internalisation due to incorporation of hydrophobic drugs.

Regarding the elucidation of possible adverse effects of free and liposomal TFL-D, these were assayed through the estimation of the cytotoxicity and the haemolytic activity. The incorporation of TFL-A6 in liposomes represents an advantage over the free derivative documented by the increase of the IC_{50} of the liposomal formulation over the free derivative revealing absence of cytotoxicity against differentiated THP-1 cells within the range of concentrations used ($IC_{50} > 50$ µM). The neglectable haemolytic activity in RBCs (< 10% at 500 µM) demonstrated by all TFL-D liposomal formulations, can be considered as an additional important advantage in regard to the standard and commercial drug miltefosine which lyses 96% RBCs at 100 µM [53].

To assess the *in vitro* anti-leishmanial activity of the free and liposomal TFL-D the intracellular amastigote form of *L. infantum* was chosen as this form is considered the more relevant parasitic stage for compound biological evaluation [104]. Both liposomal TFL-Ds proved to be active with liposomal TFL-A3 being more potent than miltefosine whereas liposomal TFL-A6 appears to have similar activity as the commercial drug [53]. In addition, these studies have also confirmed that these molecules retain their activity against *L. infantum* infecting THP-1 cells when incorporated in the liposomes.

Considering that the final aim of this chapter (part B) was to deliver TFL-D to infected MPS cells, we have studied the anti-leishmanial activity of free and liposomal TFL-A6 and TFL-A3 in a murine model of zoonotic visceral leishmaniasis where the infection is predominantly found in the liver and spleen. We intended to investigate whether the *in vitro* biological behaviour of TFL-D can be translated into *in vivo* activity against *L. infantum* infections and whether the incorporation of these derivatives into conventional liposomes has any therapeutic advantage.

The selected liposomal formulation encloses the main features needed to deliver TFL-D to the target organs: conventional liposomes prepared with rigid lipids and containing PG moieties. Liposomes, containing this phospholipid head group have been described to deliver antibiotics to MPS cells [105, 106].

The *in vivo* studies have proven that treatment of *L. infantum* infection with the new synthetic TFL-Ds reduces the parasite load in the spleen of treated mice. The higher efficacy, demonstrated by the TFL-D molecules when incorporated in conventional liposomes as compared to the free derivatives, represents a therapeutic advantage in delivering these compounds. The observed therapeutic effect was not due to the phospholipids constituents of the liposomes, as empty liposomes did not induce any noticeable therapeutic activity, but rather to their role as specific NanoDDS to the sites of infection. In addition both TFL-D liposomal formulations were more active than the standard drug Glucantime®. Nevertheless, an interesting liposomal constituent to be explored in the future is stearylamine (SA) as it is described that, alone or in combination with anti-leishmanial drugs (*e. g.* amphotericin B or sodium antimony gluconate), is active against *Leishmania* and other protozoan parasites *in vitro* and *in vivo*, without causing any adverse effect on the host [32, 37, 38, 107].

The *in vivo* activity of both TFL-Ds is in accordance with the IC_{50} values obtained against the intracellular amastigote form of *L. infantum* (Table II-B.3.6) with a superior *in vivo* activity offered by the TFL-A3 molecule over TFL-A6. The TFL-A3, after being incorporated in liposomes, was responsible for more than 95% reduction of parasite growth in the spleen in treated mice. This result confirms not only the role of liposomes as delivery systems, but also the potential of TFL-A3 as an anti-parasitic drug. Regarding TFL-A6, the incorporation in liposomes represents the best option to assess the anti-leishmanial activity of this molecule, since the activity of the free compound was not reproducible revealing a very irregular behaviour, probably due to

solubility problems. The *in vivo* results for liposomal TFL-A6 revealed a lower activity than TFL-A3 in addition to some lack of reproducibility. Further studies are required to improve the therapeutic efficacy of TFL-A6 liposomal formulations. These may include different lipid compositions, different doses, treatment schedules and routes of administration.

The different *in vivo* behaviour observed for both TFL-D tested, enhances the close relation between the substituent introduced in the TFL molecule (phenol in TFL-A6 or alcohol in TFL-A3) and its therapeutic activity. However to establish a structure/activity relationship, a systematic study of a series of related compounds would be needed.

In conclusion, these studies reinforce the idea that liposomes contribute to the efficient intracellular delivery of active TFL-D compounds with a subsequent reduction of their potential negative side effects relative to the free molecules (i.e. TFL-A6). In addition, the findings reported here have established that the design and synthesis of chemical compounds, derived from classes of bioactive agents with proven anti-leishmanial activity, is an alternative approach for the search of new active formulations for the treatment of *Leishmania* infections. The important reduction of viable parasites induced by the treatment with TLF-A3 incorporated into liposomes in this mouse model indicates that this compound can be valuable to treat zoonotic visceral leishmaniasis. It is worth mentioning that the liposomal formulations containing the TFL-D have also eliminated the need for the detergent used for the solubilisation of the free derivatives. To fully explore the anti-parasitic potential of the more active TFL-D further studies should include their incorporation in liposomes with other lipidic composition, namely the inclusion of SA in the formulation, different types of nanoparticles and the treatment of zoonotic visceral leishmaniasis in a canine model.

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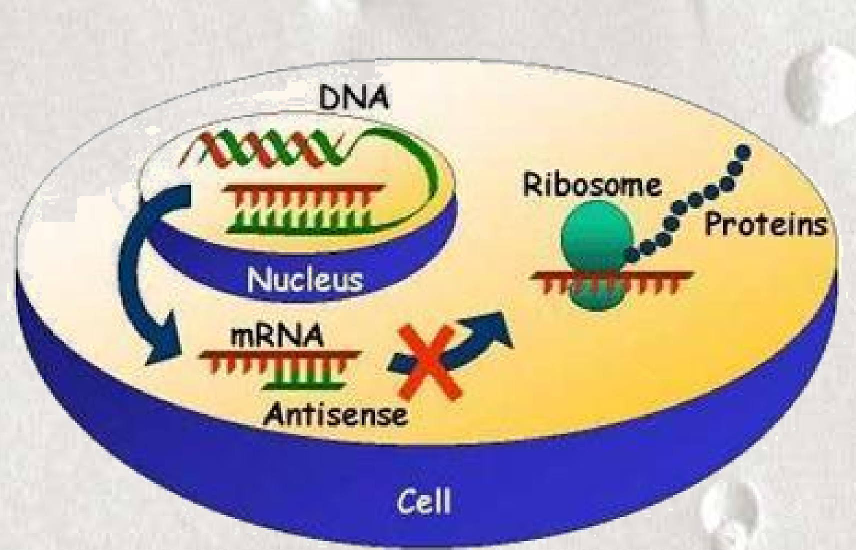
CHAPTER III

Development of antagonist G-targeted lipid-based vesicles for delivery of antisense oligodeoxynucleotides to human small cell lung cancer cells



http://www.odec.ca/projects/2004/mcgo4s0/public_html/t3/RNA.html

III-1 - Introduction

A particularly aggressive neuroendocrine histological subtype of lung cancer is the SCLC, which accounts for 15% of all pulmonary cancers. It is characterized by early and widespread metastasis and by rapid development of resistance to cytotoxic agents [1]. The high rate of SCLC patients that relapse from chemotherapy resistant disease is not exclusive of this type of cancer. In fact, most anticancer agents used in current conventional chemotherapeutic treatments lack specificity to tumour cells limiting the use of high-dose intensity therapy, due to severe systemic and organ toxicities [2] (see Chapter I). These limitations have lead to the need of novel and more selective types of anticancer agents with less side effects for the normal tissues.

Advances in molecular biology have created the concept of treating various human diseases such as cancer, infections, inflammations and genetic disorders by gene-based therapies [3, 4]. Gene silencing-based therapeutics, like antisense oligonucleotides (asODN) or small interfering RNA (siRNA) are examples of such a new class of anticancer agents that can prevent the initiation or progression of specific human cancers when targeted to appropriate molecular targets [5, 6, 7].

In cancer, malignant cells exhibit a different pattern of gene expression as compared to normal cells. These identified genetic differences, for example activated oncogenes and inactivated tumour suppressor genes, can be considered as possible targets for these new antitumor therapies [7, 8]. One of the genetic alterations observed in SCLC, which is correlated with poor prognosis, is amplification and over-expression of the *c-myc* oncogene [9]. The *c-myc* protein is important in many cellular processes such as proliferation, differentiation, and apoptosis and also in the response to antitumor agents [9]. The down-regulation of the expression of *c-myc* in cancer cells is associated with an inhibition of cell proliferation [10]. Based on these

features, the *c-myc* oncogene appears to be a good target for the treatment of SCLC, providing that, for example, appropriate asODN that target *c-myc* are available.

The asODNs are single-stranded DNA molecules that work by hybridisation to corresponding RNA inhibiting the translation of the mRNA of the target gene, through several possible mechanisms [11, 12, 13]. This results in down-modulation of gene expression at the transcriptional stage [2]. Therapeutic benefits from the use of asODNs that target *c-myc,* either alone or in combination with other drugs, have been reported in the literature in recent years [14, 15, 16, 17, 18]. Their use as therapeutic agents is however limited due to poor stability in physiological fluids, high susceptibility to nuclease degradation, unfavourable pharmacokinetics, lack of target cell recognition and limited ability to penetrate through cellular membranes [3, 19, 20, 21]. Approaches involving the chemical modification of the asODN have been used to address the instability issues. However, natural and modified asODN molecules can not cross membranes by passive diffusion and their access to the intracellular compartment is limited [22, 23]. A possible strategy to attend to this difficulty is through the engineering of appropriate asODN NanoDDS, which effectively and selectively deliver the asODN-based drugs into the target cells, upon systemic administration [24, 25, 26].

In general, the main desired benefits of the use of appropriated NanoDDS are an enhanced plasma circulation lifetime and an increased delivery to sites of the disease [27]. In addition, delivery systems for asODN-based drugs should also act as a protective enclosed space that needs to be inert and stable while in circulation. Once the tumour site is reached the carrier systems, together with the asODN, needs to be efficiently internalized by the tumour cells [28]. To reach these goals a number of different non-viral NanoDDS have been developed [reviewed in 3, 4, 13, 27].

Cationic lipid-based systems, namely cationic liposomes, have become one of the most representative non-viral NanoDDS for the intracellular delivery of nucleic acids to cells in culture and *in vivo* [29, 30]. These positively charged liposomes, most often composed of a synthetic lipid bearing a cationic head-group formulated with other neutral lipids, form spontaneously complexes with the asODN molecules due to the electrostatic interaction between negative charges of oligonucleotides and positive charges of lipids [12, 25, 31, 32]. The modification of their outer lipid membrane with the incorporation of PEG-modified lipids stabilizes and prolongs the circulation lifetime of the asODN-containing liposomes, and enhances the accumulation at the disease site [31, 33, 34]. In particular, various sorts of cationic liposomes have been developed that significantly enhance the intracellular delivery of ODN [reviewed in 29, 30, 35].

Two of the most promising PEG-grafted cationic lipid-based NanoDDS are coated cationic liposomes (CCL), developed by Stuart *et al.* [11, 36] and stabilized antisense lipid particles (SALP), developed by Semple *et al.* [24]. Both formulations have crucial requirements like long-circulation half-lifes, good stability in plasma, to properly deliver asODN. However, for systemic asODN delivery systems to be efficient in cancer therapy they ought to specifically target and deliver the nucleic acids to tumour cells and/or to multi-located tumour sites, i.e. metastasis [28 ,37].

The covalent coupling, onto the surface of these systems, of specific internalizing targeting ligands, can lead to a therapeutic advantage over non-targeted liposomes. It was described that anticancer drugs loaded into ligand-targeted liposomes show an increased cytotoxicity to tumour cells thus improving their therapeutic efficacy [38]. To covalently attach specific ligands to the reactive terminus of a hydrophilic polymer such as PEG grafted onto liposomes, several methods have been described [39, 40, 41]. One of the most simple and flexible method, termed the post-insertion

technique, was developed by Ishida *et al.* [42]. It involves the coupling of ligands (including antibodies, antibody fragments, peptides, carbohydrates, etc.) to the terminal of PEG-derivatized lipids in a micellar phase resulting in ligand-PEG conjugates. These conjugates can be easily transferred, in a simple incubation step, onto the outer layer of pre-formed, drug-loaded liposomes. This technique allows the preparation of tailor-made targeted liposomes, irrespective of the incorporated drug.

Thus, to meet a successful treatment of SCLC, lipid-based systems should be constructed from the association of antisense oligodeoxynucleotides against *c-myc* (*as(c-myc)*) with small cationic liposomes sterically stabilized with PEG. This system could be further improved by the addition of a targeting ligand for selective cellular delivery to SCLC cells. The choice of the specific targeting ligand is very important to maximize the therapeutic effect of the active agent (*as(c-myc)*). It should take into account the fact that SCLC cells secrete multiple neuropeptides growth factors whose receptor-mediated actions can be inhibited by several peptide antagonists [43, 44]. The mechanism of action is not yet clear, but studies suggest that antagonist G (H-Arg-DTrp-N^{me}Phe-DTrp-Leu-Met-NH_2) competitively inhibits the binding of neuropeptides to their receptors in the cell membrane [45]. This characteristic and the fact that it binds to a broad range of structurally unrelated neuropeptide receptors, makes antagonist G a good choice as a targeting device to be attached to the surface of liposomal formulations to improve treatment of this disease [46]. Moreira *et al.* [46, 47, 48, 49] reported the use of antagonist G, as a targeting agent for sterically stabilized liposomes containing doxorubicin in the treatment of human SCLC.

The aim of this part of the work was to construct and optimize liposomes with antagonist G as a ligand to specifically target and internalize liposomal formulations

containing an *as(c-myc)* by SCLC cells. With this intention we have selected two liposomal formulations (CCL and SALP) for the encapsulation of asODN, assessed their characteristics (comparatively), and have evaluated whether the post-insertion coupling method could be applied without harming liposome's properties. The potential of the antagonist G-targeted formulation was further assessed in terms of its cellular association towards the target cells and its biodistribution in healthy mice.

III-2 - Materials and Methods

III-2.1 – Reagents and Materials

Hydrogenated soy L-α-phosphatidylcholine, (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol)-2000] (PEG-DSPE); 1 1,2-dioleoyl-sn-glycero-3-trimethylammonium propane (DOTAP); cholesterol (CHOL), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (Mal-PEG-DSPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (PEG-CerC16) were purchase from Avanti Polar Lipids (Alabaster, USA). 2-Iminothiolane·HCl (Traut's reagent) was purchase from Fluka (Buchs, Switzerland). Sephadex G-50 and Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino)-ethanesulfonic acid (MES), RPMI 1640; penicillin-streptomycin and foetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The CHOD-POD enzymatic colorimetric assay for the determination of cholesterol was purchase from Spinreact (Sant Esteve de Bas, Spain). [1α,2α(n)-^{3}H]Cholesteryl hexadecyl ether, 1.48-2.22 TBq/mmol ([^{3}H]CHE) was purchased from Perkin-Elmer, Inc. (Walthman, USA). All other chemicals were of analytical grade.

The hexapeptide antagonist G (H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2) was synthesized by Alberta Peptide Institute (Edmonton, AB, Canada) with a purity >95%. A stock solution was prepared in water to a concentration of 5 mM. The 16-mer full phosphorothioate oligodeoxynucleotide against *c-myc*, referred to hereafter as *as(c-myc)* (5′-TAACGTTGAGGGGCAT-3′) was synthesized by TriLink BioTechnologies,

Inc. (San Diego, USA). Purity was > 95% by high-performance liquid chromatography analysis. The *as(c-myc)* is complementary to the translation region of human *c-myc* mRNA. A stock solution was prepared by the hydration of the freeze-dried powder with filtered sterile water to a concentration of 10 mg/mL. The solution was aliquoted and frozen at -70 ºC until needed.

III-2.2 - Cell lines and mice

The human classical SCLC cell line NCI-H69 (ATCC HTB-119) and the human variant SCLC cell line NCI-H82 (ATCC HTB-175) were from American Type Culture Collection. Both cell lines were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 2.5 g/L glucose and 25 mM HEPES for H69 or 10 mM HEPES for H82, pH 7.4 (full medium). Cell lines were maintained in the logarithmic phase of growth at 37 ºC in 75 cm^2 plastic culture flasks in a humidified incubator (90% humidity) containing 5% CO_2.

BALB/c mice, 6 to 8 weeks old, were purchased from Gulbenkian Institute of Science (Oeiras, Portugal), and housed at FFUL animal facilities fulfilling the European Union Council Directive (86/209/CEE), recognised by Portuguese law (DR DL129/92 and Portaria 1005/92). The animals were kept under standard hygiene conditions, fed commercial chow and given acidified drinking water ad *libitum*. All experiments were conducted, under licence, according to the local laboratory animal committee guidelines and after approval from competent authorities (DGV).

III-2.3 – Preparation of *asODN*-containing liposomes

III-2.3.1 - Coated cationic liposomes

Coated Cationic Liposomes (CCL), composed of HSPC:CHOL:DOTAP:mPEG-DSPE, in a 3:2:1:0.2 molar ratio, and containing the *as(c-myc)* were prepared in accordance with the procedure from Stuart *et al.* [36]. This method is based on the use of a reverse evaporation procedure where an outer monolayer of neutral lipids coats the exterior of the *as(c-myc)*:DOTAP particles. All the lipids used were first dissolved in chloroform to a final concentration of 10 mM. According to this method, liposomes are prepared in two steps following the process described below. A 500 µg aliquot of *as(c-myc)* (± 0.1 µmol) was dissolved in 250 µL of water. In a separate tube 510 µL of methanol were added to 1.52 µmol of DOTAP in 250 µL of $CHCl_3$. The *as(c-myc)* aqueous solution was added to this mixture and vortex mixed to form a Bligh-Dyer monophase [50] where ODN:DOTAP inverted micelles were formed. After 30 min incubation at room temperature, 250 µL of water and 250 µL of chloroform were added, vortexed and centrifuged (800 x *g*, 8 min) to separate into two phases. The upper aqueous methanol phase was removed and the absorbance measured at λ = 260 nm to determine the amount of *as(c-myc)* extracted. Under these conditions 90%–95% of the *as(c-myc)* complexed with DOTAP was recovered. The asODN:DOTAP complexes present a 1:15 molar ratio and a corresponding charge ratio of 1:1 (-/+) (each ODN molecule has 15 negative charges).

HSPC, Chol, and PEG-DSPE were added to the ODN:DOTAP inverted micelles emulsion to give a molar ratio of HSPC to Chol to DOTAP to PEG-DSPE of 3:2:1:0.2. Subsequently, 400 µL of water were added to give a lipid concentration of 20–30 mM. The mixture was vortexed and emulsified by sonication for 1 min. The organic phase was then removed under vacuum (45 °C, ~500 mmHg) on a rotary evaporator until a

gel phase was reached. Then 500 µL of water were added and additional evaporation lead to the inversion of the system from a gel to a liquid phase, which was vigorously vortex mixed and liposomes were formed. To prevent contamination with residual chloroform the milky liposomal suspension was further evaporated.

The CCL formed by this procedure were reduced to a mean size of approximately 100 nm by a sequential extrusion process through Nucleopore (Pleasanton, USA) polycarbonate filters, with pore mean size ranging from 400 to 100 nm, using a Lipex extruder (Lipex Biomembranes, Vancouver, Canada) heated at 65 ºC.

Free *as(c-myc)* was removed from CCL by filtration down a Sepharose CL-4B column equilibrated with HEPES buffer, pH 7.4 (25 mM HEPES, 140 mM NaCl).

III-2.3.2 – Stabilized antisense lipid particles

Stabilized antisense lipid particles (SALP), composed of DSPC:CHOL:DODAP:PEG-DSPE or of DSPC:CHOL:DODAP:PEG-$CerC_{16}$ in a 20:45:25:10 molar ratio, were prepared according to Semple *et al.* [24]. Lipid stock solutions were prepared in 100% ethanol to a 20 mg/mL concentration. The PEG-$CerC_{16}$ ethanolic solution was prepared at 50 mg/mL. Typically, a mixture of the appropriate amounts of lipids from stock solutions was added to a glass tube in order to have approximately 10 mg/mL (13 µmol) total lipid. In a separate glass tube, 2 mg of the *as(c-myc)* stock solution was diluted in 0.6 mL of filtered 300 mM citrate buffer, pH 4.0. Both tubes were heated at 65 ºC for 2 min and the lipids were added, drop by drop, to the *as(c-myc)* (with the help of a Pasteur pipette), while mixing constantly with vortex. The resulting emulsion composed of ~13 µmol of total lipid and 2 mg of *as(c-myc)* was sequentially extruded through polycarbonate membranes with a pore diameter of 0.2 (once) and 0.1 µm (ten times). This step was performed at 65 ºC and the resulting vesicles presented an average diameter of 100 - 120 nm. The liposomal suspension was

dialyzed (12-14 kDa cut-off) against 300 mM citrate buffer, pH 4.0 for 2 h to remove excess ethanol, leading to formation of multilamellar vesicles [24]. DODAP was neutralized by the removal of the citrate buffer from the preparation through a further overnight dialysis against HBS buffer (20 mM HEPES, 145 mM NaCl, pH 7.6). This procedure also releases any asODN associated with the outer surface of the liposomes. The non-encapsulated *as(c-myc)* was then removed by ultracentrifugation (180 000 x *g* for 2 h in a Beckman L8-60M ultracentrifuge (Beckman Instruments, Fullerton, USA).

III-2.4 - Antagonist G-targeted CCL and SALP

The CCL and SALP developed formulations were coupled with a growth factor antagonist, known as antagonist G (H-Arg-DTrp-N-^{me}Phe-DTrp-Leu-Met-NH_2). Two methods were developed for the coupling of antagonist G to CCL and SALP. In both methods the antagonist G was covalently linked to the PEG terminus using the Mal-PEG coupling method [51].

Regardless the coupling procedure, an appropriate volume of a 5 mM solution of antagonist G in water was thiolated at the N-terminus by 2-iminothiolane (20 mM), at a iminothiolane:antagonist G molar ratio of 4:1, for reactivity toward the maleimide. This reaction occurred in HEPES buffer, pH 8.0, for 1 h at room temperature with occasional mixing. The activation and coupling of antagonist G to liposomes took place in silicon-coated glassware (Sigmacote, Sigma).

III-2.4.1 - Conventional coupling method to prepare antagonist G-targeted CCL and SALP

The thiolated antagonist G was added to the CCL or SALP liposomes at an antagonist G/lipid molar ratio of 1:100 and the vial sealed under N_2 stream. The reaction took

place for 12 to 18 h at room temperature. Maleimide groups that remained free after the incubation with the antagonist G were quenched upon reaction with 2-mercaptoethanol dissolved in HEPES buffer, pH 6.5, at a Mal-PEG-DSPE/2-mercaptoethanol molar ratio of 1:5. This reaction took place at room temperature for 30 min. The G-CCL and G-SALP formulations were separated from non-coupled antagonist G by ultracentrifugation (184000 x *g*, 2 h). The pellets were suspended in HEPES buffer, pH 7.4.

III-2.4.2 - Post-Insertion method to prepare antagonist G-targeted CCL and SALP

Micelles composed of Mal-PEG-DSPE were prepared as previously described [47] by the lipid film hydration method. Lipid films containing Mal-PEG-DSPE were hydrated at a concentration of 0.5 mM in HEPES/MES buffer (25 mM HEPES, 25 mM MES, 140 mM NaCl, pH 6.5). The micelles suspension was added to the thiolated antagonist G at a 2:1 Mal-PEG-DSPE/antagonist G molar ratio. The coupling reaction was performed overnight at room temperature, in an inert N_2 atmosphere. Free maleimide groups were neutralized as described above.

The antagonist G-targeted liposomes (either CCL or SALP) prepared by the post-insertion method (PI(G-CCL)) resulted from the insertion of antagonist G-PEG-DSPE conjugates onto preformed liposomes, upon incubation of G-coupled PEG-DSPE micelles with non-targeted liposomes (either CCL or SALP), at 60 ºC for 1 h. The resulting targeted vesicles were separated by gel filtration through a Sepharose CL-4B column (Pharmacia, Sweden) equilibrated with HEPES buffer, pH 7.4.

III-2.5 – Characterization of CCL and SALP formulations

III-2.5.1 - asODN quantification

Due to their molecular structures, nucleic acids maximal absorption occurs at $\lambda = 260$ nm. A linear relationship exists between the absorption of light and a wide range of nucleic acid concentrations. According to the manufacturer data by entering the extinction coefficients into the Beer-Lambert equation, an absorbance (A) of 1.0 (at $\lambda = 260$ nm) corresponds to 30.7 µg/mL for phosphorothioate oligodeoxynucleotides. Thus the amount of asODN encapsulated in both formulations was determined by spectrophotometry in a Shimadzu UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Before quantification, CCL or SALP samples were disrupted to release all the encapsulated *asODN*. The method used consists on the following procedure:

1. take 10 - 50 µL of CCL or SALP sample;
2. add water up to 250 µL;
3. add 750 µL of chloroform:methanol (1:2.1, vol/vol);
4. add 100 µL of methanol;
5. vortex.

The absorbance of this mixture, a clear single phase, was immediately measured at $\lambda = 260$ nm against a similar blank containing 250 µL of water instead of sample. The *asODN* concentration was calculated according to the following equation:

$$[asODN]\ (\mu g/mL) = A_{260} \times 30.7\ \mu g/mL \times 1.1\ mL/\text{sample volume}\ (\mu L)$$

III-2.5.2 - Total lipid quantification

The total lipid quantification was carried out using a commercial kit (Spinreact, Spain) for an enzymatic-colorimetric determination of cholesterol (CHOD-POD) present in the samples. This method is based on the formation of a coloured complex. The intensity of the colour formed is proportional to the cholesterol concentration in the sample. The amount of total lipid is calculated from the value obtained for cholesterol using the theoretical initial molar ratios of all the lipid components for each type of formulation (CCL and SALP).

In some studies, particularly in cellular association and biodistribution studies, CCL and SALP formulations were labelled with the non-metabolizable, non-exchangeable radioactive tracer, [^{3}H]CHE, during their preparation. In these cases the total lipid concentration was determined from the specific activity counts of the [^{3}H]CHE tracer in a Beckman LS-6800 Scintillation counter (Beckman Instruments, Fullerton, USA).

III-2.5.3 - Vesicle size determinations

The mean particle diameter was measured by photon correlation spectroscopy (PCS) with a Malvern Zetasizer 3 (Malvern, UK). As a measure of the particle size distribution the polydispersity index (P.I.) was used, ranging from 0 (monodisperse) to 1.0 (polydisperse). Liposomal samples (20-50 µL) were diluted to 1.0 mL with the respective buffer prior to the determinations.

III-2.5.4 - Zeta potential determinations

Zeta potential was determined by Laser Doppler Anemometry, using a Malvern Zetasizer 3 (Malvern, UK). Liposomal samples were prepared with their filtered suspension buffer in order to have 3 mL of a 3 mM total lipid concentration.

III-2.5.5 - Antagonist G quantification

The amount of antagonist G coupled to the liposomes was determined by fluorimetry at λ_{ex} = 288 nm and λ_{em} = 330 nm in a Hitachi F-3000 Fluorescence Spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) and was based on the quantification of the tryptophan residue present in the peptide.

III-2.5.6 - Encapsulation parameters

The encapsulation parameters used to characterize all the formulations tested in this work were the Loading Capacity (L.C.), the Encapsulation Efficiency (E.E.) and the Insertion Capacity (I.C.) (when applied).

The Loading Capacity (L.C.) (g/mol) was defined as the ratio between the amount of encapsulated *asODN* (expressed in g) and the amount of Lip(expressed in mol of total lipid) in the final CCL and SALP preparations. The L.C. can be expressed by the following equation:

$$L.C. = \frac{[asODN]_f}{[Lip]_f} \text{ (g/mol)}$$

The Encapsulation Efficiency (E.E.) was expressed as the percentage of the quotient between the L.C. and the initial asODN to Lip ratio. The following equation was used to determine this parameter:

$$E.E. = \frac{L.C.}{[asODN]_i / [Lip]_i} \times 100 \text{ (\%)}$$

This parameter is a measure of the capability of the initial system (ODN and lipid) to efficiently encapsulate asODN in liposomal form.

The Insertion Capacity (I.C.), defined as the amount (in grams) of antagonist G, per mol of Lip determined after preparation, was used to characterize the antagonist G-targeted formulations. The following equation was used to determine this parameter:

$$I.C. = \frac{[\text{Antagonist G}]_f}{[\text{Total Lipid}]_f}\,(g/mol)$$

Abbreviations and other equations used are as follows:

Antisense oligonucleotides	*asODN*
Total Lipid	Lip
Initial *asODN* to Lip ratio (g/mol)	$[asODN/Lip]_i$
asODN retention (%)	$([asODN]_f/[asODN]_i) \times 100$
Lipid retention (%)	$([Lip]_f/[Lip]_i) \times 100$

III-2.6 - Cellular Association Studies

Several CCL and SALP liposomal formulations were used, including non-targeted CCL and SALP formulations, and antagonist G-targeted formulations, prepared by conventional coupling technique (G-CCL) and by the post-insertion method (PI(G-CCL) and PI(G-SALP)). All of these formulations contained *as(c-myc)* and were prepared with 55.5 kBq of $[^3H]$CHE per µmol of total lipid. The lipid concentration was determined by the specific activity (cpm/µmol TL) from β-counts using a LS-6800 counter (Beckman Instruments, Fullerton, USA).

The SCLC cell lines H69 and H82 were plated at 1×10^6 cells/well (100 µL) in 48-well plates. An equal volume of the above-mentioned liposomal formulations was added at

concentrations ranging from 0.1 to 0.8 mM. Incubation took place either at 4 ºC or 37 ºC, for 1 h in a humidified atmosphere containing 5% CO_2.

In competition experiments, sterically stabilised liposomes with or without coupled antagonist G (G-SL and SL, respectively) composed of DSPC:CHOL:PEG-CerC16:Mal-PEG-DSPE in a 2:1:0.08:0.02, were prepared by the lipid film hydration method and downsized by extrusion to a mean vesicle size below 120 nm as previously described [46]. H82 cells were pre-incubated either with G-SL, corresponding to 0.6 µg antagonist G/well or with SL liposomes (at lipid concentration that matched the one for G-SL), for 30 min at 37 ºC. In experiments with endocytosis inhibitors H82 cells were pre-incubated for 30 min at 37 ºC with 0.45 M sucrose/well. After the pre-incubation period, SALP and PI(G-SALP) formulations were added and the incubation proceeded at 37 ºC in a humidified atmosphere containing 5% CO_2 in air for 1 h.

After incubation, 750 µL of cold (kept at 4°C) PBS buffer, pH 7.4 (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4) was added to each well and the plates were centrifuged at 1000 rpm for 10-12 min. The supernatant was aspirated and an additional 1 mL of the same buffer was added. This procedure was repeated once more, for a total of three washes, and the cells were resuspended in 0.5 mL PBS, pH 7.4. The cellular suspension of each well was then transferred to scintillation vials with 5 mL of aqueous counting scintillant (ACS). Cellular association of liposomes was calculated from the specific activity of the lipid label [^{3}H]CHE in liposomes and was expressed as nmol of total lipid (TL)/10^6 cells.

III-2.7 – Tissue Distribution Studies

Male BALB/c mice, (20-25 g) received injections via tail vein (i.v.) of a single dose of SALP liposomes (targeted or non-targeted), in a total volume of 0.2 mL (1 µmol Lip/mouse). At selected time points (30 min, 2, 6 and 24 h post-injection) groups of

four mice per liposomal formulation were anesthetized and sacrificed by cervical dislocation. A blood sample was withdrawn from the retro orbital sinus before sacrifice, and the liver, spleen and lungs were excised. The blood (50 µL) and organ (50 µg) samples were discoloured with 0.2 mL of hydrogen peroxide and 0.1 mL of perchloric acid overnight in an oven at 50 ºC. Samples were then neutralized with 0.1 mL of acetic acid and transferred to 10 mL Hionic Fluor scintillation fluid. Total radioactivity was measured using a Beckman LS-6800 Scintillation beta counter (Beckman Instruments, Fullerton, USA). Aliquots of the injected liposomes (10 µL) before and after being subjected to the same treatment as the samples were simultaneously counted to correct the physical decay. The results were expressed as percentage of the injected dose per mL of blood or gram of tissue.

III-3 - Results

Ligand-targeted liposomes have the potential to increase the therapeutic efficacy of anti-cancer drugs. As such, one of the aims of this work was the development of a targeted liposomal formulation formed by cationic lipids, sterically stabilized with poly(ethylene glycol)-derivatized lipids and encapsulating an *as(c-myc)* oligonucleotide, to selectively target SCLC cell lines and with adequate features for systemic administration.

III-3.1 – Physicochemical characterisation of non-targeted and antagonist G-targeted CCL and SALP liposomes

With the intent of establishing whether antagonist G could be coupled to the surface of liposomes we have adapted and prepared the previously described CCL and SALP liposomal formulations containing an *as(c-myc)* oligonucleotide. The preparation of the antagonist G-targeted CCL and SALP liposomes was assayed using either conventional coupling techniques, or the post-insertion approach. The influence of the coupling method on the targeting efficiency of the resulting formulations to SCLC cell lines was assessed.

To ensure a valid evaluation of all liposomal formulations, the encapsulation parameters, already used in the previous chapter, L.C. and E.E., the mean diameter and zeta potential properties of the final preparations were systematically determined. Particular attention was paid in keeping the mean size of the particles around 100 to 150 nm, while achieving a maximization of the encapsulation parameters, specially the L.C.

The encapsulation parameters and properties of non-targeted CCL and SALP and the antagonist G-targeted CCL and SALP formulations, prepared either by the conventional

(G-CCL and G-SALP) or the post-insertion (PI(G-CCL) and (PI(G-SALP)) coupling methods, are displayed in Table III.3.1.

Table III.3.1 – Encapsulation parameters for non-targeted and antagonist G-targeted CCL and SALP liposomes.

Formulation	L.C. (g/mol)	E.E. (%)	I.C. (g/mol)	Particle Size (nm)
CCL	78 ± 13	87 ± 15	n.a.	140 ± 12
G-CCL	27 ± 4	33 ± 5	3 ± 1	177 ± 16
PI(G-CCL)	56 ± 10	68 ± 9	4 ± 0.1	189 ± 23
SALP	125 ± 34	71± 22	n.a.	106 ± 10
G-SALP	48 ± 6	31 ± 4	2.4 ± 1	89 ± 17
PI(G-SALP)	58 ± 8	53 ± 5	1.7 ± 0.5	118 ± 8

Non targeted: CCL [HSPC:DOTAP:CHOL:PEG-DSPE (30:10:20:2)]; SALP [DSPC:CHOL:DODAP:PEG-$CerC_{16}$ (20:45:25:10)]; Targeted: CCL [HSPC:CHOL:DOTAP:PEG-DSPE:Mal-PEG-DSPE (30:20:10:1.6:0.4); SALP [DSPC:CHOL:DODAP:PEG:DSPE:Mal-PEG-DSPE] (20:45:25:8:2). Both formulations were coupled at a 1:100 antagonist G/Lip molar ratio.
n.a. – not applicable.

The non-targeted CCL and SALP formulations show an intraliposomal concentration of the asODN higher than 75 g of *as(c-myc)* per mol of total lipid. Both formulations present suitable particle size and a membrane charge characteristic of a neutral formulation.

The coupling of antagonist G to both CCL and SALP formulations led to a reduction of the encapsulation parameters. This reduction was higher when the targeted formulations were prepared by the conventional method. In particular for G-CCL, the L.C. and E.E. decreased by 65% and 62% respectively, as compared to the non-targeted formulation. The sizes of the targeted vesicles prepared by both coupling methods suffered an increase of 40 to 50 nm. When antagonist G-targeted

SALP liposomes were prepared by either coupling methods, a reduction in the L.C. and E.E. was also obtained as compared with the non-targeted SALP. The highest reductions were observed for G-SALP formulation with a 62% reduction in the L.C. and 56% for the E.E. parameter. For the PI(G-SALP) an average reduction of 54% was obtained for the L.C. The average size of the liposomes remained in the same range.

The I.C. values obtained for antagonist G-targeted CCL prepared either by conventional or post-insertion methods were comparable corresponding to an average of 3.5 g of antagonist G/mol of lipid. As for the SALP formulation these values were lower, specially in the case of PI(G-SALP).

Based on the above results among the CCL targeted formulations studied, we selected the PI(G-CCL) formulation as a candidate for *in vitro* studies with SCLC cells, as evidencing the highest incorporation parameters.

In respect to the targeted SALP formulation, as there was a significant reduction in the encapsulation characteristics, additional studies were performed before testing these formulations with SCLC cell lines.

III-3.2 - Optimisation of the PI(G-SALP) formulation

Several studies were conducted in order to optimise the PI(G-SALP) formulation to carry the *as(c-myc)* to the SCLC cells. These studies are described below.

III-3.2.1 - Effect of the preparation method on the as(c-myc) *encapsulation parameters*

In a first stage the insertion of the antagonist G-PEG-DSPE conjugates was attempted in different steps of the SALP preparation process (Table III.3.2). In addition, these formulations were compared with G-SALP.

Table III.3.2 – Characterization parameters of PI(G-SALP) containing *as(c-myc)* prepared by modifications of the original SALP procedure in comparison with G-SALP.

Formulation	Insertion of G-PEG-DSPE conjugates	L.C. (g/mol)	E.E. (%)	I.C. (g/mol)	Particle Size (nm)
PI(G-SALP)1	After extrusion (Citrate buffer, pH 4.0)	84 ± 11	72 ± 17	3.2 ± 0.3	97 ± 10
PI(G-SALP)2	After dialysis against Citrate buffer, pH 4.0	63 ± 3	51 ± 8	2.9 ± 0.5	96 ±14
PI(G-SALP)3	After dialysis against HBS buffer, pH 7.5	33 ± 5	23 ± 6	1.2 ± 0.8	81 ± 10
G-SALP	Conventional coupling	48 ± 6	31 ± 4	2.4 ± 1.0	89 ± 17

Antagonist G:Mal-PEG-DSPE = 1:2 molar ratio; antagonist G:Lip = 1:100 molar ratio
I.C. = Insertion Capacity = amount (gram) of antagonist G per mol of Lipid
$[Lip]_i$ = 13 µmol/mL; $[as(c\text{-}myc)]_i$ = 2 mg/mL

As shown above the transfer of antagonist G-PEG-DSPE conjugates to SALP after extrusion (PI(G-SALP)1) resulted in the highest encapsulation parameters for *as(c-myc)* and the best I.C. for antagonist G. On the other hand the PI(G-SALP)3 formulation presented the lowest encapsulation parameters with an E.E. of 23% and a L.C. of only 33 g of *as(c-myc)* per mol of lipid and also a I.C. value of 1.2 g of antagonist G per mol of lipid. These values were also lower than the ones obtained for the G-SALP formulation. The mean sizes of these liposomes were not affected either by the preparation or coupling methods.

III-3.2.2 - Effect of PEG-DSPE versus PEG-CerC$_{16}$

The following experiments were conceived to elucidate the effect of two PEG-derivatized lipids on the encapsulation parameters of the PI(G-SALP) liposomes containing *as(c-myc)*. The two PEG-derivatized lipids involved in this study were the PEG-DSPE having a negative charge on the phosphate moiety at physiological pH and a

neutrally charged PEG-lipid in which the PEG moiety is linked to a ceramide (PEG-CerC_{16}). Table III.3.3 presents the results for both the PI(G-SALP) formulations composed either by DSPC:CHOL:DODAP:PEG-DSPE or DSPC:CHOL:DODAP:PEG-CerC_{16} and named PI(G-SALP)[DSPE] and PI(G-SALP)[Cer], respectively. These formulations were prepared by inserting the G-PEG-DSPE conjugates after the liposome extrusion (as in PI(G-SALP)1, from Table III.3.2). The PI(G-SALP)[Cer] presented higher L.C., with similar E.E. and I.C., than liposomes containing PEG-DSPE, and comparable vesicle sizes.

Table III.3.3 – Characterization parameters of PI(G-SALP) prepared with two different PEG-derivatized lipids (PEG-DSPE and PEG-CerC_{16}).

Formulation	L.C. (g/mol)	E.E. (%)	I.C. (g/mol)	Particle Size (nm)
PI(G-SALP)[DSPE]	65 ± 15	38 ± 17	2.2 ± 0.2	113 ± 7
PI(G-SALP)[Cer]	90 ± 3	49 ± 13	2.6 ± 0.4	109 ± 6

$[Lip]_i$ = 13 μmol/mL; [*as(c-myc)*]I = 2 mg/mL. Antagonist G was coupled in a peptide/total lipid molar ratio of 1:100.

III-3.2.3 – Effect of the ratio between PEG-CerC_{16} and Mal-PEG on the as(c-myc) *encapsulation*

The effect of the concomitant variation of PEG-CerC_{16} and Mal-PEG-DSPE on the encapsulation parameters of antagonist G-targeted SALP liposomes was assessed. For these experiments five PI(G-SALP) formulations were prepared with different amounts and molar ratios of total PEG-derivatized lipids. The formulations were labelled according to the molar ratio of the two different PEG-derivatized lipid included in the bilayer (Table III.3.4). The PEG-CerC_{16}:Mal-PEG-DSPE molar ratio varied between 0.66:1 and 4:1. For these formulations the molar ratio between the Mal-PEG-DSPE and the antagonist G was kept constant at 2:1. Consequently, the more Mal-PEG-DSPE was

included in the SALP formulation, the more antagonist G was initially present during the coupling procedure.

Table III.3.4 - Characterization parameters of PI(G-SALP) liposomes prepared with different amounts of PEG-derivatized lipids (mol% PEG-CerC$_{16}$: mol% Mal-PEG-DSPE).

PEG-CerC$_{16}$: Mal-PEG-DSPE (molar ratio)	Total PEG (mol% Lip)	L.C. (g/mol)	E.E. (%)	I.C. (g/mol)
(8:6)	14	37 ± 8	34 ± 2	8 ± 3
(8:4)	12	46 ± 7	42 ± 3	7 ± 2
(8:2)	10	61 ± 11	56 ± 12	8 ± 3
(6:4)	10	66 ± 7	62 ± 9	9 ± 2
(4:6)	10	48 ± 8	45 ± 6	5 ± 2

As(c-myc) was encapsulated in SALP liposomes composed of DSPC:CHOL:DODAP:PEG (20:45:25:X molar ratio), where X= 10, 12 or 14 mol% of total PEG (PEG-CerC$_{16}$ + Mal-PEG-DSPE). Antagonist G was coupled in peptide/Lip molar ratio of 1:100. The mean particle sizes of all the resulting liposomes ranged from 100 – 120 nm.

The (8:2) and (6:4) formulations presented the highest encapsulation parameters, both with a total of 10 mol% of PEG-derivatized lipids. In this case the reduction of the amount of the pre-existing PEG-CerC$_{16}$ had no influence on the physical parameters of the liposomes. This may denote that the SALP structure remains stable with less pre-existing PEG-CerC$_{16}$ (6 mol%) during the post-insertion procedure, allowing the insertion of antagonist G-PEG-DSPE conjugates without an increase in asODN leakage. The L.C. values of the remaining PI(G-SALP) formulations were one third lower suggesting the occurrence of some asODN leakage. The I.C. parameter presented similar values for all the PI(G-SALP) formulations. Antagonist G-targeted SALP formulations prepared by the post-insertion method with the inclusion of

between 4 and 8 mol% PEG-CerC_{16} present good physicochemical characteristics for its application as a target system.

The results from all the studies described above have demonstrated that the post-insertion coupling method applied both to CCL and SALP liposomes containing *as(c-myc)*, results in antagonist G-targeted formulations with the necessary characteristics to be evaluated in *in vitro* studies for the delivery of asODN to SCLC.

III-3.3 – Cellular association of antagonist G-targeted liposomes

Cellular association experiments were carried out to determine whether the presence of the antagonist G, covalently attached to the terminus of PEG, at the surface of both CCL and SALP liposomal formulations containing *as(c-myc)* would increase the *in vitro* internalization of these liposomes into H69 and H82 SCLC cell lines.

III-3.3.1 – Effect of total PEG on the extent of cellular association to SCLC cell lines

This set of experiments aimed at establishing whether different PEG-CerC_{16} to Mal-PEG-DSPE ratios and/or total amount of PEG present in the liposomal surface affects the cellular association of PI(G-SALP). Formulations described in Table III.3.4 (section III.3.2.3) were radiolabeled with [^{3}H]CHE during their preparation and incubated with the H82 cell line at appropriated conditions.

Results in Figure III.3.1 show that the cellular association levels of PI(G-SALP) liposomes decreased when the amount of total PEG-derivatized lipids increases from 10 to 14 mol%, at the expenses of increasing amounts of Mal-PEG-DSPE. In particular,

the inclusion of 6 mol% of antagonist G-Mal-PEG-DSPE micelles into formulations containing 8 mol% PEG-Cer-C_{16} reduced approximately by half, the association as compared to the others. In contrast, no PEG coating effect was observed when different PEG-CerC_{16} to Mal-PEG-DSPE molar ratios were tested, while keeping constant the amount of total PEG-derivatized lipids at 10 mol%.

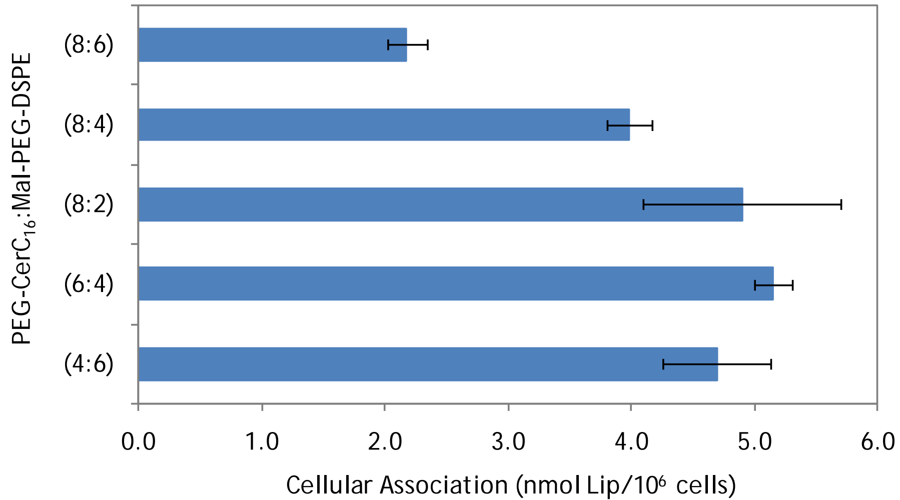


Figure III.3.1 – Cellular association of [^{3}H]CHE-labelled PI(G-SALP) formulations with SCLC H82 cells. Antagonist G-targeted SALP liposomal formulations were prepared with different amounts of total PEG-lipid derivatives (PEG-CerC_{16}:Mal-PEG-DSPE) at 0.8 mM Lip/well and incubated with 1 x 10^6 H82 cells for 1 h at 37 °C. Data was expressed as nmol of Lip/10^6 cells. Each point is the mean of three samples, ± SD, from one representative experiment.

The association results are in agreement with the similar I.C. values (Table III.3.4) obtained for all the PI(G-SALP) formulations assayed. The only exception is the formulation with 14 mol% total PEG, which has a low association level and an I.C. value of 8 g/mol, the same as PI(8:2) with high binding. In this particular case the high amount of total PEG in the PI(8:6) formulation, may partially shield the antagonist G in the outer surface so that it is not available for binding.

III-3.3.2 - Effect of the presence of antagonist G on the extent of cellular association of CCL and SALP liposomes

The cellular association of PI(G-CCL) and PI(G-SALP) liposomes, containing *as(c-myc)*, were assessed in a classical (H69) and a variant (H82) human SCLC cell lines respectively, and compared to non-targeted liposomes of similar composition. All formulations (with or without antagonist G) were labelled with [^{3}H]CHE and incubated with the SCLC cells at various concentrations and at two different temperatures (4 ºC and 37 ºC). The former temperature is non-permissive to endocytosis and thus provides an estimate of the specific and non-specific binding to the cell surface. On the other hand, at 37 ºC endocytosis mechanisms are active and internalization of liposomes is possible. Hence, this temperature gives a measure of cell surface binding and internalization of bound liposomes [46]. Data is shown in Figure III.3.2 for CCL and in Figure III.3.3 for SALP formulations.

The significant increase in the extent of cellular association observed for PI(G-CCL) and PI(G-SALP) liposomes compared to the corresponding non-targeted formulations demonstrated the ability of the coupled antagonist G to improve the cellular association of targeted liposomes to the tested SCLC cell lines, in a lipid dose-dependent manner. The higher cellular association levels obtained for targeted formulations in the experiments carried out at 37 ºC when compared to those achieved at 4 ºC, strongly suggest that antagonist G-targeted liposomes were being actively internalized by SCLC cells.

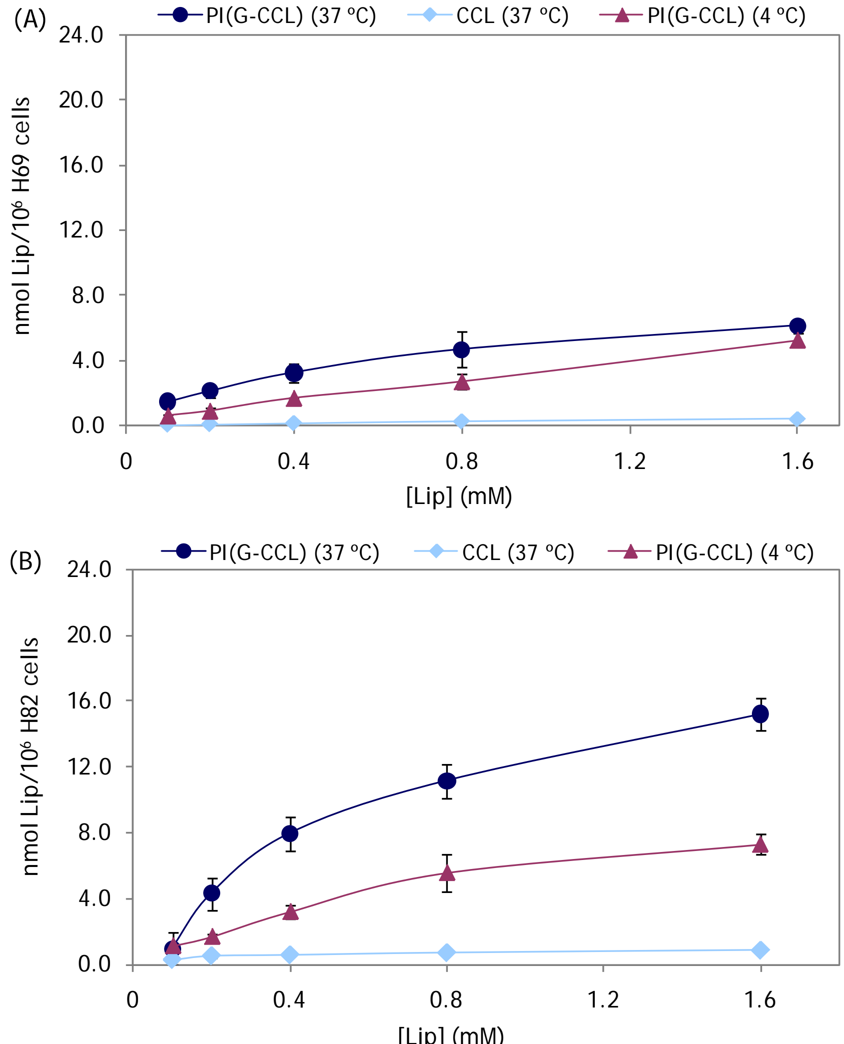


Figure III.3.2 – Cellular association of PI(G-CCL) and non-targeted CCL liposomes to SCLC cells. **(A)** Classical SCLC H69 cells or **(B)** Variant SCLC H82 cells. CCL liposomes were composed of HSPC:CHOL:DOTAP:mPEG-DSPE:Mal-PEG-DSPE at 30:20:10:1.6:0.4 molar ratio. Both cell lines (1 x 10^6 cells/well) were incubated with increasing concentrations (0.1 to 1.6 mM well) of [^{3}H]CHE-labelled PI(G-CCL) or CCL liposomes either at 37 °C or 4 °C for 1 h. Data are expressed as nmol Lip per million cells. Results are the mean of 4 samples ± standard deviation, from one representative experiment.

Results for PI(G-CCL) liposomes in the H69 cell line (Figure III.3.2 (A)) showed a maximum level of association of 6.2 nmol Lip/10^6 cells at 1.6 mM lipid (37 °C). The extent of PI(G-CCL) associated at 4 °C was 5.3 nmol lipid per million cells for the same lipid concentration. These results indicate that the level of internalization was low, nevertheless the binding of these liposomes was 15-fold greater than non-targeted

CCL implying that the antagonist G mediated cell binding but liposomes were not internalised. Results obtained for this same formulation in H82 cells (Figure III.3.2 (B)) showed a level of association that was more than two-fold higher (15 nmol Lip/10^6 cells) compared to H69. For the H82 cell line the presence of antagonist G increases the liposomes uptake 18-fold over CCL for the highest lipid concentration tested. Also, based on the results at 4 ºC, it may be inferred that the internalization ranges between 0.1 and 7.9 nmol Lip/10^6 cells within the range of lipid concentrations used.

Cellular association results for PI(G-SALP) liposomes (Figure III.3.3) followed the same general profile as the ones described above for PI(G-CCL). However, the extent of association of the targeted SALP liposomes was higher than for the CCL, for both cell lines, with a maximum of 18.8 and 19.1 nmol Lip/10^6 cells for H69 and H82 respectively, at 1.6 mM Lip. At this concentration the uptake of PI(G-SALP) was around 17-fold greater than for non-targeted SALP, for both cell lines (Figure III.3.3 (A) and (B)). Again, this increased uptake suggested that the peptide mediated internalization. At 4 ºC, an average 2.1-fold decrease in the cellular association of PI(G-SALP) was observed in both cell lines. This difference indicates that a part of the cell-liposome association observed at 37 ºC corresponds to liposome internalization by the tumour cells.

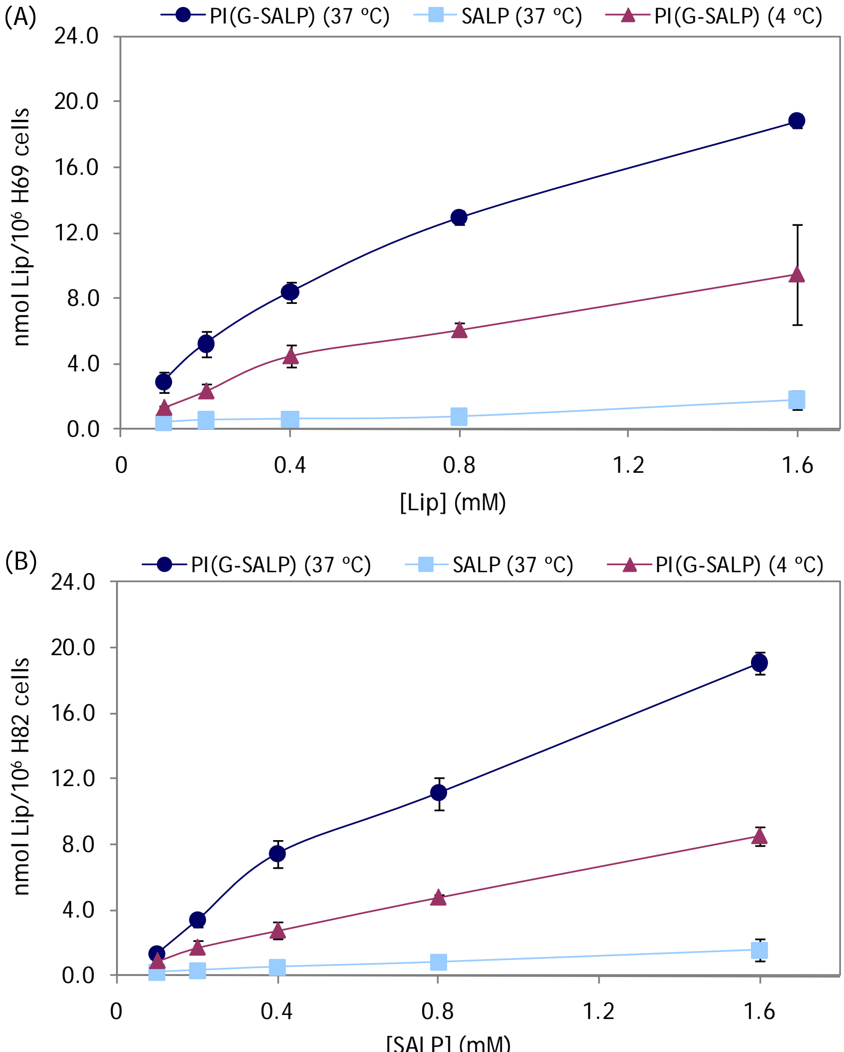


Figure III.3.3 - Cellular association of PI(G-SALP) and non-targeted SALP formulations to SCLC cells. **(A)** Classical SCLC H69 cells or **(B)** Variant SCLC H82 cells. SALP formulations were composed of DSPC:CHOL:DODAP:PEG-CerC$_{16}$:Mal-PEG-DSPE at 20:45:25:8:2. Both cell lines (1 x 10^6 cells/well) were incubated with increasing concentrations (0.1 to 1.6 mM/well) of [3H]CHE-labelled PI(G-SALP) or SALP liposomes either at 37 °C or 4 °C for 1 h. Data are expressed as nmol Lip per million cells. Results are the mean of 4 samples ± standard deviation, from one representative experiment.

In an attempt to shed light on the mechanism of internalization of PI(G-SALP) liposomes, H82 cells were pre-treated with 0.45 M sucrose, known to selectively inhibit clathrin-mediated endocytosis by blocking clathrin-coated pit formation [52]. Furthermore, to confirm the peptide specificity of the cellular association, competition experiments were performed in these SCLC cells with non-radiolabeled

sterically stabilized liposomes (SL) and antagonist G-coupled sterically stabilized liposomes (G-SL). In all these experiments, before the addition of PI(G-SALP) liposomes, H82 cells were incubated with the various compounds and formulations. Data is shown in Figure III.3.4.

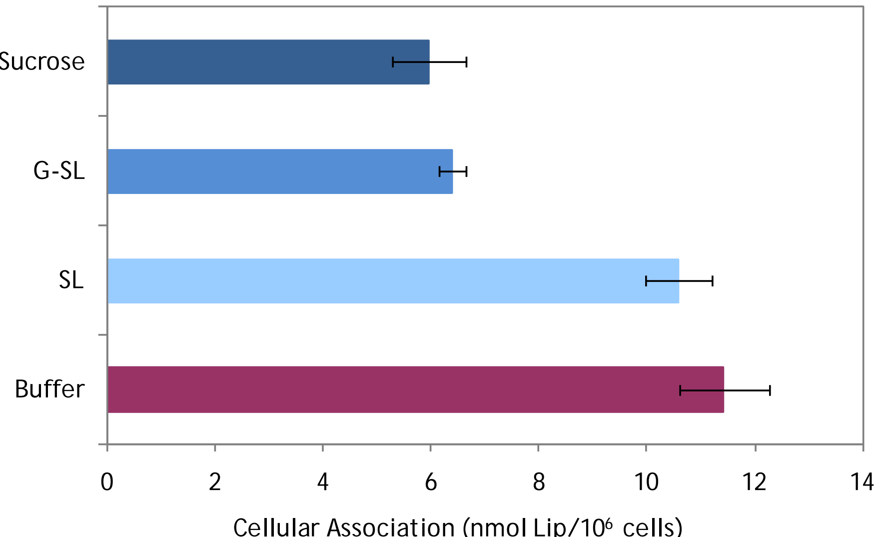


Figure III.3.4 – Effect of pre-treatment with an endocytosis inhibitor and competitive inhibition of the cellular association of [^{3}H]CHE labelled PI(G-SALP) with SCLC H82 cells. H82 cells (1x10^{6} cells) were pre-incubated at 37 ºC for 30 min with either 0.45 M sucrose/well or 0.56 µg of antagonist G coupled to non-radiolabeled SL (G-SL) and also non-targeted SL (5 mM/well). Inhibition was determined by adding [^{3}H]CHE-PI(G-SALP) (0.8 mM Lip/well), at 37 ºC for 1 h. Cellular association of liposomes was expressed as nmol of Lipid/10^{6} cells. Each point is the mean of four samples, ± standard deviation, from one representative experiment.

A decrease of 47% in the cellular association level of PI(G-SALP) was observed when H82 cells were treated with sucrose, as compared to the absence of endocytosis inhibitor. This result suggests that the liposomes were internalized by receptor-mediated endocytosis.

In competition experiments the SL liposomes without coupled antagonist G did not interfere with the association of PI(G-SALP) to SCLC cells. This result was expected as

SL are neutral liposomes with unmodified surface. On the contrary, the association of PI(G-SALP) with H82 cells was competitively inhibited when cells were pre-incubated with non-radiolabeled G-SL. This formulation was responsible for a 43% inhibition, and is a clear indication that internalization of antagonist G-targeted liposomes is receptor-mediated.

Competition experiments where free antagonist G (5 µg/well) was pre-incubated for 30 min did not competitively inhibit the binding of [^{3}H]CHE-PI(G-SALP) (0.4 mM lip), at 37 ºC (data not shown). The amount of the free peptide added was 20 times higher than the amount of antagonist G present at the surface of PI(G-SALP) and almost 10 fold higher than the amount on the surface of G-SL.

III-3.4 - Biodistribution studies of targeted and non-targeted SALP liposomes

The aim of these studies was to evaluate the biodistribution of PI(G-SALP) and non-targeted SALP containing *as(c-myc)*. The major characteristics of the liposomes used in these experiments are shown in Table III.3.5. The experiments were carried out in BALB/c mice with [^{3}H]CHE-labelled formulations administered as a single bolus dose of 1.5 µmol of Lip.

Table III.3.5 - Characteristics of the [^{3}H]CHE-labelled PI(G-SALP) and SALP liposomes

Formulation	L.C. (g/mol)	E.E. (%)	I.C. (g/mol)	Particle Size (nm)
PI(G-SALP)	45 ± 8	60 ± 9	7 ± 2	130 ± 3
SALP	55 ± 7	71 ± 5	n.a.	140 ± 3

Targeted and non-targeted SALP liposomes composed of DSPC:CHOL:DODAP:PEG-CerC$_{16}$:Mal-PEG-DSPE at 20:45:25:8:2 molar ratio were prepared with 1 mg of *as(c-myc)* per 13 µmol (10 mg) of total lipid.

The blood clearance of PI(G-SALP) after i.v. injection into mice was compared with that of SALP (Figure III.3.5). An increased initial clearance, especially for the first 6 h after infection, was observed for the non-targeted SALP, with less than 15% of the injected dose at this time point, in comparison with the 40% still present for the PI(G-SALP), after the same period of time. After this time point, the rate of clearance decreased for both formulations, but at different pace. At 24 h post-injection, blood levels were still quantifiable with around 12% of the injected dose for PI(G-SALP) and only about 2% for the SALP formulation. These results demonstrate that the presence of the antagonist G at the surface of SALP does not affect the long-circulation characteristics of the SALP liposomes.

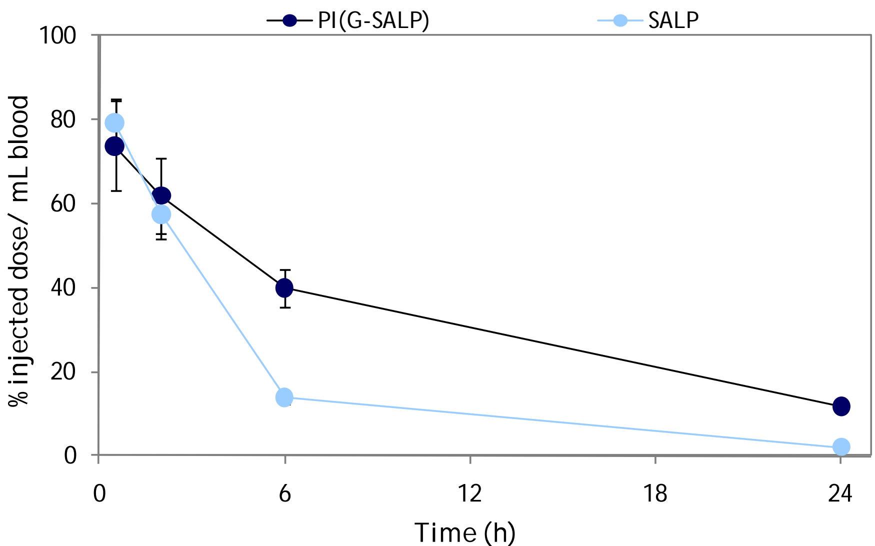


Figure III.3.5 - Blood clearance of PI(G-SALP) and SALP, in naïve BALB/c mice. SALP liposomes composed of DSPC:CHOL:DODAP:PEG-CerC$_{16}$:Mal-PEG-DSPE at 20:45:25:8:2 molar ratio, with or without coupled antagonist G labelled with [^{3}H]CHE were injected i.v. in the tail vein at a single bolus dose. At different post-injection times blood was collected and digested. The resulting samples were counted for ^{3}H. Data are the mean ± standard deviation of 4 animals/time point and are expressed as the percentage of injected dose per mL of blood.

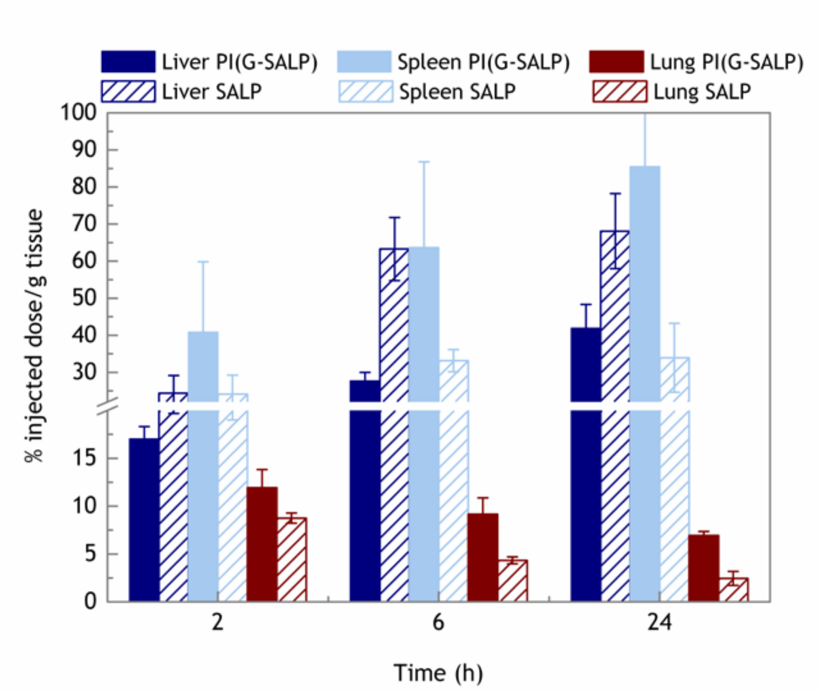


Figure III.3.6 – Tissue distribution of PI(G-SALP) and SALP, in naïve BALB/c mice. SALP liposomes composed of DSPC:CHOL:DODAP:PEG-CerC$_{16}$:Mal-PEG-DSPE at 20:45:25:8:2 molar ratio, with or without coupled antagonist G labelled with [^{3}H]CHE were injected i.v. in the tail vein at a single bolus dose. At different post-injection times liver, spleen and lungs were collected and digested. The resulting samples were counted for ^{3}H. Data are the mean ± standard deviation of 4 animals/time point and are expressed as the percentage of injected dose per gram of tissue.

The tissue distribution of SALP and PI(G-SALP) formulations in the liver, spleen and lungs at different times is reported in Figure III.3.6. The pattern of biodistribution of these formulations in the examined tissues showed a preferential accumulation of SALP liposomes in the liver whereas the PI(G-SALP) formulation presents a tendency to accumulate in the spleen. In fact the amount of PI(G-SALP) accumulated in the spleen, 24 h post-injection is 2.5-fold higher than that of SALP. As for the lung, our organ of interest, a preferential accumulation of PI(G-SALP) as compared to SALP was observed for all time points and at 24 h is still around 3-fold higher, showing the possible interest in the use of this formulation *in vivo*.

III-4 - Discussion

Several studies have established that the covalent attachment of appropriated targeting ligands to the surface of liposomes significantly increases the site specific delivery and internalization of these drug delivery systems into their target cells [39, 53].

In the work presented in this chapter, we have comparatively developed antagonist G-targeted CCL (adapted from Stuart *et al.* [11]) and antagonist G-targeted SALP (adapted from Semple *et al.* [24]) liposomes, tailored to promote specific targeting and efficient intracellular delivery of an *as(c-myc)* to SCLC cells. A similar CCL formulation was previously used with success for the incorporation of an asODN against a multidrug-resistant human B-lymphoma cell line causing a reduction in the expression of the P-glycoprotein *in vitro* [36]. As well, SALP type formulations were used as lipid-based systems for the incorporation of different nucleic acid molecules [24, 54, 55, 56].

Our choice for the targeting moiety was a growth factor antagonist, named antagonist G. Our strategy, similar to that described by Moreira *et al.* [46], consisted in the covalent attachment of antagonist G to the surface of *as(c-myc)* containing CCL and SALP formulations. The literature describes different coupling methods for attaching ligands to the surface of liposomes [57, 58]. We have selected and compared two of them: a so called conventional coupling technique previously used to couple different targeting ligands (i.e. anti-GD_2 mAb and anti-CD19 mAb) to the surface of CCL containing similar asODN [8, 10, 36, 59], and the post-insertion technique developed by Ishida *et al.* [42] for stealth liposomes (SL) containing anti-cancer drugs [38, 42, 47]. Considering the attachment of antagonist G to the CCL formulation, the characterization parameters obtained for G-CCL suggested liposomal destabilization and *as(c-myc)* leakage during the conventional coupling procedure. On the other hand

the net increase in the particle size (~40 nm) obtained by the post-insertion technique (PI(G-CCL)) when compared to the CCL formulation, could be an indication that antagonist G-PEG-DSPE conjugates were inserted into the lipid bilayer. A similar size increase was observed by other authors after the transfer of IgG-PEG-DSPE into SL liposomes [42]. A considerable amount of antagonist G was present at the surface of the PI(G-CCL). This fact together with the high encapsulation parameters, made this system suitable to be tested *in vitro*, in cellular association studies with SCLC cell lines.

Another lipid based system that was tested was SALP and along with its development several innovative features were found. The work reported here is one of the few studies on the attachment of a targeting device to the surface of SALP liposomes and one of the first to study the features of the SALP formulation and aspects of the preparation method that might affect the insertion of ligand-PEG-DSPE conjugates by the post-insertion method [60]. Similar work on the use of antagonist G-targeted SALP type formulations was also reported by Santos *et al.* [49] and Mendonça *et al.* [61]. The target of the SALP formulations, by both coupling methods done in pre-formed SALP, suggested that the reduction of the encapsulation parameters observed for both the G-SALP and PI(G-SALP) formulations (Table III.3.1) was caused by a destabilization in the SALP bilayer structure leading to the leakage of the encapsulated *as(c-myc)*. This destabilization was most probably caused either by the long (12 to 18 h) incubation time (at room temperature) of SALP with antagonist G in the conventional coupling method; or by the 1 h incubation time at a high temperature (60 ºC) of SALP with antagonist G-PEG-DSPE conjugates in the post-insertion method. As both these reactions occurred at physiological pH, the ionisable cationic lipid DODAP is neutral (pKa 6.6 [62]). Thus, at this point, the electrostatic interactions between the *as(c-myc)* and DODAP were no longer present to retain the asODN molecules inside the

liposomes. In a parallel study by other authors differing only in the asODN molecule [49], the same conclusion was drawn for equivalent results. To overcome this problem the authors replaced the ionisable aminolipid DODAP with DOTAP (1,2-dioleoyl-sn-glycero-3-trimethylammonium propane), allowing them to work at a pH closer to neutrality (pH 6) and still having the cationic lipid completely protonated during post-insertion. In another example, Zhou *et al.* [25] added the pre-formed folate-PEG-DSPE conjugate together with the other lipids, in the 100% ethanol solution, before mixing it all with the asODN. Our strategy was to include the "targeting step" in the SALP preparation procedure consisting on the insertion of the antagonist G-PEG-DSPE conjugate into the SALP after the extrusion. This method seems to be valuable as it led to a significant retention of the encapsulated *as(c-myc)* (approx. 70%), maintaining the original SALP characteristics. At this stage of the SALP preparation, the DODAP/asODN complexes together with the neutral and PEG-derivatized lipids are still able to form the final vesicles that occur after the dialysis. This way, the addition of a new component or step does not create significant destabilisation of the structure in formation.

Based on the above, the optimised post-insertion method was selected as the coupling procedure for the preparation of the targeted CCL and SALP formulations. In the case of the PI(G-CCL) liposomes, the insertion of the antagonist G-PEG-DSPE conjugate at the end of the CCL preparation had no effect on the physicochemical characteristics obtained as compared to the corresponding non-targeted formulation. As for the SALP liposomes, the proper inclusion of the targeting step during the preparation procedure also led to the preparation of a targeted formulation with similar characteristics as the non-targeted.

Other modifications involving the PEG-coating were assayed in the PI(G-SALP) formulation. One modification resulted in the substitution of the pre-existing PEG-

DSPE lipid with an exchangeable PEG-derivatized lipid in which the PEG is linked to a ceramide anchor containing a palmitoyl acyl group. The advantages of the presence of a PEG-coating on the outer surface of liposomes include an extended circulation lifetime in blood resulting in increased delivery to target sites [62, 63]. In the case of SALP formulations, the incorporation of PEG is also described as a requisite to stabilize the particles preventing aggregation and assisting in the formation of uniform, small mono-disperse particles [24, 64]. It has however been reported that the stable steric barrier produced by the use of PEG-DSPE may lower the liposomal-cell membrane interaction [31, 56, 65] slowing down the endosomal release of antisense to the cytoplasm [34]. Thus, steric stabilization has to be transient for efficient transfection to occur [31]. It has been shown that the PEG-ceramide exchange out of the cationic liposome at a rate that is mainly determined by the size of the ceramide acyl chain, with small chains exchanging faster [31, 63, 66, 67, 68, 69]. Wheeler *et al.* [63] tested the effect of the PEG-ceramide acyl chain length in SPLP formulations (similar to SALP). Their findings showed that SPLP containing PEG-ceramide conjugated with $CerC_8$ and $CerC_{14}$ chains were released from the liposomes in a matter of minutes (1.2 min and 70 min, respectively), while the same SPLP with PEG-$CerC_{20}$ stayed attached to the bilayer for up to 13 days. Transfection efficiencies *in vitro* were also dependent on the length of the ceramide acyl with higher transfection level for SPLP containing PEG-$CerC_8$ and lower for PEG-$CerC_{20}$ (tested in various cell lines). In addition pharmacokinetics studies [67] showed that SPLP containing either PEG-$CerC_8$ or PEG-$CerC_{14}$ were cleared in less than 1 h while SPLP containing PEG-$CerC_{20}$ remained in the circulation for hours. Accordingly, in this work all the PI(G-SALP) formulations used in the cellular association and tissue distribution studies were prepared with PEG-$CerC_{16}$.

Another modification consisted in the establishment of the more accurate PEG-CerC$_{16}$ to Mal-PEG-DSPE (antagonist G-PEG-DSPE conjugates) molar ratios in a SALP formulation containing PEG-lipids, since it is expected that the presence of PEG-CerC$_{16}$ in the preformed SALP might reduce the transfer of additional antagonist G-PEG-DSPE into the liposomes [42]. However, except at the highest molar ratio of both PEG-lipids (8:6), adequate transfer of antagonist G onto the preformed liposomes occurred resulting in PI(G-SALP) formulations with high cellular association levels. The fact that the "targeting step" was introduced in an initial stage of the SALP procedure, when particles were still forming, may reduce the inhibition of the insertion of antagonist G-PEG-DSPE onto the liposomes.

We have demonstrated that the post-insertion technology could be successfully applied to the preparation of PI(G-CCL) and PI(G-SALP) liposomes. With this work we have also established that the post-insertion is a simple and flexible methodology, in accordance to previous work [53], and appropriated for the rapid preparation of targeted-liposomes for eventual clinical applications. Then we investigated whether these systems expressed the features required to become efficient delivery systems for the systemic administration of nucleic acids. For this purpose we have compared the cellular association of *as(c-myc)* encapsulated in PI(G-CCL) and PI(G-SALP) to the corresponding non-targeted liposomes. Both antagonist G-targeted formulations were found to promote an increase in cellular uptake, in the two cell lines, when compared to the non-targeted liposomes demonstrating that the presence of antagonist G at the surface of these liposomal formulations mediate the *in vitro* specific recognition and internalization of liposomes into H69 and the H82 SCLC cell lines. The differences in cellular association in the experiments carried out at 4º and 37ºC is additional evidence that antagonist G-targeted liposomes were being internalized by both cell lines. Results from the competition experiments where antagonist G coupled to SL

inhibited the binding of PI(G-SALP), provided yet further support that internalization of PI(G-SALP) was receptor mediated. This conclusion is in agreement with previous reports [46, 47, 48] that demonstrated that antagonist G coupled to SL either by a conventional technique (SLG), or by the post-insertion approach (PLG), lead to an increased binding and internalization of antagonist G-targeted SL into human SCLC cell lines, on a peptide- and cell-specific manner, to deliver an anti-cancer drug.

It is accepted that for targeted-cationic liposomes to gain access to tumour sites and deliver their entrapped nucleic acids *in vivo*, long circulation times are required [53]. For this reason we have developed PI(G-SALP) formulations with blood clearance profiles of long-circulating formulations [70].

From the biodistribution studies we can conclude that both targeted and non-targeted SALP formulations have comparable profiles, however the main organs of accumulation are different, the liver for SALP and spleen the for PI(G-SALP). The lung also presents a reasonable value, in particular for the targeted formulation. An interesting result was the preferred spleen uptake of PI(G-SALP). The cause for this observation is not known, but some specific binding of the antagonist G may be occurring in this organ. In effect, Moreira *et al.* [46] have suggested that antagonist G may stimulate vasopressin receptor-mediated splenic uptake of antagonist G-targeted liposomes. Knowing that vasopressin receptors are expressed in the spleen [71], the observed higher PI(G-SALP) uptake is consistent with the hypothesis of an enhanced vasopressin receptor-mediated endocytosis of this formulation in the spleen.

Nevertheless what was more remarkable to observe in these studies was the superior accumulation of the PI(G-SALP) in the lung, our organ of interest as compared to the SALP. The possibility of achieving a considerable accumulation in the target organ, as demonstrated by our results, points to the need of evaluating the biodistribution studies in appropriated SCLC tumour-bearing mice. In fact in diseased animals with

enhanced vascular permeability and angiogenesis, it is expected further accumulation in the tumour tissue, by the EPR mechanism.

In conclusion, it was demonstrated that the post-insertion technique can be applied to different types of pre-formed liposomes generating ligand-targeted lipid particles that showed high loading efficiency, high extent of *in vitro* cell internalization, and characteristics of long-circulating profiles *in vivo*. As a consequence, our results advocate that PI(G-CCL) and PI(G-SALP) liposomes are valid candidates to selectively deliver *as(c-myc)* to SCLC cells, and in general as proved by others [49, 61], to deliver to tumour cells, any nucleic acid molecule against cancer-related genes. There is however, the need to determine the actual fate of the nucleic acids after their release in the cell cytoplasm and the role these systems may have inside the cell to mediate the down-regulation of the protein expression.

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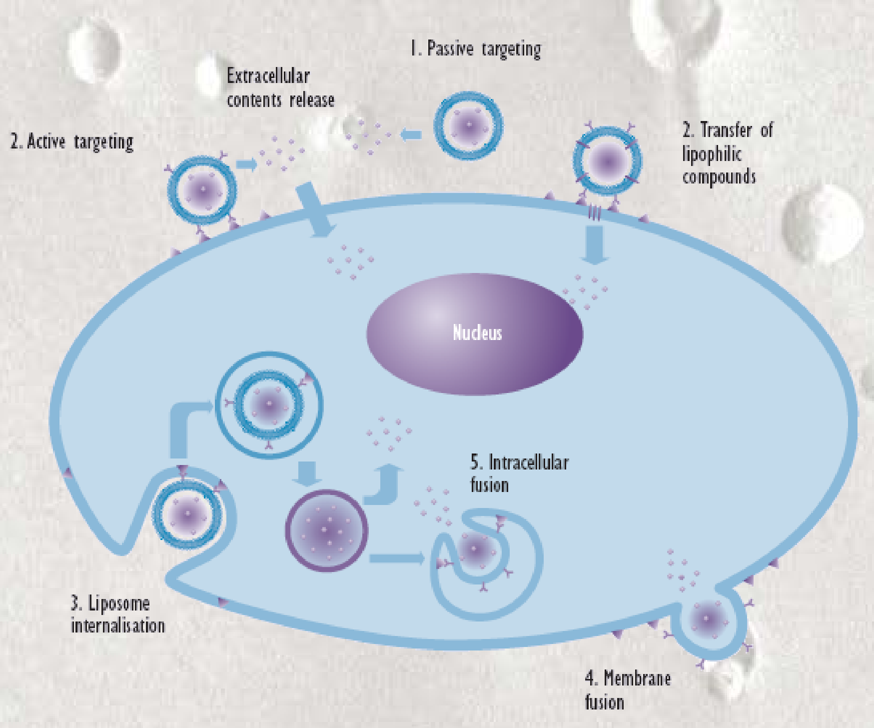
CHAPTER IV

Conclusions and Prospects



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IV - Conclusions and Prospects

The design of efficient NanoDDS to transport and deliver bioactive agents to their therapeutic targets *in vivo* has been widely investigated as an innovative strategy in drug discovery and development. Liposomes, the most frequently employed NanoDDS, have improved the pharmacological and therapeutic properties of many bioactive agents. The design of tailor-made liposomes able to accumulate at the disease sites, to be specifically recognised by the target cells or tissues and, above all, to provide an efficient intracellular delivery, are currently the most challenging goals in drug targeting as part of a therapeutic approach.

The work described here confirms the versatility of liposomes as NanoDDS to accommodate very different types of bioactive agents and their relevant use in the treatment of two diseases with distinct characteristics. In leishmaniasis, one of the selected diseases, macrophages are the main cellular targets, while in SCLC, an aggressive form of lung cancer, malignant cells are the targets. Although it was obvious that different bioactive agents with different pharmacological actions would have to be employed, our challenge was to use the same type of strategy to stabilise, transport and deliver the various drugs. To reach this goal we designed and developed two types of liposomes: conventional and ligand-targeted sterically stabilised, with appropriated characteristics for the incorporation and *in vivo* delivery of either low molecular weight molecules or macromolecules.

In Chapter II our aim was to evaluate the validity of dinitroanilines, a non-conventional class of compounds, as anti-leishmanial agents and the efficiency of liposomes as macrophages directed systems for their *in vivo* delivery. As described in that chapter, conventional liposomes were used to generate delivery systems for commercial (TFL) and hemi-synthetic (TFL-D) dinitroanilines. These low molecular

weight hydrophobic molecules were successfully incorporated with high encapsulation efficiencies and loading capacities. After a systematic study of the effect of formulation factors we were able to efficiently incorporate TFL in liposomes composed by phospholipids of low T_c, with charged polar head groups (PG) and without cholesterol. With the addition of a cryoprotectant it was possible to produce a stable TFL liposomal system for its maintenance/storage during a significant time after production [1]. Moreover, these systems kept their properties upon scale-up. The evaluation of the therapeutic activity of liposomal TFL in a visceral model of *Leishmania* infection led us to the conclusion that their activity was dependent on the lipid composition, dose and number of administrations, reaching 70% parasite load inhibition [2]. In a very aggressive type of cutaneous leishmaniasis model, around 60-fold smaller dose of liposomal TFL than the standard drug, Glucantime® reduced up to 2-fold the size of lesions. Liposomal TFL also improved the clinical condition of naturally and experimental infected dogs, reduced their parasite load and promoted the induction of protective immune response with the increment of protective cytokines [3].

With the aim to further increase TFL activity as an anti-parasitic agent, new synthetic TFL-D molecules, with *in vitro* anti-leishmanial activity, were selected and efficiently incorporated in selected conventional liposomes. These formulations conjugated *in vitro* high anti-leishmanial activity against promastigotes (*L. infantum* and *L. donovani*) in culture and against the intracellular form of *Leishmania* with irrelevant cytotoxicity and haemolytic activity. These findings are of particular relevance when compared with the performance of the standard drug miltefosine that evidenced signs of both negative side effects. Of the liposomal TFL-D tested in a murine model of zoonotic visceral leishmaniasis, treatment with the one containing TFL-A3 reduced significantly (97%) the number of viable parasites in the spleen of

infected mice. When used in the free form, this derivative, apart from displaying a lower activity (44% parasite load inhibition), also presented the inconvenience of needing detergent dissolution to be administrated.

The conclusions drawn from these studies are that conventional liposomes are a powerful NanoDDS to overcome the difficulties of handling and administering problematic drugs and to efficient delivery to MPS cells in different tissues. The use of liposomes has contributed to a dramatic increase in the water solubility of these drugs allowing the systemic administration of therapeutic doses with no signs of toxicity and without the use of organic solvents. These observations together with their preferential uptake by macrophages resulted in an enhancement of their anti-leishmanial activity as compared to the free drugs. The conjugation of the two strategies used: design and hemi-synthesis of specific new TFL-D and their association to macrophage-targeted liposomes, represented an innovative approach for the treatment of *Leishmania* infections.

For NanoDDS to be a commercially viable alternative tool in the treatment of poverty associated disease, like leishmaniasis, they must increase the treatment efficacy and tolerance, reduce treatment duration and cost, limit the emergence of drug resistance, and prove to be superior to current treatment modalities. Thus to fully explore the potential of TFL and in particular the more active TFL-D in the treatment of *Leishmania* infections, the incorporation of these molecules in more cost-effective lipid-based systems should be comparatively studied. The use of Solid Lipid Nanoparticles (SLN) is one of the most attractive alternatives to liposomes, which should be exploited in the future [4].

Independent of the NanoDDS used, the design of bioactive agents that target exclusively *Leishmania* parasites is of crucial importance to combat this infectious disease. In this work, bioactive agents were successfully used to target the parasite

tubulins. However, going a step further, it is intended to take advantage on the availability of the complete DNA sequence of the *Leishmania* genome. This approach, consisting on the delivery of an asODN complementary to the β-tubulin mRNA is expected to result in the inhibition of β-tubulin synthesis leading to the arrest of the multiplication of intracellular parasites [5]. This new strategy appears to be a very interesting perspective for future research.

In Chapter III we aimed to use a gene silencing strategy, based on the molecular differences or abnormalities observed in cancer cells relatively to normal cells, for the treatment of SCLC. The identification and characterization of these differences has strongly contributed to the emergence of new molecular targets for cancer therapy. Once the molecular targets have been defined, bioactive agents that specifically modulate their activity can be designed. An example of such a class of bioactive agents is asODNs when used as gene silencing agents. The use of NanoDDS is crucial to promote the efficient intracellular delivery of the associated asODNs to cancer cells. As described in this chapter, antagonist G targeted liposomal formulations were used to generate a NanoDDS to deliver an *as(c-myc)* to the cytosol of SCLC cells. After a comparative study between two coupling methods and two types of long circulating liposomes, we selected the post-insertion as the method to prepare the targeted formulations and the SALP as the liposomal system. The encapsulation of *as(c-myc)* in antagonist G-targeted SALP liposomes produced a system with high encapsulation efficiency and loading capacity, while keeping an appropriated small particle size. The antagonist G was capable of promoting the internalisation of this system allowing the intracellular accumulation of the encapsulated *as(c-myc)*. To take full advantage of this targeting strategy, we had to prepare a NanoDDS that, when *in vivo*, circulates long enough to give ligands the opportunity to bind to the cancer cell surface. This

role was played by the PEG-coating and by the small particle size (up to 150 nm) that characterises our PI(G-SALP) formulation. These liposomes, apart from accumulating in the liver and spleen, they also accumulate in the lung, the organ of interest, with a reasonable percentage of the injected dose (15%) still present 24 h after injection. Interestingly the PI(G-SALP) accumulate in this organ in a higher extent than the non-targeted liposomes, indicating that the presence of the antagonist G did not alter the long circulation characteristics of these liposomes.

With the results obtained from this work, the observed cellular internalisation and the blood circulation profile of the PI(G-SALP), it is possible to conclude that the use of targeted sterically stabilised liposomes can contribute, with a targeting approach, for the delivery of asODN to cancer cells. However for PI(G-SALP) to be considered as a therapeutic tool in the treatment of SCLC it needs to be validated. This validation implies demonstrating the down-regulation of the *c-myc* protein expression and parallel decrease in SCLC cellular viability, after treatment with PI(G-SALP) containing the *as(c-myc)*. Because there is some controversy in the literature about the therapeutic value of some molecular targets (e.g. Bcl-2) in SCLC [6, 7], it is pertinent to evaluate the down-regulation of the *c-myc* protein mediated by the use of an *as(c-myc)* and establishing whether this protein is a valid target. Carrying out such studies represents an interesting challenge for future research. It will be also interesting to examine if the observed cellular internalisation of the PI(G-SALP) shows a correlation with the reduction in the protein expression or if some of the problems encountered by other authors are partially due to an inadequate release of the nucleic acids from the liposomes after receptor-mediated endocytosis. In this case, some improvements in the delivery system must be considered.

Overall it appears that the main aims of this work were fulfilled, and several challenging questions were arisen that will be addressed in future work.

Chapter IV - Reference List

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