Departamento de Farmacia y Tecnología Farmacéutica

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"Design and *in-vitro/in-vivo* evaluation in colon cancer cells of targeted oxaliplatin liposomes to epidermal growth factor receptor by conjugation of different ligands"

Trabajo presentado por Sara Zalba Oteiza para obtener el Grado de Doctor

Fdo. Sara Zalba Oteiza

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DÑA. MARÍA JESÚS GARRIDO CID, Investigadora y Profesora contratada Doctora del Departamento de Farmacia y Tecnología Farmacéutica,

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Que el presente trabajo, titulado "**Design and *in-vitro/in-vivo* evaluation in colon cancer cells of targeted oxaliplatin liposomes to epidermal growth factor receptor by conjugation of different ligands**", presentado por DÑA. SARA ZALBA OTEIZA para optar al grado de Doctor en Farmacia, ha sido realizado bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Navarra. Considerando finalizado el trabajo autoriza su presentación a fin de que pueda ser juzgado y calificado por el Tribunal correspondiente.

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Las investigaciones realizadas en el presente trabajo se han desarrollado dentro del proyecto *Desarrollo y caracterización farmacocinética-farmacodinámica de liposomas de oxaliplatino dirigidos a receptores de membrana en el cáncer de colon* financiado por el Instituto de salud Carlos III, Ministerio de Ciencia e Innovación (Ref. PS09 / 02512-FISS).

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CONTENTS

CHAPTER 3

Targeting of EGFr with different ligands coupled to oxaliplatin PEG-liposomes

ABBREVIATIONS / ABREVIATURAS

Abs Antibodies / Anticuerpos

Ara-C Cytosine arabinoside / Citosina arabinosa

AUC Area under the curve plasma concentrations/ Área bajo la curva de las concentraciones plasmáticas

CA19-9 Cancer antigen 19-9 / Antígeno de cáncer 19-9

CDDP Cisplatin / Cisplatino

CEA Carcinoembryonic antigen / Antígeno carcinoembrionario

CH/Chol Cholesterol / Colesterol

CHEMS Cholesteryl hemisuccinate / Colesteril hemisuccinato

CRC Colorectal cáncer / Cáncer colorectal

CTR1 Copper transporter-1 / Transportador de cobre-1

CTX Cetuximab / Cetuximab

DACH Diamminocyclohexane / Diaminociclohexano

Dil 1,1′-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate / Perclorato 1,1´-Dioctadecil-3,3,3´,3´-tetrametilindocarbocianina

DLT Dose-limiting toxicity / Dósis tóxica limitante

DMEM Dulbecco's modified Eagle's medium / Medio Eagle modificado de Dulbecco

DMPC Dimyristoyl phosphatidylcholine **/** Dimiristoil fosfatidilcolina

DMPG Dimyristoyl phosphatidylglycerol **/** Dimiristoil fosfatidilglicerol

DMSO Dimethyl sulfoxide / Dimetil sulfóxido

DNA Deoxyribonucleic acid / Ácido desoxiribonucléico

DOPE Dioleoylphosphatidylethanolamine / Dioleoilfosfatidiletanolamina

DOTAP 1,2-Dioleoyl-3-trimethylammonium-propane / 1,2-Dioleoil-3-trimetamonio-propano

DPPC Dipalmitoylphosphatidylcholine / Dipalmitoilfosfatidilcolina

DPPG Dipalmitoylphosphatidylglycerol / Dipalmitoilfosfatidilglicerol

DSPC 1,2-Distearoyl-sn-glycero-3-phosphatydilcholine / 1,2-Distearoilglicero-3-fosfatidilcolina

DSPE-PEG1000 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine conjugated with PolyethyleneGlycol 1000 / 1,2-Distearoil-glicerol-3-Fosfoetanolamina conjugada con Polietilenglicol 1000

DSPE-PEG2000 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine conjugated with PolyethyleneGlycol 2000 / 1,2-Distearoil-glicerol-3-Fosfoetanolamina conjugada con Polietilenglicol 2000

DSPE-mMPEG2000 Methoxypolyethylen glycoldistearoylphosphatidylethanolamine 2000 / Metoxipropilenglicol distearoilfosfatidiletanolamina 2000

EE Efficiency of encapsulation / Eficiencia de encapsulación

EGF Epidermal growth factor / Factor de crecimiento epidérmico

EGFr Epidermal growth factor receptor / Receptor del factor de crecimiento epidérmico

EPR Enhance permeability and retention effect / Efecto de retención y mejora de la permeabilidad

Fab´ Monovalent Antibody binding fragment / Fragmento monovalente de unión del anticuerpo

FBS Fetal bobine serum / Suero fetal bovino

Fc´ Cristallizable fragment of antibody / Fragmento cristalizable del anticuerpo

FDA Food and Drug Administration / Agencia de Alimentos y Medicamentos

FM Film method / Método del film

GC Guanine-cytosine / Guanina-citosina

HAMA Human anti-chimeric monoclonal antibodies / Anticuerpos humanos anti Anticuerpos quiméricos monoclonales

HEPES 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid / Ácido 4- (2-Hidroxietil)-1-piperacinaetanosulfónico

HER Human EGF-related receptor / Receptor similar al EGF humano

HM Heating method / Método de calor

HSPC Hydrogenated soy phosphatidylcholine / Fosfatidil colina de soja hidrogenada

Hz Hydrazine / Hidracina

IC_{50} Drug concentration able to inhibit 50% of control growth / Concentración de fármaco capaz de inhibir el 50% del crecimiento del control

i.p. Intraperitoneal administration / Administración intraperitoneal

i.v. Intravenous administration / Administración intravenosa

L-NDDP Aroplatin / Aroplatino

LP Liposome / Liposoma

LP-CTX Liposomes with Cetuximab / Liposomas con Cetuximab

LP-Fab´ Liposomes with Fab´ fragment / Liposomas con el fragmento Fab´

LP-N Non targeted liposomes / Liposomas no dirigidos

LTSL Thermosensitive liposomes of cisplatin / Liposomas de cisplatino termosensibles

LV Leucovorin / Leucovorina

mAb Monoclonal antibodies / Anticuerpos monoclonales

Mal Maleimide/ Maleimida

MMR Mismatch repair system **/** Sistema de reparación de bases desemparejadas

MSPC Monostearoylphosphatidylcholine / Monostearoilfosfatidilcolina

MTD Maximum tolerated dose / Dósis maxima tolerada

MWCO Molecular weight cut off / Límite de corte peso molecular

NER Nucleotide excision repair system / Sistema de reparación-excisión de nucleótidos

NSCLC Non-small cell lung cancer / Cáncer de pulmón no microcítico

PARP-1 Poly ADP-ribose polymerase 1 / Poli ADP ribosa polimerasa 1

PBS Phosphate buffered saline / Tampón fosfato

PC Phosphatidylcholine / Fosfatidilcolina

PDP Pyridylditiopropionoylamino / Piridiltiopropionoilamino

PE Phosphatidylethanolamine / Fosfatidiletanolamina

PEG Polyethylenglycol / Polietilenglicol

PDI Polydispersity index / Índice de polidispersión

P-gp P glycoprotein / Glicoproteína P

pHi Internal ph / pH interno

pHo Outside pH / pH externo

PK Pharmacokinetic / Farmacocinética

RES Reticuloendothelial system / Sistema retículo endotelial

REV Reverse-phase evaporation **/** Evaporación en fase reversa

RLU Relative light units / Unidades relativas de luz

RNA Ribonucleic acid / Ácido ribonucléico

RPMI Roswell Park Memorial Institute medium / Medio Roswell Park Memorial Institute

RT Room temperature / Temperatura ambiente

s.c. Subcutaneous administration / Administración subcutánea

scFv Single chain antibody variable regions / Regiones variables de anticuerpo de cadena simple

SD Standard deviation / Desviación estándar

SDS Page Sodium dodecyl sulfate polyacrylamide gel electrophoresis / Electroforesis en gel de poliacrilamida con dodecil sulfato sódico

SPC-3 Soy phosphatidylcholine / Fosfatidil colina de soja

Stealth Sterically stabilized liposome / Liposomas estéricamente estables

Tc Transition temperatura / Temperatura de transición

TEM Transmission electron microscopy / Microscopía de transmisión electrónica

TGF Transforming growth factor / Factor de crecimiento transformante

5-FU 5 Fluorouracil / 5 Fluorouracilo

INTRODUCTION

INTRODUCTION

1. - Colorectal cancer

The Colorectal carcinoma (CRC) is the second form of cancer in the European population. It is the second type of cancer diagnosed in women after breast cancer, and the third in men, after prostate and lung cancer. The mortality rate is around 33% in the developed world [1].

1.1. - CRC development and characteristics

The colorectal carcinogenesis develops from years to decades. The adenoma-carcinoma presents a sequenced process of tumorigenesis. The tumors can be characterized by its location, mucinous histology, poor differentiation, and lymphocytic infiltration. Table 1 lists the difference stages of the CRC when it is spreading through the intestines (Figure 1).

***Table 1**.Tumor stages of CRC.*

0	No evidence of primary tumor
I	Tumor invades submucosa
II	Tumor invades muscularis propia
III	Tumor invades through the muscularis propia into subserosa or into nonperitonealised pericolic or perirectal tissues
IVa	Tumor penetrates the surface of visceral peritoneum
IVb	Tumor invades other organs

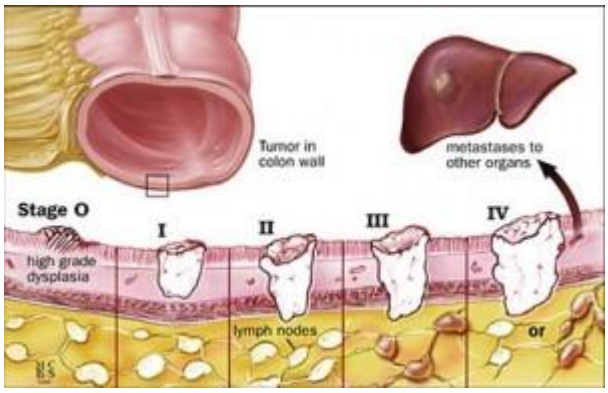

***Figure 1**. Stages of CRC.*

Adapted from: http://www.radiologysingapore.com/lectures/virtual-colonoscopy/

1.2. - Risk factors

Currently, several factors have been associated with CRC development.

1.2.1. - Genetic factors

The relationship between CRC and DNA inestability is clearly demonstrated in several works. In fact, there are specific phenotypes related to CRC. About 85% of cases present an allelic imbalance at several chromosomal loci (including 5q, 8p, 17p, and 18q), and chromosome amplification and translocation, contributing to tumor aneuploidy. The remaining 15% have high-frequency of microsatellite instability phenotypes [1**].**

On the other hand, a type of CRC affecting 2 or 3 people in a family represents 20% of the incidence on this type of cancer. Moreover, the risk to develop CRC in extended family members is 2 or 3 times higher than for the general population. This means that those people are forced to a periodical screening [1 - 3].

1.2.2. - Enviromental and demographic factors

Most of CRC cases arise sporadically. However, several factors such as age, sex, previous colonic polyps and environmental factors like lifestyle, diet and unhealthy habits,

are related to the presence of CRC. In the same way, some diseases including intestinal alterations like inflammatory bowel disease, ulcerative colitis and Crohn's disease are associated with a greater risk to develop CRC [1, 2].

1.3. - Diagnosis

The CRC diagnosis at the early stages of the disease represents a challenge in oncology. In fact, its diagnosis at the beginning of the disease provides a significant increment in the survival rate of the patients, as is shown in Table 2. A percentage higher than 70% of people CRC diagnosed at the first stages reach a survival rate of 5 years. However, this rate decreases dramatically, 10%, if the metastasis is detected [1-3].

Based on this data, it is recommended a screening as soon as possible especially for high risk groups. The aim is to detect a premalignant adenoma and then, prevent the development of advanced carcinomas, since these adenomas can become tumors in 80% of people with this pathology.

***Table 2**. Five years relative survival rate for CRC patients depending on the stage.*

Stage I	*97.1 %*
Stage IIa	*87.5 %*
Stage IIb	*71.5 %*
Stage IIIa	*87.7 %*
Stage IIIb	*75.0 %*
Stage IVa	*50.5 %*
Stage IVb	*27.1 %*

The main methods or approaches for screening and CRC diagnosis are [1 - 3]:

1. The traditional method which is the most widely used is the guaiac-based faecal occult blood test. Although, it is considered as the least sensitive test, several trials have demonstrated that could reduce the mortality of the patients by 16%. Positive results in this test recommend a colonoscopy. Currently, the introduction of immunochemical advances in the faecal occult blood test has provided significant improvements compared to the traditional test.

2. The endoscopic test allows for the examination of sigmoid colon and rectum. 60% of adenomas and cancers are located in this part of the intestine. Results from epidemiological studies suggest that this technique is able to provide a reduction from 60 to 80% of the mortality in CRC patients. One of the advantages of this technique is the removal of small polyps during the endoscopy. However, if advanced adenomas are found in this screening, the next test is the application of a colonoscopy.

3. The colonoscopy is a technique to confirm an advanced adenoma. Its use is associated with serious risks and complications. A biopsy of the tissues is needed to support the positive images found in this test.

4. Computed tomographic (CT) colonography consists in a virtual colonoscopy, which requires the radiation to confirm in the presence of cancers and large adenocarcinomas. In this case, the removal of the detected lesions is by colonoscopy.

5. Serum biomarkers like the carcinoembryonic antigen (CEA) and CA19-9 (a carbohydrate antigen recognized by the monoclonal antibody NS19-9), are commonly used as markers for early CRC diagnosis. Although they are considered

as a useful tool, their clinical importance for diagnosis, prognosis and survival, remains unclear [3].

6. Currently, new sophisticated techniques such as DNA microarrays and proteomic assays are emerging in order to find new biomarkers for CRC diagnosis.

Based on the results found in the previous tests, patients are examined by techniques like CT, magnetic resonance image (MRI), positron emission tomography (PET) or ultrasounds to localize the precise place of the tumors, and to detect other metastatic lesions in the chest, abdomen and pelvis [1].

1.4. - Treatment

1.4.1. - Surgery

The first approach in the treatment of this type of cancer is the complete resection including adequate margins of the tumor and a lymphadenectomy. Surgery depends on the stage of the adenocarcinoma, and in some of these patients, a partial resection of the organs, like liver and lung affected by the metastases, is also needed. With this approach, it is possible to improve the survival rate of patients in 5 or 10 years [1 - 3].

1.4.2. - Radiation

There are several strategies focused on the radiation therapy. In this treatment, the radiation should be used to prevent the relapse and as an adjuvant for chemotherapy. Conventional chemoradiation regimens combine with radiation antitumor drugs such as 5-FU or capecitabine (a 5-FU prodrug used for oral administration), although in recent studies, the combination with oxaliplatin has provided promising results to control and inhibit the metastasis [1].

1.4.3. - Chemotherapy

It has been proved that systemic chemotherapy improves the survival rate in CRC patients. The most common drugs applied in this therapy are 5-FU or capecitabine, combined with oxaliplatin and Leucovorine (LV). This regimen, known as FOLFOX regimen, improves the time of the free disease and overall survival [4]. In case of metastatic disease, irinotecan and some tumor targeted molecules are also included in that combination. These last biomolecules have demonstrated an enhancement of the efficacy of the conventional drugs, and the improvement of the quality life of patients. One of the most popular molecules associated with oxaliplatin in CRC, is Cetuximab. It is a monoclonal antibody able to bind and inhibit the activity of the epidermal growth factor receptor (EGFr). However, its use in clinic depends on the status of K-Ras, an oncogen that becomes resistant to the activity of Cetuximab when it is mutated. Therefore, Cetuximab is only applied to patients with wild type oncogen.

2. - Oxaliplatin

Oxaliplatin, a third generation platinum derivative, is currently the first line of chemotherapy in metastatic CRC. It was approved by the Food and Drug Administration (FDA) in 2002 as adjuvant in metastatic colorectal cancer treatment in stages III and IV. It is combined with 5-FU and LV in the FOLFOX regimen [4].

Oxaliplatin is the best tolerated of the platinum derivatives. Its main side effect is the neurotoxicity which becomes reversible after stopping treatment. This represents a clear advantage regarding the main side effects, nephrotoxicity and hematotoxicity, of cisplatin and carboplatin respectively [5].

Regarding the mechanism of action of platinum derivatives, their native forms are reduced to mono-, di-chloro or diaquated metabolites in aqueous environment [6]. These metabolites are highly reactive to different cellular targets such as DNA, RNA, proteins and other molecules that contain cysteine, methionine or sulphydryl groups, among others. They are able to form intrastrand (99%) and interstrand cross-links (1%) in DNA chains, as is shown in Figure 2. These adducts are responsible to induce cell cycle arrest triggering the apoptosis. Although this mechanism is common among platinum derivatives, the cell line sensitivity for oxaliplatin is different from cisplatin, because it has demonstrated efficacy in several cisplatin resistant cell lines or in patients with cisplatin refractory effect. In fact, for equipotent doses the amount of adducts formed by oxaliplatin is lower than for cisplatin [7].

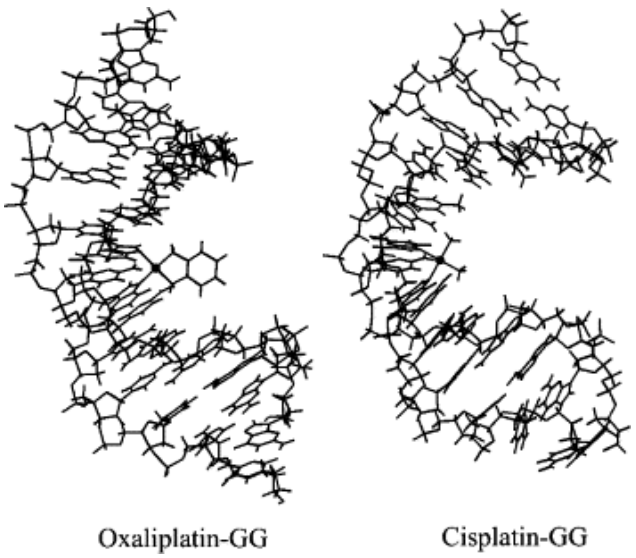

Figure 2. *DNA adducts of oxaliplatin and cisplatin [9]*.

Free Oxaliplatin shows a kinetic profile in plasma characterized by two or three compartmental model, with a rapid initial distribution phase and a slow terminal

elimination phase. Thus, after an intravenous infusion, oxaliplatin binds in a covalent form to several plasma proteins and erythrocytes. This means that only the 15-30% of the administered dose is in the free form. This biding to erythrocytes reaches the maximum at 2 h post-treatment, and represents the 37% of the dose. For the rest of the dose, the 80-85% is bound to plasma proteins. Therefore, the half-life of this platinum drug depends on the degradation of blood cells and plasma proteins. Based on that data, the levels of the platinum free fraction corresponding to the biologically active molecule are very low. This fact supports the low efficacy of this drug [6, 8].

3. - Liposomes

Nanotechnology is a very useful tool to develop new controlled drug release systems or carriers for drug delivering to selective tissues. In this way, liposomes represent a group of nanocarriers biocompatible with the cellular membranes. They allow the encapsulation of different types of drugs, hydrophilic and hydrophobic, protecting them from the enzymatic degradation. These systems have demonstrated a huge impact on the pharmacokinetic (PK) properties of the encapsulated drug, increasing its bioavailability and biodistribution [10, 11].

The major draw-back of the conventional liposomes is their rapid uptake by phagocytic cells of the reticuloendothelial system (RES) after their systemic administration [12]. The organs with major liposomes accumulation are liver and spleen, due to their rich blood supply and the abundance of phagocytic cells. In order to reduce their clearance by RES and prolong the half-life in plasma, these systems have been modified at the surface by a conjugation with synthetic phospholipids or polymers, like polyethylenglycol (PEG). These PEG-coated liposomes are known as sterically stabilized or Stealth liposomes. The

stabilization effect of PEG is due to the presence of highly hydrated groups that can inhibit the hydrophobic and electrostatic interactions with the blood components. This effect provides a significant delay in their RES uptake, prolonging their circulation half-life [12, 13].

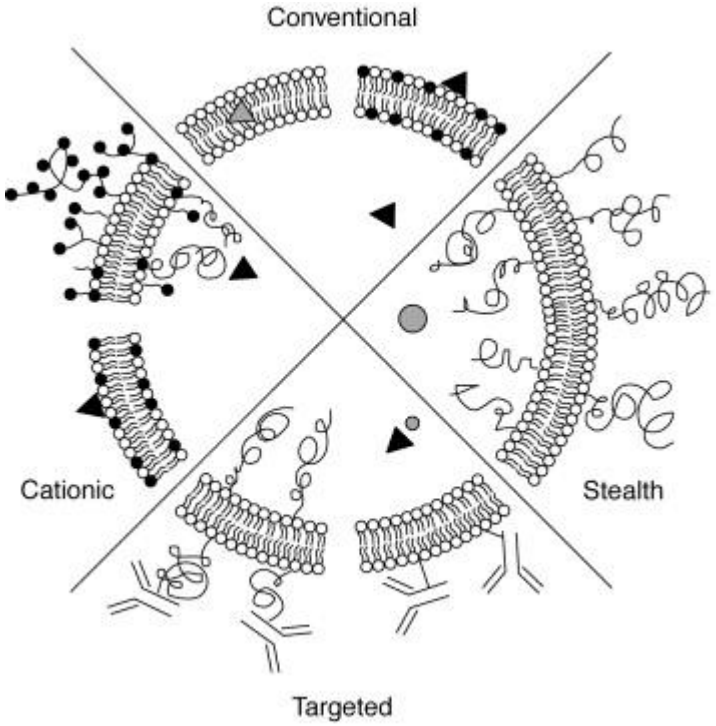

Figure 3. *Scheme of the different types of liposomes.*

Adapted from http://www.sciencedirect.com/science/article/pii/S1461534798000078.

This characteristic represents an advantage achieving the "passive" targeting in the tumor tissue of these formulations (Figure 3). This passive targeting is caused by the formation of new vessels in the tumor area, which is associated with defective endothelial cells showing wide fenestrations. This, together with the lack of effective lymphatic drainage, allow the extravasation of molecules with a high particle size (from 10 to 500 nm), leading to a greater accumulation in the interstitial space of the tumor tissue than in normal tissue. This phenomenon is known as enhanced permeability and retention effect (EPR) or passive targeting, and it is present in a wide variety of solid tumors [14, 15].

Nevertheless, this EPR effect shows a high variability between different types of solid tumors. Even for the same tumor it is considered a heterogeneous process. In addition, the accumulation of molecules is localized in the tumor interstitium but the antitumor efficacy of the encapsulated drug is not always successfully reached as the cell uptake is slow and no very effective [16].

3.1. - Oxaliplatin liposomes

Taking into account that the main limitation for the efficacy of oxaliplatin is due to its pharmacokinetic properties associated with a large volume of distribution, short half-life and very low levels of the free drug available, its incorporation into liposomes might represent a good strategy to modulate that [17].

There are few oxaliplatin liposomal formulations described in the literature. Lipoxal is one of these formulations, and it is being assayed in phase I/II clinical trials [18]. Other formulations developed with anionic and cationic lipids have been published by different authors, but they are still in the experimental stage [19 - 22].

On the other hand, increasing the circulation time of liposomes would result in more drug-depleted liposomes to the tumor cells but would not implicate a higher amount of drug delivered. Therefore, an improvement in the therapeutic effect it is not always achieved.

Based on that, there is currently a new challenge in the development of liposomes. The interest is in the use of ligand-mediated in the formulations as a strategy to increase the therapeutic effectiveness of antitumor drug. This strategy is known as “active targeting” [23]. To formulate this type of targeted liposomes, monoclonal antibodies (mAb), peptides or growth factors have to be conjugated at the surface of these stealth liposomes. These ligands are characterized by their high affinity to bind selectively to

tumor-expressed surface receptors or antigens, inducing the internalization of the complex, formulation and receptor.

4. - Epidermal growth factor receptor (EGFr)

To achieve the active targeting strategy, the coupling to the liposomes of a specific ligand for a tumor-specific antigen or receptor is needed. One of these receptors is the epidermal growth factor receptor (EGFr). Its over-expression is very common in most of the solid tumors including CRC, as is listed in Table 3 [24].

Table 3. *Relative percentage of the EGFr over-expression in several types of tumors.*

Adapted from: http://www.licor.com/bio/products/reagents/irdye_680rd_egf/irdye_680rd_egf.jsp

Tumor Type	% of Tumors Over-Expressing EGFR
Head and Neck	80-100
Renal Cell	50-90
Non-small-cell Lung	40-80
Glioma	40-50
Ovarian	35-70
Bladder	31-48
Pancreatic	30-50
Colon	25-77
Breast	14-91

This ErbB receptor family includes a group of transmembrane tyrosin kinase proteins. The ErbB-1, also called epidermal growth factor receptor, and ErbB-4, are the main proteins implicated in the cell proliferation, migration or differentiation. These processes occur when a growth factor ligand, EGF, TNFα and others, bind to them. In that

moment, the receptors undergo dimerization which activates the signalling downstream cascades, Ras/Raf/MAPK, STAT and PI3K/AKT, generating all of the cellular processes commented before (Figure 4). The other two receptors, ErbB-2 (also called HER2 in human and Neu in rodents) which acts as an heterodimeric complex with other, and ErbB-3 which cannot generate signals in isolation, are able to induce potent intracellular signals together. Interestingly, each of theses receptors are able to bind to different ligands. This mechanism is also facilitated by an autocrine activation of the tumor itself [25].

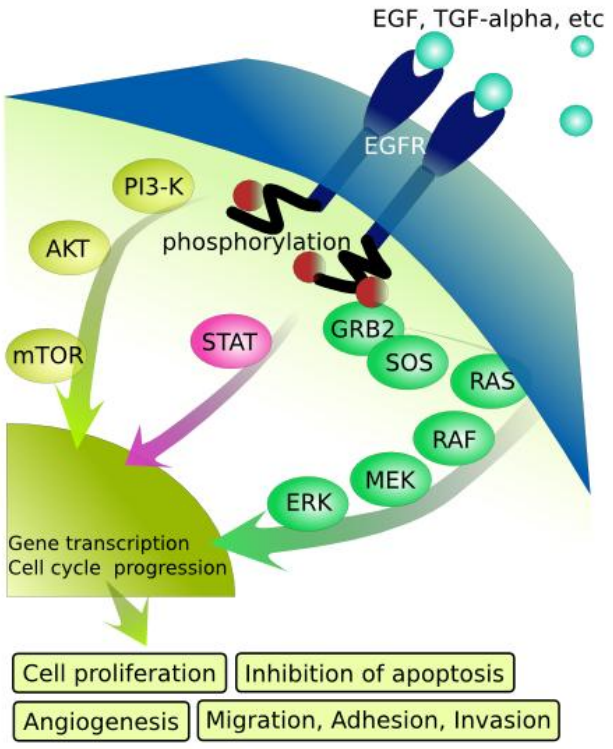

Figure 4. *Schematic representation of the EGFr activation.*

Adapted from http://biomarkings.com/category/conditions.

Note that in some clinical studies, the EGFr overexpression in CRC has been associated with a poor prognosis in patients, suggesting a more aggressive stage of the tumor [26].

Different strategies have been developed in the last two decades in order to inhibit the function of EGFr:

1. ***Development of Monoclonal antibodies (mAb)***

   The treatments based on mAb are the most advanced strategies to inhibit in a selective manner the EGFr activity. In general, they show a higher affinity for the receptors than the natural ligands.

   Some examples are:

- Trastuzumab: Herceptin® (Roche), a full humanized antibody, binds to the extracellular domain of HER2 [27]. It was the first mAb against the EGFr approved by the FDA, in 1998, in the treatment of metastatic breast cancer in combination with chemotherapy, such as paclitaxel or doxorubicin [28]. This combination represents an enhancement of the response rates, the median time of the progression and the median of overall survival in patients. The antiproliferative and cytotoxic effects of Trastuzumab are described as a combination of the inhibition of the extracellular cleavage, a decreasing the DNA repair and an antiangiogenic effect. However, its administration is associated with a resistance phenomenon.
- Cetuximab: Erbitux® (Merk), a chimeric humanized mouse mAb, was approved by the FDA in 2006 in the treatment of metastatic colorectal carcinoma and squamous cell carcinoma of the head and neck. It binds to the receptor in the extracellular domain, with higher affinity than EGF or TGFα, blocking their binding to the receptor [29]. Cetuximab, administered in combination with chemotherapy and radiotherapy, is able to enhance the anti-tumor effect of those treatments. However, its efficacy is associated with a wild type K-Ras profile, reserving its use to patients with this phenotype. Its antitumor mechanism includes the internalization and down regulation by degradation of the receptor, leading to the inhibition of the

proliferation of tumor cells. That inhibition involves the accumulation of cells in G1 phase and an inhibitory effect on the angiogenesis. Interestingly, this molecule is also able to exert its cytotoxicity by the complement activation associated with antibody-dependent cell-mediated toxicity [30].

- Matuzumab: EMD-72000 (Merk), a humanized anti-EGFr mAb, is currently in phase I/II clinical trials. It binds to EGFr in a competitive manner with the natural ligands, EGF or TGFα [29]. Few articles have been reported in literature regarding this mAb.
- Panitumumab: Vectibix® (Abgenics), a fully human mAb currently in phase II in clinical trials [31]. It binds to the external domain of the EGFr to prevent the receptor activation induced by the ligands. It promotes the receptor internalization inhibiting the tyrosine kinase activity, but does not induce the receptor degradation. This fact suggests a recycling of the receptor to the surface. Its activity is related with the cell arrest and the inhibition of antiangeogenic factors. Since this mAb is fully human its immunogenical reactions in patients are lower than those for chimeric or humanized mAb. Panitumumab does not induce antibody-dependent cellular toxicity [29]. A selection based on tumor K-Ras status increases its therapeutic benefits.
- Pertuzumab: Ormnitarg, 2C4 (Genetech) is a Trastuzumab-based antibody. Currently is in phase I-III clinical trial studies in combination with other compounds [32].
- T-DM1: (Genetech) is a new approach which combines the Trastuzumab with an anti-microtubule agent (emtansine). Its efficacy is being tested in phase III trials in combination with other chemotherapeutic agents [32].

Despite the advantages of these biomolecules, the development of resistances promoted by mutations on the target-epitopes and the production of human anti-chimeric monoclonal antibodies (HAMA), especially after their repeated administration, represents serious limitations during their use. The presence of the HAMAs antibodies have been reported as a consequence of the Fc' fragment recognition of the chimeric and humanized antibodies, by the immune system of the patients. This induces an activation of the complement system together with the activation of RES uptake [33].

2. Tyrosin kinase inhibitors

Another strategy applied to block the intracellular tyrosin kinase domains of the receptor, is the administration of molecules that bind to these domains. This binding leads to the inhibition of the signal propagation in an appropriate manner blocking the proper biological response.

Some tyrosin kinase inhibitors such as Gefitinib (Iressa®), Erlotinib (Tarceva®) or Lapatinib (Tykerb) have been included as adjuvants in the cancer therapy with conventional antitumor drugs [32]. The treatment of advanced non small cell lung cancer or metastatic pancreatic cancer, are the most common clinical use [34].

For Neratinib or Afatinib, they are currently enrolled in different clinical trials.

This strategy, as the case of mAbs, shows several limitations related to the selective inhibition of EGFr-tyrosin kinase activity, and the development of resistances due to the mutations in the phosphorilated domains of the receptor [30].

5. - The magic bullet

Paul Ehrlich developed the concept of the "magic bullet" for an ideal therapeutic agent with selective target-disease properties. This concept may be applied to the mAbs because they provide a specific binding to a specific epitope of a molecule. Therefore, a significant advantage in oncological therapeutic application would be the combination of an antitumor drug entrapped in a nanoparticle and coated with a specific mAb. In this way, the targeted-liposomes, loaded with therapeutic drugs and coupling to a specific ligand which is able to recognize an antigenic molecule in cells, tissues or organs, is the most complex approach to a treatment. This strategy is known as active targeting in opposition to passive targeting [23].

5.1. - Methods to obtain targeted liposomes

There are two main methods to obtain targeted liposomes [35]:

1. ***The conventional method***

   In this method, the composition of liposomes includes lipid and the PEG polymer functionalized with a crosslinker to conjugate the ligand. Some of these functionalized polymers are the pyridylditiopropionoylamino (PDP-PEG), hydrazine (Hz-PEG) or maleimide (Mal-PEG). In this method, the ligand has to be modified in order to incorporate several linkage groups such as the thioether, disulfide, carboxamide, amide or hydrazone to couple with the polymer.

2. ***The post-insertion method***

   For this method, the ligand is modified as it was commented in the conventional method, and coupled to the lipid-PEG derivative. The difference is that the mixture ligand-lipid-PEG is in a micellar phase that is incubated with the liposomes loaded

with the therapeutic drug, previously developed. This incubation takes place using a temperature above the lipid transition temperature. Under this condition, the micelles are incorporated in the bilayer of the loaded liposomes.

5.2. - Ligands

Active targeting of liposomes to the tumor tissue involves the use of specific ligands with a high affinity towards a certain receptor or domains of that receptor [36].

Immunoliposomes consist of liposomes, loading an active agent, conjugated with a mAb or antibody fragment, as targeting ligands. They represent the most promising strategy in cancer [37].

The whole monoclonal antibodies may induce some immunogenic effect due to the presence of Fc' fragments. This region is specifically recognised by phagocytic cells allowing their blood circulation clearance. In this way, the smaller fragments of mAbs, Fab' fragments or single chain antibody variable regions (scFv) [38], which maintain the specificity to receptor recognition, reduce the immune-reactions and prolong the blood residence time of these immunoliposomes. Figure 5 shows the different types of antibody-derivative ligands used for coupling to liposomes.

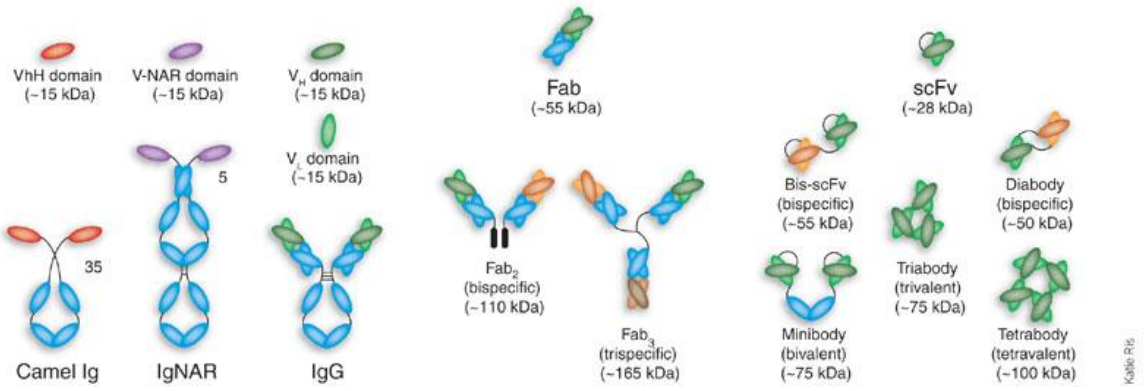

Figure 5. *Different fragments of antibody.*

Adapted from: http://www3.imperial.ac.uk/people/m.deonarain.

Small fragments are becoming the most popular alternative to the whole mAb [38]. Some of these fragments are produced using microorganisms like phages. Thus, they are easy to produce and the modification of specificity and affinity to a selected target is allowed by this technology.

Over the last years, new molecules known as "affibodies" have been incorporated in the active targeting. They are relatively small proteins with a high stability, solubility and easy to produce. They are also able to mimic the activity of specific antibodies.

Other alternatives for active targeting are: i) the use of the natural or endogenous agonists of the receptors like hormons, vitamins, growth factors, etc [23]. For example, the epidermal growth factor, EGF, a molecule that binds with a high affinity to EGFr to induce cell proliferation, represents a ligand for coupling to liposomal formulation. This type of structure has been previously reported by several authors [39]. It is able to produce a downregulation of the receptor associated with a cytotoxic effect due to the release of the entrapped drug in the liposome; ii) the use of some synthetic peptides are able to recognize the antigen binding site or a specific part of the receptor [23, 40, 41].

The development of targeted liposomes using ligands as specific molecules, represent a promising strategy in the treatment of cancer. Therefore, the aim of this work was to develop oxaliplatin liposomes conjugated with Cetuximab, and its Fab' fragment, to investigate their therapeutic tumor activity *in-vitro* and *in-vivo,* using nude mice solid tumor model of colorectal cancer.

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CHAPTER 1

LIPOSOMES, A PROMISING STRATEGY FOR CLINICAL APPLICATION OF PLATINUM DERIVATIVES

CHAPTER 1

LIPOSOMES, A PROMISING STRATEGY FOR CLINICAL APPLICATION OF PLATINUM DERIVATIVES

Sara Zalba[1], María J. Garrido[1]*

[1]*Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona 31008, Spain*

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***Corresponding author**: Address: Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain. Tel.: +34 948425600; Fax: +34 948425649. E-mail address: mgarrido@unav.es (María J. Garrido)

OBJECTIVES

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Oxaliplatin is currently used in several types of solid tumors, but its low therapeutic efficacy is due to its PK characteristics. To modify that limitation, oxaliplatin has been encapsulated in nanocarrier systems like liposomes. They represent tissue-selective carriers, able to improve the tumor accumulation modifying the PK of encapsulated drug.

The over-expression of EGFr in many solid tumors as CRC has led to use this molecule as a new approach for cancer therapy. This therapy is based on the treatment with specific monoclonal antibodies. The combination of these biomolecules with conventional chemotherapy is increasing as in the case of oxaliplatin administered in combination with Cetuximab, a mAb against EGFr.

Therefore, the main objective in this work was to develop EGFr targeted liposomes loaded with oxaliplatin to investigate the behaviour of these systems in *in-vitro* and *in-vivo* models. To reach this general objective, three different steps were followed:

1. The development of pegylated liposomes of oxaliplatin to evaluate their behavior in *in-vitro* and *in-vivo* studies.
2. The development of EGFr targeted liposomes of oxaliplatin using different ligands.
3. The evaluation of the *in-vitro* cellular uptake mechanism for targeted and non-tageted liposomes and their *in-vivo* antitumor efficacy.

OBJETIVOS

El oxaliplatino se utiliza actualmente en el tratamiento de diversos tumores de tipo sólido. Sin embargo, presenta una baja eficacia terapéutica debido a sus características farmacocinéticas. Su incorporación en sistemas de transporte como los liposomas ha ayudado aumentar su eficacia. Estos sistemas permiten un transporte selectivo al tumor con una mayor acumulación del fármaco en este tejido y por tanto modificar las propiedades farmacocinéticas del compuesto encapsulado.

El avance de las nuevas terapias mediante el uso de los Anticuerpos monoclonales, han hecho que receptores de membrana sobre-expresados en tumores, como el receptor del factor de crecimiento epitelial (EGFr), representen un nuevo objetivo terapéutico. La combinación de esas biomoléculas con la quimioterapia convencional está aumentando en clñinica. Un ejemplo es el caso del oxaliplatino administrado en combinación con el Cetuximab, un anticuerpo monoclonal específico del EGFr.

Por tanto, el principal objetivo de este trabajo fue desarrollar y evaluar en sistemas *in-vitro/in-vivo,* liposomas de oxaliplatino dirigidos frente al EGFr para investigar el comportamiento de esos sistemas en modelos *in-vitro* e *in-vivo*. Para conseguir este objetivo general, se llevaron a cabo los siguientes pasos:

1. Desarrollar liposomas de oxaliplatino pegilados para evaluar su comportamiento en estudios *in-vitro* e *in-vivo*.
2. Desarrollar liposomas de oxaliplatino dirigidos frente al EGFr utilizando diferentes ligandos.
3. Evaluar el mecanismo de internalización celular de los liposomas dirigidos y no dirigidos *in-vitro* y su eficacia antitumoral *in-vivo.*

CHAPTER 2

APPLICATION OF DIFFERENT METHODS TO FORMULATE PEG-LIPOSOMES OF OXALIPLATIN: EVALUATION *IN-VITRO* AND *IN-VIVO*

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APPLICATION OF DIFFERENT METHODS TO FORMULATE PEG-LIPOSOMES OF OXALIPLATIN: EVALUATION *IN-VITRO* AND *IN-VIVO*

Sara Zalba [a], Iñigo Navarro [b], Iñaki F. Trocóniz [a], Conchita Tros de Ilarduya [a], María J. Garrido [a]*

[a]*Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona 31008, Spain;* [b]*Departament of Analytical Chemistry, University of Navarra, Pamplona, 31008, Spain*

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***Corresponding author**: Address: Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain. Tel.: +34 948425600; Fax: +34 948425649. E-mail address: mgarrido@unav.es.

ABSTRACT

In this work the Film Method (FM), Reverse-Phase Evaporation (REV) and the Heating Method (HM) were applied to prepare PEG-coated liposomes of oxaliplatin with natural neutral and cationic lipids, respectively. The formulations developed with the three methods showed similar physicochemical characteristics except in the loading of oxaliplatin, which was statistically lower ($P < 0.05$) using the HM.

The incorporation of a semi-synthetic lipid in the formulation developed by FM, provided liposomes with a particle size of 115 nm associated with the lowest polydispersity index and the highest drug loading, 35%, compared with the other two lipids, suggesting an increase in the membrane stability. That stability was also evaluated according to the presence of cholesterol, the impact of the temperature and the application of different cryoprotectants during the lyophilization. The results indicated long-term stability of the developed formulation because after its intravenous *in-vivo* administration to HT-29 tumor bearing mice it was able to induce an inhibition of tumor growth statistically higher ($P < 0.05$) than the inhibition caused by the free drug.

In conclusion, the FM was the simplest method in comparison with REV and HM to develop *in-vivo* stable and efficient PEG-coated liposomes of oxaliplatin with a higher loading than the reported for REV.

1. - Introduction

Liposomes are considered as efficient carriers for drugs, vaccines, nutrients, diagnostics, and other biomolecules [1–6]. This is due to some advantages, such as the ability to incorporate water and lipid soluble agents, high versatility in terms of fluidity of liposomal membrane, size and superficial charge [7]. The new generation of liposomes by the insertion of polyethylenglycol (PEG)-derivatized phospholipids into liposomal membrane, leads to obtain sterically stabilized (stealth) liposomes [3, 8–10]. The main characteristics of these liposomes are the decrease in their clearance [11, 12] and their increase accumulation in affected organ sites [13, 14]. Therefore, this system is able to alter the pharmacokinetics and biodistribution of the encapsulated drug [15].

In this way, oxaliplatin is a third generation of platinum (Pt) antitumor drugs, used as first-line chemotherapy for metastatic colorectal cancer [16–19]. This platinum derivative shows higher tolerability of adverse effects than cisplatin or carboplatin [20–22]. However, its efficacy is relatively low due to its pharmacokinetic properties, such as high irreversible binding to plasmatic and tissue proteins and erythrocytes among other components. For this reason, the encapsulation of oxaliplatin represents a strategy to overcome these limitations, delivering in a selective manner the drug into the tumor.

On the other hand, the methods used to prepare liposomes have a significant impact in some physicochemical characteristics such as size or efficiency of encapsulation of the agent. In this way, Film Method (FM) [23] and Reverse-Phase Evaporation (REV) method [24] have been selected by several authors as two conventional methods to prepare liposomes. However, in the last years other methods have been described in literature and one of them is the Heating Method (HM) [25]. This new method is characterized by the absence of organic solvent for the solubilization of lipids, representing an advantage in terms of toxicity. In general, all methods have advantages and disadvantages.

FM is characterized by the fact that it can be used for all different types of lipid combinations and it is very easy to perform. The main step is the hydration of the lipids, and the acceptable encapsulation rates that can be obtained [26]. For REV the main step is in the oil/water emulsion, which is diluted with further aqueous phase for liposomes formation. This method is very popular due to a high encapsulation rate, up to 50%; however, the problem is the remaining solvent and the high polydispersity index (PDI) in the particle size. In both cases, to formulate a homogeneous population of liposomes regarding the particle size it is needed the application of a homogenization technique.

Finally, the HM has not been widely applied, because few examples are only reported in the literature with 5-FU and DNA [27–29].

Taking into account that most of the publications about liposomes of oxaliplatin have used REV method, the aim of this work is the development of PEG-coated liposomes of oxaliplatin using different methods and lipids. It is also evaluated the stability of the formulation under different conditions. In addition, the cytotoxicity and antitumor effects, respectively, were assayed in *in-vitro* and *in-vivo* models with colorectal cancer cell lines.

2. - Material and methods

2.1. - Materials

Oxaliplatin was purchased from Sigma (Barcelona, Spain). Phosphatidylcholine (PC), cholesterol (Chol), soy hydrogenated L-α-phosphatidylcholine (HSPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine- N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG 2000) were purchased from Avanti polar lipids Inc. (Alabaster, Alabama, USA).

2.2. - Methods

2.2.1. - Oxaliplatin liposomes preparation

Three different methods were carried out to develop oxaliplatin- loaded liposomes.

2.2.1.1. - Film Method

Liposomes containing oxaliplatin were prepared employing the thin film hydration method following the basic specifications described by Bangham and Lea [23]. Briefly, lipids were dissolved in chloroform forming a mixture. The organic solvent was then removed by rotary evaporation under reduced pressure (Büchi-R144, Switzerland) at room temperature (RT) to obtain a film on the wall of the flask. The dry lipid film was hydrated with a solution of oxaliplatin dissolved in glucose 5% (2 mg/ml). The dispersion of the lipid was facilitated by mechanical shaking in an ultrasound bath for 1 min. To control the particle diameter the emulsion was extruded through a polycarbonate membrane (Mini-Struder Set, Avanti Polar Lipids Inc (Albaster, Alabama, USA)) with a pore size of 100 nm.

2.2.1.2. - Reverse-Phase Evaporation method

This method, described by Szoka and Papahadjopoulos [24], is used to prepare liposomes with a large internal aqueous space. Lipids solubilized in a mixture of chloroform:diethyl ether (1:2, v/v) were added to the aqueous phase containing oxaliplatin (4 mg/mL) dissolved in glucose 5%, in a ratio 3:1 (v/v) between organic and aqueous phase. The mixture was sonicated at RT for 5 min and placed on the rotary evaporator to remove the organic solvent under reduced pressure (200 mm Hg). At this point, the material forms a viscous gel which becomes an aqueous suspension by shaking in a vortex. The liposomes were extruded following the method described earlier.

2.2.1.3. - Modified Heating Method

In the last technique, the Heating Method [25] was combined with a gradient of pH [30]. The lipids were hydrated in a citrate solution (pH 4) and mixed in a bath-ultrasound for 1 min. The mixture was extruded as it has been described in the previous methods, and the excess of citrate was removed by ultrafiltration using an Amicon (10,000 MWCO) (Millipore, Billerica, USA).

The incorporation of oxaliplatin (2 mg/mL) dissolved in glucose 5% was achieved by adding the drug solution together with Hepes solution (pH 7.8). This mixture was heated at the corresponding lipid transition temperature, 60°C, for 30 min. Afterwards it was cooled at 4°C.

In all methods, the amount of non-encapsulated oxaliplatin was removed from the formulation by ultrafiltration using the Amicon devices (10,000 MWCO). The final formulation was washed at least two times with 3 ml of PBS and ultrafiltered again.

To evaluate the efficiency of this method a fixed concentration of free oxaliplatin (1 mg/mL) was added to empty liposomes. This mixture was shaking for 30 min at room temperature and it was ultrafiltered using the Amicon system (10,000 MWCO) at 2,200 g for 30 min. After the ultrafiltration, both aliquots were collected, the liposomes and the ultrafiltered solution, to measure the levels of oxaliplatin by the atomic absorption spectrometry technique.

Liposomes without oxaliplatin, empty formulation, were prepared following the same procedure but adding glucose 5%.

These methods were carried out with two different types of lipids: neutral, such as PC and cationic, DOTAP, in order to study the influence of them in the physicochemical characteristics of the liposomes developed and in the efficiency of encapsulation (EE) of oxaliplatin.

2.2.2. - Characterization of liposomes

The particle size, polydispersity index (PDI) and Zeta potential of liposomes were analyzed by laser diffractometry using a Zetasizer Nano-Z (Malvern Instruments, UK). Formulations were diluted 1:100 (v/v) in deonized water in order to ensure a convenient scattered intensity on the detector.

The oxaliplatin encapsulation was measured by atomic absorption spectrometry using a validated method. Then, the EE expressed in percentage (%) was calculated by dividing the drug to lipid ratio recovered after ultrafiltration in the final formulation by the initial amount of oxaliplatin and lipid.

The phospholipid concentration was quantified following the Zöllner and Kirsch method [31].

2.2.3. - Stability of liposome formulations

Stability is a critical factor that must be considered during formulation design and development. Physical or colloidal stability based on size distribution under storage conditions as well as in a biological medium must be considered. Based on the results found during liposome formulation, HSPC liposomes developed with FM were selected to characterize the stability of the liposomes formulated without and with cholesterol [HSPC:Chol:DSPE-PEG2000]. Chol was used at 40% in the lipid mixture.

In addition, other different approaches were followed to complete this study.

2.2.3.1. - Drug release

This study was carried out at two different temperatures: 4°C, used to storage the formulation and 37°C used for *in-vitro* and *in-vivo* studies. Then, 100 µl of formulation mixed with 900 µl of complete cell medium was incubated at 37°C in continuous shaking.

Samples collected at different times, 0, 1, 4, 7, and 24 h, were ultrafiltered using the Amicon system (10,000 MWCO) to obtain the liposomes.

The encapsulated and released oxaliplatin levels were quantified by atomic absorption spectrometry. In addition, the parameters particle size, PDI, and Zeta potential, were also characterized in these samples.

2.2.3.2. - Lyophilization assay

Formulations were lyophilized following three different strategies: without cryoprotectant, with Trehalose (4:1, w/w sugar:lipid), or with L-arginine (4:1, w/w aminoacid:lipid). After the lyophilization process, the formulation was again characterized by determining size, PDI and Zeta potential.

2.2.4. - Transmission electron microscopy (TEM)

Liposomes formulated without and with DSPE-PEG2000 were analyzed by TEM [32]. The measurements were carried out by means of a LIBRA-Zeiss 120 electron microscope operating at 80 kV, equipped with an electron spectrometer filtering out inelastic electrons for better imaging.

Ten microliters of the sample were incubated with OsO4 (1%) during 30 min. Twenty microliters of the mixture were deposited over carbon-coated copper grids with 200 mesh during 1 minute and dried. Negative contrast staining was carried out using 2% aqueous phosphotungstic acid solution. The samples were visualized 24 h later.

The same protocol was followed for the negative control corresponding to a sample without liposomes. Images were analyzed using iTEM Olympus Soft Imaging Solutions GmbH 5.1 software.

2.2.5. - Antiproliferative activity in cultured cells

The human colorectal cancer lines HCT-116 and HT-29 (purchased from ATCC) were cultured in McCoy's Medium modified, completed with Fetal Bovine Serum (10%) and Penicillin–Streptomicin (1%), at 37°C in a humidified atmosphere containing 5% CO_2. All cells were used under sub-confluence conditions. Cells were seeded into 96-well microtiter plates at a density of $10x10^3$ cells/well/200 µl of medium.

After 24 h, cells were treated with several concentrations (ranged from 0.1 to 50 µM) of free oxaliplatin, empty liposomes or oxaliplatin liposomes, for 72 h. The survival cells after each treatment were measured with the colorimetric Neutral Red Assay [33]. The optical density was read at 540 nm (Labsystems iEMS Reader MF).

2.2.6. - *In-vivo* study

Twenty-four female athymic nude mice weighing 20–25 g (aprox. 4 weeks old) were purchased from Harlan (Barcelona, Spain). Animals were housed in microisolator cages under positive pressure ventilation and maintained in closed-shelf laminar flow racks to avoid contact with pathogens, odors or noises and kept under standard laboratory conditions. Sterilized food and water were available *ad libitum*.

To induce the tumor, 100 µl of PBS containing $1.5x10^6$ HT-29 cells were subcutaneously injected into the right flank of the mice. Tumor growth expressed as volume (V) was calculated by $V= 4/3\pi\ (A^2B/2)$, where *A* and *B* correspond to the smallest and the largest diameter, respectively [34].

One week after cells injection and when the volume of the tumor was around 200 mm^3, animals were randomly divided into four groups with six animals per group: Group I, control (PBS), Group II, animals treated with empty liposomes at corresponding dose of

oxaliplatin, Group III, free oxaliplatin (5 mg/kg) and Group IV, oxaliplatin encapsulated in liposomes (5 mg/kg).

The dose of liposomes was calculated based on the quantity of oxaliplatin encapsulated per lipid, avoiding a concentration higher than 1.25 mg of lipids in each injection. All animals received two consecutives cycles of treatment with 5 days apart. In each of them, two doses of 2.5 mg/kg every 48 h were administered. At the end of the study, animals were sacrificed by cervical dislocation.

The tumor was removed to quantify the oxaliplatin levels. This organ was weighted and homogenized with nitric acid 0.1 N overnight (100 mg tissue/1 mL acid) and diluted with 5 mL of deonized water. Then, the samples were measured by atomic absorption spectrometry.

Toxicity was also evaluated by measuring the body weight at the same time that the tumor. The protocol of the study was approved by the Animal Experimentation Committee of the University of Navarra and is in accordance with the applicable European guidelines.

2.2.7. - Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The statistical study was performed using SPSS, version 15.0 for windows. All data were analyzed with the non-parametric Kruskall–Wallis test followed by the Mann–Whitney U test. The level of significance was set at $P < 0.05$.

3. - Results

3.1. - Characterization of PEG-coated liposomes of oxaliplatin

3.1.1. - Film Method

The results found with FM are shown in Table 1. The particle size was similar for the two formulations, as it was expected, due to the use of the extrusion technique with the same type of membrane. The Zeta potential was negative for PC due to the combination of lipids and positive for DOTAP, a typical cationic lipid. In relation to the EE, the percentages were similar between both lipids, although it was slightly higher for PC than for cationic lipid ($P > 0.05$). However, this value was higher than those reported by other authors [35–38].

Table 1. *Physicochemical characterization of two liposomal formulations using three different methods. Data represent the mean of three independent measurements with SD in parentheses.*

	Size (nm)	*Z potential (mV)*	*PDI*	*EE (%)*	*Oxaliplatin(µg)/ mg lipid*
Film Method					
PC	*112.4 (9.5)*	*-21.4 (2.2)*	*0.161 (0.02)*	*36.74 (6.4)*	*65.61 (7.28)*
DOTAP	*119.3 (14.7)*	*23.8 (3.5)*	*0.185 (0.02)*	*30.10 (8.2)*	*39.02 (5.6)*
Reverse-Phase Evaporation					
PC	*160.1 (7.8)*	*-29.3 (2.0)*	*0.223 (0.03)*	*30.44 (12.1)*	*59.30 (3.9)*
DOTAP	*132.7 (4.1)*	*34.0 (0.2)*	*0.226 (0.06)*	*26.78 (2.1)*	*41.00 (5.8)*
Heating Method					
PC	*108.3 (3.2)*	*-20.4 (2.0)*	*0.106 (0.002)*	*22.15 (0.6)*	*45.83(8.24)*
DOTAP	*117.9 (4.4)*	*33.8 (6.1)*	*0.083 (0.021)*	*27.08 (1.8)*	*57.07 (3.6)*

PC, Phosphatydilcholine; DOTAP, 1,2-dioleoyloxy-3-[trimetryammonio] propane; PDI, polydispersity index; EE, efficiency of encapsulation.

3.1.2. - Reverse-Phase Evaporation method

REV is the most common technique used to encapsulate platinum derivatives. Table 1 shows the results found for liposomes of oxaliplatin where all the parameters were very similar for both lipids except the Zeta potential, as it was expected. The EE for drug was slightly lower for DOTAP, but this difference was not significant ($P>0.05$), suggesting that the method ruled in the same way with independence of the ionic charge of the lipid.

3.1.3. - Heating Method modified

This method has been modified adding citrate and Hepes buffers to reach a gradient of pH. This change was because the amount of oxaliplatin incorporated into liposomes without difference of pH between inside/outside was very low, 6% approximately. However, when the oxaliplatin was in a solution of pH 7.8, the encapsulation rate was higher than 20%. Although the size and Zeta potential obtained in these formulations were similar to those observed with the other two methods, the EE was lower ($P < 0.05$) (Table 1).

No differences were observed between liposomes with vs. without oxaliplatin in each of the methods (data not shown). The three methods can be considered for manufacturing liposomes of oxaliplatin. However, the REV was one of the most complex methods due to the number of steps. In fact, several initial conditions of oxaliplatin/lipid ratio were tested to increase the loading but this value did not increase when the amount of oxaliplatin was higher than 4 mg/mL. In addition, the liposome suspension showed quite high polydispersity in terms of size. The reason could be that to obtain homogeneous

population of liposomes the extrusion technique could be applied with two types of membranes before the evaporation step.

On the other hand, although HM has the advantage to produce liposomes without volatil organic solvents to dissolve the lipids, the EE for oxaliplatin was the lowest compared with FM or REV. The main step is the incorporation of the drug into the liposomes previously formulated by heating at temperatures not lower than the transition temperature (Tc) of the lipids. This Tc was around 60°C, which is not high enough to modify the stability of the platinum molecule.

Based on the results found in Table 1 the FM appeared to be the simplest method to prepare liposomes with an adequate size, PDI and EE of oxaliplatin. Since PC and DOTAP were used as two basic lipids for selecting the method, another semi-synthetic hydrogenated lipid, HSPC, was included. This lipid was of interest because is one of the main components of several marketed liposomal formulations. Lipids with different degree of saturation in their aliphatic chain seem to provide a higher stabilization effect to the liposomal membrane. The physicochemical parameters of this HSPC formulation were similar to those obtained for the liposomes formulated with PC. Thus, the particle size for this formulation was 115.6 ± 2.0 nm, with a Zeta potential of -18.4 ± 3.9 mV, the EE was 34.23 ± 2.9% and a loading of 68.5 ± 4.2 μg/mg of lipid. Moreover, the PDI was lower than the PC formulation, 0.034 ± 0.01 vs. 0.161 ± 0.02, suggesting that HSPC contributed to increase the stabilization of the membrane. Therefore, HSPC–DSPE-PEG2000 liposomes were selected for the next studies.

3.2. - Stability assay

Previous studies showed that at 4°C the formulations were stable in relation to size, Zeta potential and EE at least for 1 month. The impact of several factors such as the

inclusion of Chol, the temperature, different solutions for the incubation and freeze-drying processes, were also investigated to evaluate the retention of oxaliplatin into the liposomes.

Table 2 shows that at 37°C the inclusion of the sterol increased the stability of the membrane, although the difference in the retention of oxaliplatin between both formulations, with vs. without Chol, was only 10%. However, the drug release in the culture cell medium was slower for liposomes with Chol, suggesting that this release takes time because Chol is able to stabilize the lipid bilayers. Therefore, a depot effect in tumor area could be achieved using this pegylated liposomal formulation. This effect represents an advantage to oxaliplatin because the drug would be more stable in the blood circulation and could be released slowly at the tumor site. In addition, the PDI presented a higher value for liposomes without Chol. This observation is compatible with an aggregation of the particles.

Table 2. *Influence of Chol in the stability of the formulations developed by FM and incubated at 37°C with culture cell medium. Data represent the mean of three independent measurements with SD in parentheses.*

	Time (h)	*Size (nm)*	*PDI*	*Zeta potential (mV)*	*ED (%)*
HSPC–PEG2000	*0*	*91.5 (0.4)*	*0.190 (0.03)*	*-17.2 (1.6)*	*100*
HSPC–Chol–PEG2000	*0*	*122.9 (2.1)*	*0.050 (0.03)*	*-22.2 (1.0)*	*100*
Complete medium					
HSPC–PEG2000	*1*	*74.1 (1.7)*	*0.203 (0.01)*	*-14.0 (0.7)*	*86.2 (0.15)*
HSPC–Chol–PEG2000	*1*	*118.6 (1.8)*	*0.081 (0.01)*	*-21.7 (1.3)*	*95.0 (0.01)*
HSPC–PEG2000	*4*	*73.7 (0.5)*	*0.231 (0.02)*	*-11.8 (2.0)*	*79.1 (0.01)*
HSPC–Chol–PEG2000	*4*	*116.7 (0.5)*	*0.098 (0.05)*	*-20.7 (1.6)*	*87.9 (0.01)*
HSPC–PEG2000	*7*	*76.1 (0.7)*	*0.248 (0.02)*	*-12.6 (1.1)*	*72.2 (0.01)*
HSPC–Chol–PEG2000	*7*	*115. (0.6)*	*0.103 (0.01)*	*-23.2 (1.4)*	*78.9 (0.01)*
HSPC–PEG2000	*24*	*82.1 (1.3)*	*0.250 (0.01)*	*-11.6 (1.2)*	*64.9 (0.1)*
HSPC–Chol–PEG2000	*24*	*111.7 (1.1)*	*0.064 (0.01)*	*-17.3 (3.9)*	*72.8 (0.01)*

HSPC, soy hydrogenated $_L$-α-phosphatidylcholine and ED, encapsulated drug.

Since the structural integrity of the liposomes for long period of time is one of the objectives to optimize the formulation, the effect of the dehydration/reconstitution process was assayed in this work. Two different cryoprotectants, Trehalose and L-arginine were used to prevent the thermodynamic instability evaluated by the changes in the size and Zeta potential. L-arginine was included with the thought of the possible problems of diabetic patients. The results listed in Table 3, showed that the presence in the formulation of Chol together with Trehalose or L-arginine was the best combination to obtain a stable formulation. Both cryoprotectants displayed similar behavior, supporting the fact that both could be used.

Table 3. *Influence of lyophilization process in the size, PDI and surface charge of liposomes associated with two cryoprotectants. Data represent the mean of three independent measurements with SD in parentheses.*

	Size (nm)	*PDI*	*Zeta potential (mV)*
HSPC–Chol–PEG2000 [fresh]	*122.9 (2.1)*	*0.050 (0.03)*	*-22.2 (1.0)*
HSPC–Chol–PEG2000 [lyophilized]	*384.8 (35.0)*	*0.479 (0.08)*	*-17.6 (0.7)*
HSPC–Chol–PEG2000/trehalose	*102.3 (0.4)*	*0.169 (0.05)*	*-16.2 (1.5)*
HSPC–Chol–PEG2000/L-arginine	*131.7 (2.9)*	*0.138 (0.01)*	*-31.6 (0.8)*

3.3. - Transmission electron microscopy (TEM)

Figure 1 shows that HSPC:Chol of liposomes were small vesicles with a concentric interior space. In the case of liposomes associated with DSPE-PEG2000, a white coated film was observed in the surface [32].

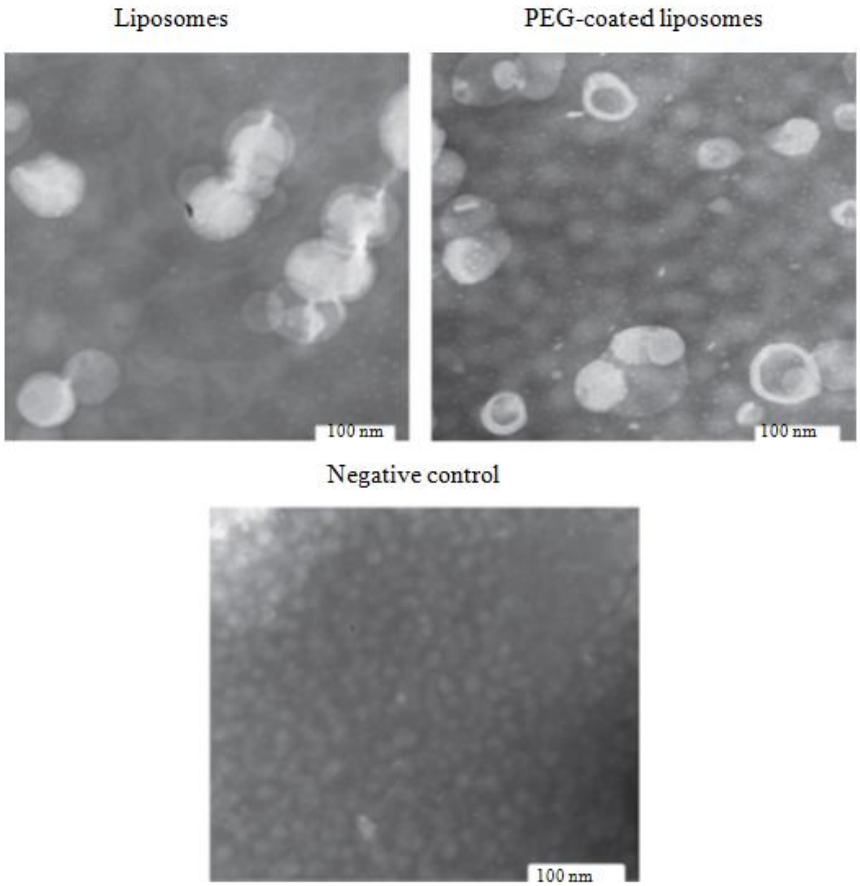

Figure 1. *Photographs of transmission electron microscopy. Left panel shows nonpegylated liposomes (HSPC:Chol (2:1)), whereas right panel shows pegylated liposomes (HSPC–Chol–PEG2000 (2:1:0.2)). Bars represent 100 nm. The negative control is in the low panel.*

3.4. - Cytotoxic studies in colon cancer cell lines

The two cell lines were sensitive for oxaliplatin with IC_{50} values between 9.2 and 2.8 µM for the free drug. Oxaliplatin-loaded liposomes showed a reduced cytotoxicity. This effect was observed for the three types of lipids used in the formulation. Table 4 lists the IC_{50} values found for all treatments in both cell lines, HT-29 and HCT-116.

Table 4. *IC_{50} values of oxaliplatin and oxaliplatin encapsulated in different types of liposomes. The values are expressed in µM.*

	Free oxaliplatin	*PC-LP*	*DOTAP-LP*	*HSPC-LP*
HCT-116	*2.8 ± 0.6*	*9.3 ± 2.4*	*4.5 ± 1.9*	*8.9 ± 1.7*
HT-29	*9.2 ± 2.2*	*16.5 ± 2.8*	*9.5 ± 2.5*	*15.1 ± 3.2*

PC-LP, Phosphatidylcholine-liposomes; DOTAP -LP, 1,2-dioleoyloxy-3-[trimetryammonio] propane-liposomes; HSPC-LP, soy hydrogenated $_L$-α-phosphatidylcholine-liposomes.

HCT-116 was more sensitive to oxaliplatin, free and encapsulated, than HT-29. The cytotoxic effect was higher in both cell lines for free than for the encapsulated oxaliplatin in anionic liposomes, PC-LP and HSPC-LP. However, in the case of the cationic liposomes the value of the IC_{50} was very similar to the value for the free drug. This difference could be explained by the effect of the empty DOTAP liposomes. They were able to reduce the proliferative effect about 30% compared with the control group. PC and HSPC liposomes without oxaliplatin did not affect the cell proliferation.

3.5. - *In vivo* study

The formulation, [HSPC:Chol:DSPE-PEG2000] of oxaliplatin liposomes, was intravenously administered to HT-29 tumor bearing mice. The dose was selected based on previous results found in our group (data not shown) and on the dose reported by Abu Lila et al. [35–37], although the cell lines were not the same. Figure 2 show that pegylated liposomes suppressed tumor growth more efficiently than free oxaliplatin. This inhibition reached statistical significance ($P<0.05$) during the second cycle. This enhanced antitumor activity of the liposomes is in line with those results reported by several authors for different antitumor drugs encapsulated in pegylated liposomes.

The levels of oxaliplatin found in the tumor at the end of the study were three times higher for encapsulated drug in comparison with free drug: 560 ± 200 vs. 190 ± 101 ng/mg tissue, respectively. In addition, this dose schedule was compatible with a low toxicity for both treatments (Figure 3). So, the encapsulation of oxaliplatin exhibited an improvement in the therapeutic effect of the drug.

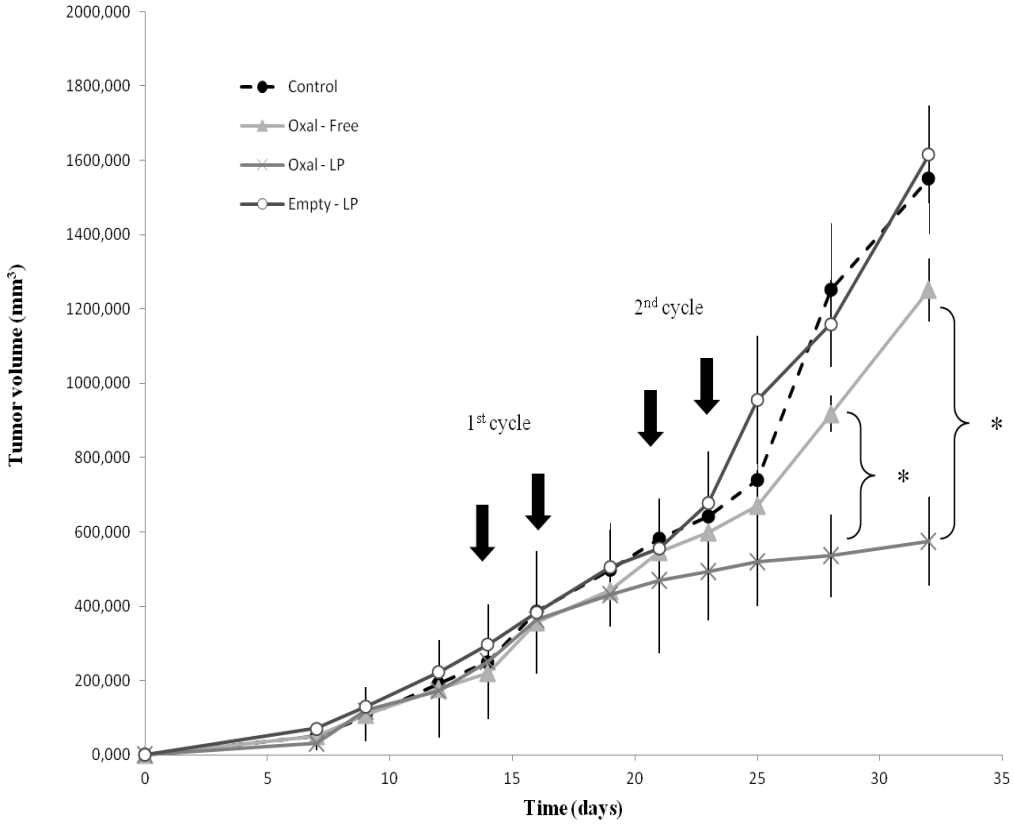

Figure 2. *Time profiles of the tumor growth after two cycles of treatments with free and encapsulated oxaliplatin. Each symbol represents the mean of six animals and the bars, the corresponding standard deviation. Oxal-LP, liposomes without oxaliplatin (*P< 0.05).*

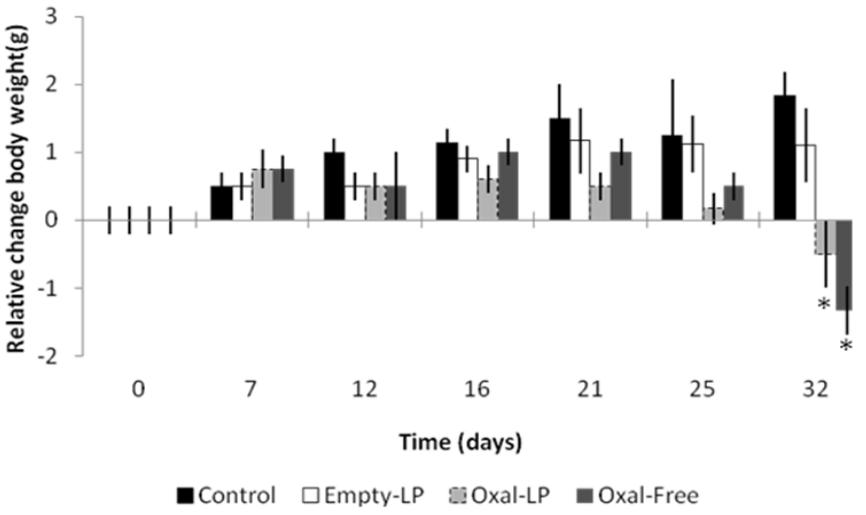

Figure 3. *Body weight changes in mice treated with PBS (control), empty liposomes, oxaliplatin liposomes, and free drug. The results represent the mean ± SD (*P < 0.05).*

4. - Discussion

In this work, pegylated liposomes of oxaliplatin have been developed using different methods. FM and REV were selected as the most common methods used to prepare conventional liposomes [4, 5]. The HM was chosen as one of the novel methods introduced in the last years. The absence of volatile organic solvents in this last method is the main characteristic. It represents a potential benefit in relation to the toxicity exerted by these components in *in-vivo* [25]. In addition, two types of lipids have been used to evaluate their influence in the physicochemical characteristics of the formulations obtained for each type of method.

The results showed that the lipid did not have any influence in the studied characteristics. However, in the case of the REV method, the particle size was slightly higher than in the FM and HM, even when all methods were associated to the extrusion technique with the same type of polycarbonate membrane. This difference could be

explained by the fact that REV is one of the most complex methods, moreover compared to FM and HM. For example, the main step is the formation of a viscous gel which is responsible of the spontaneous formation of liposomes dispersion [24]. The characteristics of these liposomes depend on the lipid–water mixture, in the emulsion and in the time of the evaporation, among other steps. This leads to a higher variability in the final formulations than with the other two methods, which are methodologically simpler. In the case of the HM, the encapsulation of oxaliplatin dissolved in glucose 5% was extremely low (aprox. 6%). However, when a gradient of pH was reached between inside (pHi 4) and outside (pHo 7.8) of the liposomal formulation, the efficiency of encapsulation achieved levels of 20%. This result is in the same order as those values reported by other authors for oxaliplatin using the REV method [35–38]. In general, the transbilayer transport of weak acids and weak bases is more efficient in the presence of a pH gradient but little or none encapsulation is observed in the absence of this gradient [30], as it was demonstrated for doxorubicine. Finally, the FM associated with the extrusion technique allowed the formulation of a homogenous population of liposomes following very simple steps.

Although some liposomal formulations with other antitumor drugs have been developed using this method, in the case of oxaliplatin most of them have been formulated with REV. The main advantage of this method is the encapsulation rate that can be up to 50%. Nevertheless, a significant difference is found in relation to the particle size and EE of oxaliplatin depending on the author. For example, with REV and neutral lipids, Abu Lila *et al* [35–37] have reported liposomes with a particle size of 200 nm and an EE of 20%, higher than the EE found by Suzuki *et al* [38]. In the last year, Yang *et al* [39] have described the methodology to develop PEG-liposomes of oxaliplatin with a particle size of 150 nm and an EE of 40%. Note that it has not been described how that value was calculated. In this work, the particle size was reduced to 115.3 ± 3.5 nm obtaining an EE of

34.2 ± 2.9%. This loading drug was calculated as Abu Lila and coworkers have described. Although several concentrations of oxaliplatin (2, 4 and 5 mg/mL) were assayed the loading did not change between 4 and 5 mg/mL.

On the other hand, different techniques have been found in the literature for the removal of the non-encapsulated drug: the dialysis technique against 5% of dextrose [35–37] or the ultrafiltration [39]. In our study, the applied ultrafiltration technique showed that the 99.72% of the free drug added to empty liposomes was in the ultrafiltered solution justifying its use. The three methods have similar behavior for neutral and cationic lipids as it was previously reported by Abu Lila *et al* [40]. This data show that each method to encapsulate a specific drug had a similar behavior without a dependence of the superficial charge of the lipid.

Taking into account that the formulations obtained with the three methods were very similar, the FM was selected to study several factors that influence the stability of the formulations. One of them is the use of semi-synthetic lipid such as HSPC, a component of many marketed liposomal formulations such as Doxil or Caelyx for doxorubicin (HSPC/Chol/DSPE-PEG2000); Ara-c (HSPC/Chol/DSPE-PEG2000); Lurtotecan (HSPC/Chol); Ambisome (HSPC/Chol/DSPG) [5, 41, 42]. Although the physicochemical characteristics of HSPC liposomes were similar to those found for PC, the PDI was lower and the EE slightly higher. The degree of saturation of the aliphatic chain of the lipid confers a more dynamic structure of the membrane. This property should represent an advantage for entrapping more of the drugs with a low permeability across the cellular membranes, as in the case of oxaliplatin [7].

Finally, HSPC-LP was selected to study other factors that influence the stability of the formulation. These factors were the temperature at 37°C, the medium of the liposomes incubation, and the presence of Chol in the membrane. In the case of the incorporation of

Chol, this factor did not influence significantly the amount of oxaliplatin released from the formulation. After 24 h of incubation at 37°C, both types of formulations with and without sterol, release oxaliplatin with a difference of 10%. This difference in drug concentration suggests a minimum impact in the effect. Nevertheless, the most important aspect in the *in-vivo* activity is the time release of the drug. Therefore, this point could be a limitation for the formulation for further studies.

In this work, the retention time of oxaliplatin into the formulation assayed in culture cell medium was slightly higher than the value found by Abu Lila *et al* [37] in plasma. Plasma does not have the same compositions of culture cell medium, but its complexity suggests that the behavior of the formulation could be similar to plasma. Then, the Chol exerted its function as a stabilizing agent of the liposomal membrane, which was reflected in the lower PDI compared with the PDI of formulations without Chol.

Therefore, the final formulation using the mixture HSPC:Chol:DSPE-PEG2000 was selected to assay its *in-vivo* activity.

The results found with the lyophilization technique represent a promising strategy to provide a stable formulation for a long period of time. Sugars have been reported to act as protective agents during the dehydration/reconstitution of liposomes by preventing vesicle fusion and enhancing the retention of the encapsulated compounds within the liposomes [43–46]. Because aminoacids have been reported to exhibit similar lypoprotective effect as sugars, L-arginine was assayed regarding the problems for patients with diabetes [47]. The application of amino acids or sugars as potential cryoprotectants did not show significant differences, although in both cases, the presence of Chol lead to a marked stability of liposomes during the freeze-drying process. This effect has been previously reported by Popova and Hincha [48]. They have reported an interaction between phospholipids and sugars due to the presence of Chol. This sterol could increase

the lipid space leading the sugars to interact with lipid head-groups. Nevertheless, more studies are required to optimize the use of cryoprotectants in the lyophilization of liposomes, because the EE of the reconstituted liposomes decreased in 6.1 ± 2.9%.

The *in-vitro* antitumor activity showed that HCT-116 had a higher sensibility to oxaliplatin compared with HT-29. This result was supported by Kalimutho et al. [49], because they have reported that the status of p-53, wild-type in HCT-116 and mutated in HT-29, could be involved in this phenomenon explaining that difference. In this study, the free oxaliplatin led to a better antiproliferative effect than the encapsulated (Table 4). These results are according to the results reported by several authors regarding the IC_{50} for free drug *vs.* liposomal formulation [50]. In the case of the cationic formulation, its effect could be explained by the additional cytotoxicity found for empty liposomes discarding these liposomes for further studies.

Additionally, the *in-vivo* toxicity of DOTAP liposomes has been reported in the literature [51, 52]. On the other hand, the *in-vivo* study carried out with the HSPC:Chol:DSPE-PEG2000 liposomes, showed an efficient antitumor activity in the murine tumor-xenograft model, reflecting the stability and ability of the formulation to reach the tumor area. This result suggests that PEG-coated liposomes could act as a depot of oxaliplatin in the tumor area, delaying its RES uptake due to the presence of the PEG in the surface of liposomes [13–15]. The antitumor activity for this formulation was more evident *in-vivo* than *in-vitro* which is according to the results found by other authors [38].

Therefore, PEG-coated liposomes of oxaliplatin developed by the FM, the simplest method, provided a potent antitumor activity compared with the free drug. In our knowledge, this is the first study where PEG-coated liposomes of oxaliplatin have been developed using several methods, FM, REV and HM, to compare the impact of them in the physicochemical parameters of the formulations, including the efficiency of encapsulation.

Moreover, the effect of the inclusion of a semisynthetic lipid and the Chol led to obtain a stable formulation during the incubation at high temperature and the lyophilization process.

Finally, the *in-vivo* antitumor efficiency was characterized by a reduction of the growth rate, followed by a stabilization of the tumor size. This effect, together with the oxaliplatin levels found in this organ, suggests a long-time stability of the formulation.

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CHAPTER 3

TARGETING OF EGFr WITH DIFFERENT LIGANDS

COUPLED TO OXALIPLATIN PEG-LIPOSOMES

CHAPTER 3

TARGETING OF EGFr WITH DIFFERENT LIGANDS COUPLED TO OXALIPLATIN PEG-LIPOSOMES

Sara Zalba[1], Azadeh Haeri[2], Iñigo Navarro[3], Gerben Koning[2], María J. Garrido[1]*

[1]Department of Pharmacy and Pharmaceutical Technology, University of Navarra, 31008 Pamplona, Spain; [2]Department of surgical oncology, Erasmus Medical Center, Rotterdam 3015 GE, The Netherlands; [3]Department of Chemistry and Edaphology, University of Navarra, 31008 Pamplona, Spain

Keywords: EGFr targeted liposomes, oxaliplatin, colorectal cancer, Cetuximab, Fab' fragment, *in-vitro/in-vivo*

***Corresponding author**: Address: Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain. Tel.: +34 948425600; Fax: +34 948425649. E-mail address: mgarrido@unav.es.

ABSTRACT

Oxaliplatin is a platinum derivative with high tolerability but low anti-tumor efficacy, due to its elevated covalent binding to erythrocytes and other components like tissues and plasmatic proteins. To reach a successful tumor targeting, a selective delivery system has been developed using liposomal formulations. Due to many solid tumors over-express some receptors such as the epidermal growth factor receptor (EGFr), these formulations were conjugated with Cetuximab, the monoclonal antibody against that receptor, or with its Fab' fragment, to deliver the oxaliplatin in an EGFr tumor selective way.

Two types of studies were carried out, *in-vitro* using four colorectal cancer (CRC) cell lines expressing different levels of EGFr, and *in-vivo* by a xenograft model.

The cell uptake for targeted liposomes was higher than for non-targeted and the degree of this uptake was dependent on the cell line receptor level expression. This mechanism could be demonstrated by a competition with Cetuximab administered as a pretreatment to the cells and additionally, using a cell line that expresses very low levels of EGFr. That result was also supported by imaging with confocal microscopy. In a xenograft model, the liposomal formulations improved the antitumor effect of the free drug, being the targeted liposomes more efficient than the non-targeted. Besides, liposomes conjugated with the monovalent Fab' fragment showed a higher efficacy and accumulation in tumor tissue, suggesting that the whole antibody might be easily cleared from circulation by recognition of the cristallizable fragment, Fc', and that the random orientation of the antibody within the conjugate structure might limit the receptor binding.

Therefore, the results suggest that EGFr targeted liposomes with Cetuximab, LP-CTX, or its Fab' fragment, LP-Fab', might be a promising carrier for oxaliplatin treatment in CRC that over-express this receptor. The liposomes conjugated with the fragment were the formulation that provided better *in-vivo* efficiency, due to an improvement in the passive and active targeting for delivering oxaliplatin to the EGFr positive tumor.

1. - Introduction

Liposomes, defined as nanocarriers, are basically formed by lipid bilayers. In oncology, this type of formulations represents a strategy to enhance the effectiveness of anticancer chemotherapy, reducing the risk of the side effects [1].

Platinum derivatives represent the chemotherapeutic drugs more widely used in the treatment of cancer, 50 - 70% of cancer patients. In this family, oxaliplatin is a third generation platinum derivative used in first line of colorectal cancer (CRC) treatment [2]. It is characterized by a high tolerability in the patients. However, its pharmacokinetic (PK) properties represent a serious limitation to reach a good efficacy as the free fraction is low and rapidly cleared [3]. Liposomal carriers have a strong impact on the PK of the encapsulated drug. The selective distribution of these formulations to the tumor is facilitated by the passive extravasation in this area. This phenomenon, together with the chaotic blood circulation and a defective lymphatic system, is called enhance permeability effect or EPR [4].

Pegylated liposomes or stealth liposomes are characterized by the presence of the polyethylen glycol (PEG) in the surface, providing a longer half-life in the plasma compartment. This is because the polymer allows a significant delay in the uptake of liposomes by the reticulo-endothelial system [5]. That property, together with the EPR effect, provides a higher accumulation of the liposomes in the tumor tissue, modifying the biodistribution of the drug. The pegylated liposomes of oxaliplatin have previously shown a higher antitumor efficacy than the free drug [6, 7].

The new generation of liposomes is represented by ligand-conjugated pegylated liposomes used to achieve a selective tumor targeting, and including different types of ligands such as small molecules, peptides or even monoclonal antibodies [8]. These ligands

are related to some receptors or molecules over- or specific-expressed in some types of cancer cells.

The epidermal growth factor receptor (EGFr) is a member of the tyrosin kinase family. It is implicated in the cell proliferation and survival processes, and is over-expressed in many solid tumors as CRC [9].

Cetuximab (CTX) is a chimeric human-mouse antibody that binds to the EGFr, inhibiting the receptor activation [10]. This binding induces an inhibition of the basal phosphorilation, avoids the binding of the EGF or TGF-α and leads to a receptor down-regulation, although this process is very slow. In this way, the second mechanism for this type of molecules is the elimination of tumor-cells by an immune-mediated effect. Repeated administrations of chimeric monoclonal antibodies (mAb) are associated with the presence of certain immunogenic reactions, due mainly to the presence of Fc' fragment.

In the last 5 years, several works have reported the development of functionalized nanocarriers with (mAb), including CTX [11]. However, to diminish the immune side effect of these molecules, the coupling of the Fab' as a ligand instead the whole antibody is the new strategy used by some authors.

Moreover, in CRC therapy CTX is administered in association with Oxaliplatin to potentiate the efficacy of the antitumoral drug.

Thus, the aim of this work was the conjugation of pegylated oxaliplatin liposomes with different EGFr target ligands, CTX and Fab', and their *in-vitro* and *in-vivo* evaluation.

2. - Materials and methods

2.1. - Drugs and chemicals

3-(2-Pyridyldithio)propionic-acid-N-hydroxysuccinimide-ester, Tris (2-carboxy ethyl) –phosphine -hydrochloride, dialysis tube (12,000 MWCO), 1,1′-Dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (Dil), Vivaspin tubes (300,000 MWCO) and β-Mercaptoethanol were purchased from Sigma (Barcelona, Spain). The monoclonal antibody Cetuximab (Erbitux®) and the Oxaliplatin (Eloxatin®) was provided by the University Clinic of Navarra (Pamplona, Spain). Hydroxiphosphatydilcholine (HSPC), cholesterol (CH), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxy (polyethylenglycol)-2000] (DSPE-PEG2000) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[Maleimide (polyethylene glycol) 2000] (DSPE-PEG2000-Mal) were purchased from Avanti Polar Lipids Inc. (Alabaster, Albama, USA). Amicon tubes with membranes of 10,000, 30,000 and 50,000 MWCO were obtained from Millipore Corporation (Billerica, MA, USA).

2.2. - Oxaliplatin liposomes

Liposomes of oxaliplatin were developed using the film technique, following the methodology previously described by Zalba et al. [6]. Briefly, the lipids HSPC:CH:DSPE-PEG2000:DSPE-PEG2000-Mal, in a molar ratio of 1.85:1:0.12:0.03, were dissolved in a solution of chloroform:methanol [9:1 (v/v)]. The organic solvents were evaporated at 65ºC under pressure (Büchi-R144, Switzerland). The lipid film was hydrated with a solution of oxaliplatin (5 mg/ml) in glucose 5% to obtain a final solution of 10 mM of lipids. The same procedure was followed to prepare the non-loaded or blank liposomes, but adding glucose 5% without oxaliplatin. These mixtures, with and without drug, were hydrated in

continuous agitation during 1 hour at 65°C. Then, the solution was extruded through a 100 nm polycarbonate membrane (Mini-Extruder Set, Avanti Polar Lipids Inc., U.S.A.) to obtain an homogeneous population of liposomes (LP-N).

Non-encapsulated oxaliplatin was removed by ultrafiltration at 2,200 g for 60 minutes using the Amicon system (10,000 MWCO). The liposomes were washed with Hepes saline solution (pH 6.7). This procedure was carried out in triplicate and the final formulation was stored at 4°C until use.

To prepare the labelled liposomes, the fluorescent probe Dil (λ_{exc}: 549; λ_{emm}: 565 nm) was added to the lipid mixture (1% w/w) in the step previous to the evaporation of the organic solvents.

2.3. - Development of immunoliposomes

2.3.1. - Coupling of Cetuximab to the liposomes

The method followed to develop the immunoliposomes has been previously described by Songs and coworkers (2009) [12]. Briefly, the CTX was mixed for 1 h at room temperature with 3-(2-Pyridyldithio)propionicacid-N-hydroxysuccinimide-ester (propionate) at 1:12 molar ratio under orbital shaking. Afterwards, the excess of propionate was removed from the mixture by ultrafiltration at 2,200 g for 60 minutes using the Vivaspin system (300,000 MWCO). The ultrafiltered solution was incubated for 1 h with Tris(2-carboxyethyl)phosphine-hydrochloride (1:625 molar ratio) at room temperature (RT) under agitation. These two reactions allow the thiolization of CTX generating a reactive crosslinker with the maleimide molecule present in the DSPE-PEG2000-Mal of the liposome, forming a thioether bond. This process was carried out overnight at 10ºC. The same procedure was followed to obtain fluorescent or blank immunoliposomes using Dil or non-loaded liposomes, respectively.

The formulation was purified by ultrafiltration at 2,200 g for 60 minutes using the Vivaspin system followed by a washout process with Hepes saline to eliminate the non-bound ligand. To reduce the particle agregation, the final liposomes were incubated with 1 mM of L-cysteine to quench the free radicals and prevent the disfuphide bonds. The immunoliposome (LP-CTX) was stored at 4°C until use.

2.3.2. - Fab' fragment of Cetuximab coupling to liposomes

CTX was hydrolized using a pepsin solution (1:20 w/w) prepared in sodium acetate (100 mM, pH 3.7), to obtain the antibody-binding fragments, Fab'. This process was carried out at 37°C for 2 h under shaking. The elimination of cristallizable fragment, Fc', from the previous mixture was carried out by ultrafiltration at 2,200 g for 30 minutes using the Vivaspin system (50,000 MWCO). This step was followed by two washing processes with Hepes saline and ultrafiltrations. Afterwards, the collected Fab2 fragments were reduced by incubation at 37°C for 30 minutes with a solution of 15 mM of β-Mercaptoethanol. To collect the single Fab' fragment and eliminate the β-Mercaptoethanol, two steps of filtration/washing were applied.

A SDS page with a gel of 8% was assayed to verify the control for each of the steps: whole mAb, the non-reduced Fab2 fragments and the single Fab' fragment.

In the last reduction step, a thiol group is formed in the structure of the Fab'. This functional group allows the conjugation with DSPE-PEG2000-Mal of the liposome by incubation at 10ºC overnight. The final formulation (LP-Fab') was purified by elimination of the non-coupled fragments. This was carried out by three washing/ultrafiltration cycles with Hepes saline solution and Vivaspin tubes. In this formulation the incubation with 1 mM of L-cysteine was also applied, to store the liposomes at 4°C until use. The same protocol was followed to formulate the fluorescent and blank immunoliposomes.

To quantify the efficiency of conjugation for the two different ligands, the MicroBCA™ kit (Thermo Scientific, U.S.A.) was used following the manufacturing instructions.

2.3.3. - Study to optimize the ratio ligand/lipid

In order to optimize the ligand/lipid ratio, different concentrations of ligands were tested: 5, 10, 20, 30, 40 and 50 µg of ligand per mmol of lipid. Fluorescent liposomes were developed following the protocol described above and using the different amounts of the ligands. In the final formulations the efficiency of the coupling was measured using the MicroBCA™ kit. That effiency was calculated by a comparison between the initial and the final amount of the ligand incorporated in the liposomes.

In a parallel study, the previously formulated liposomes were assayed for their uptake in HCT-116 cells. This human CRC cell line was purchased from ATCC and maintained at 37°C, 5% CO_2 in RPMI containing 10% (v/v) fetal bovine serum and a 1% (v/v) of Penicillin-Streptomycin.

One day before the study, the cells were harvested by trypsinization, counted and seeded in 96-well black plates at a density of $15x10^3$ cells/well. 24 hours later, the medium was replaced for another containing a total lipid concentration of 100 µM for each of the different fluorescent formulations previously developed. The plates were incubated at 37°C for 24 h. Afterwards, the treatments were removed and the plates were washed twice with PBS. The intensity of the fluorescence present in each well was measured with the Tecan GENios fluorimeter (Tecan Group Ltd, Maennedorf, Switzerland) and compared with the corresponding control group.

2.4. - Characterization of liposomes

The particle size and Zeta potencial of the formulations were analyzed by laser diffractometry using a Zetasizer Nano Series (Malvern Instruments, UK). Formulations were diluted 1:50 (v/v) in deionized water in order to ensure a convenient scattered intensity on the detector. The efficiency of oxaliplatin encapsulation was measured by atomic absorption spectrometry using a previously validated method [6]. The concentration of the lipid in each formulation was quantified using the phosphate assay method [13].

2.5. - Evaluation of the oxaliplatin release from the liposomal formulations

Oxaliplatin release rate from the liposomes was determined through a dynamic dialysis procedure [14]. Aliquots of the targeted formulations corresponding to 0.5 mg of oxaliplatin loaded in those liposomes were transferred into a dialysis tube (12,000 MWCO). The liposomes placed into the tubes were dialyzed against 0.5 L of phosphate buffered saline (PBS, pH 7.4) at 37°C for 24 h. Several samples of PBS were collected at 0, 2, 5, 10, 20, 30, 60 minutes and 3, 5, 7 and 24 h to measure the drug released. This assay was carried out by triplicate, and it was also performed for the free drug used as a control release rate.

To study the impact of the serum proteins in these release profiles, a parallel study was also carried out using the same protocol in presence of FBS. In this study, the aliquots of the liposomes prepared with FBS 50% (v/v), were placed into the dialysis tubes and dialyzed against PBS, as it was described above.

The released oxaliplatin was quantified by atomic absorption spectrometry. The accumulative platinum release was expressed as a percentage of release (% R) applying the following formula:

$$\%R = \left(\frac{Qa}{Qt} \right) \times 100$$

where, *Qa* represents the amount of oxaliplatin measured in the collected sample, and *Qt*, the total amount of the drug placed in the dialysis tube.

2.6. - *In-vitro* studies

2.6.1. - Cytotoxicity study

Four human colon cancer cell lines, HCT-116, HT-29 and SW-480, SW-620 were purchased from ATCC and routinely maintained at standard conditions in RPMI and DMEM respectively, containing 10% (v/v) fetal bovine serum and a 1% (v/v) of Penicillin-Streptomycin.

After the detachment of the cells by trypsinization, each cell line was seeded in 96 well microtiter plates at a density of $5x10^3$ cells/well. One day later, the cells were exposed at different concentrations, 0.1, 0.5, 1, 2, 5, 10, 20, 30, 40, 50, 80 and 100 µM of oxaliplatin, free and encapsulated in the different formulations, LP-N, LP-CTX and LP-Fab', for two different times, 1 and 4 h. After each time, the cells were rinsed with PBS and wells were refilled with complete growth medium free of drug. Cell viability was measured by Neutral Red Assay [15] at 72 h after each treatment to obtain the IC_{50} parameter. The different blank liposomes were also tested in the same conditions of lipid amount and exposure times.

2.6.2. - Cell uptake of liposomes

The cell uptake of the three liposomal formulations, LP-N, LP-CTX and LP-Fab', was quantified at different times of incubation, 0.5, 1, 3, 5 and 24 h, in the four cell lines

mentioned using fluorescent liposomes. This study was carried out in parallel at 37ºC and 4ºC [16].

The cells were seeded in 96 well microtiter black plates at a density of $15x10^3$ cells/well. After 24 h, 100 µM of lipid corresponding to each fluorescent formulation was added to the cell medium. At different times, the supernatant was removed and the cells were washed twice with PBS to eliminate the cell medium. The intensity of the fluorescence present in each well was measured with the Tecan GENios fluorimeter (Tecan Group Ltd, Maennedorf, Switzerland) and compared with the corresponding control group.

The role of the EGFr was assayed by a pretreatment with Cetuximab (37ºC for 1 h with 100 µg/mL) followed by the treatments with the different liposomal formulations. This approach was evaluated by the quantification of the fluorescensce incorporated into the cells after each treatment and by the imaging study using confocal microscopy. For this experiment, 250,000 cells were seeded in 35 mm glass plates (MatTek Corporation, U.S.A.). After 24 h, a final concentration of 1 µM of each type of formulation was added at the corresponding glass plates, and incubated for 4 or 24 h. After drug treatment, each glass plate was washed twice with PBS and then, fixed for 20 min with 4% formaldehyde in PBS. The cells were washed and incubated with TOPRO®-3 as a contrast staining. Cells were analyzed on a confocal laser-scanning microscope (LSM 510 META, Carlzeiss, Germany).

2.7. - *In-vivo* study

Fifty four female athymic nude mice weighing 20-25 g (Harlan, Barcelona, Spain) were housed in plastic cages under standard and sterile conditions (25°C, 50% relative humidity, 12 hours dark/light), with water and food *at libitum*. All the experiment were

performed according to European animal care regulations and the protocol was approved by Ethical comitee of the University of Navarra (075/07).

A subcutaneous tumor was induced by inoculating $6x10^6$ SW-480 cells in the right flank of the mice. Tumors were measured by an electronic caliper every 2 days, and the volume was calculated according to the following formula:

$$V(mm^3) = 4/3\pi(d^2 x D/2)$$

Where, *d* and *D* are respectively the smallest and the largest tumor diameters [6].

When tumors reached aproximately a volume of 200-300 mm^3, mice were randomly divided into different groups: group 1: control; group 2: free oxaliplatin; group 3: LP-N; group 4: LP-Fab'; group 5: LP-CTX; group 6: LP-N blank; group 7: LP-Fab' blank; group 8: LP-CTX blank; group 9: free CTX.

Mice from group 2 to 6 were intravenously treated with 2.5 mg/kg of oxaliplatin. Group 9 was treated with free CTX at an equivalent dose of CTX coupled to liposomes from group 5. Every 2 days animals were weighted and their tumor measured.

Four days after the first dose, mice receive a second dose [17]. The tumor growth was represented as relative tumor growth (*RTG*) following the formula:

$$RTG\ (\%) = St/Si$$

Where *St* is the size at each time point and *Si* the initial size measured.

Just after dose administration, mice from each group were randomly divided in two sub-groups. The first sub-group was sacrified at 4 h postreatment and the second at 24 h. Several organs, liver, spleen, kidney and tumor, were collected to measure the platinum

levels. These organs were weighted and homogenized with nitric acid overnight and after centrifugation, platinum levels were quantified using the atomic absorption technique.

2.8. - Statistical analysis

All data were expressed as the mean ± SD. The statistical analysis was performed using Student´s t-test or Kruskall-Wallis test followed by the U of Mann-Whitney test. The significance was set at $p<0.05$.

3. - Results

3.1. - Cetuximab Fab' fragment

Figure 1 shows the different molecules obtained during the fragmentation of CTX. The electrophoresis allowed the identification of a band in line 3 at 150 kDa of protein size, corresponding to the whole CTX. In line 4, the Fab2 fragments and pepsine are represented by the corresponding two bands while the band for the antibody is absent. The single fragment of Fab' is identified in line 5 as a band around 50 kDa of protein size. In this last line, a unique band can be observed suggesting that the protocol with the enzymatic digestion followed by the β-Mercaptoethanol treatment for reducing the disulfide bonds in the hinge region, was highly efficient to obtain the single Fab' fragments.

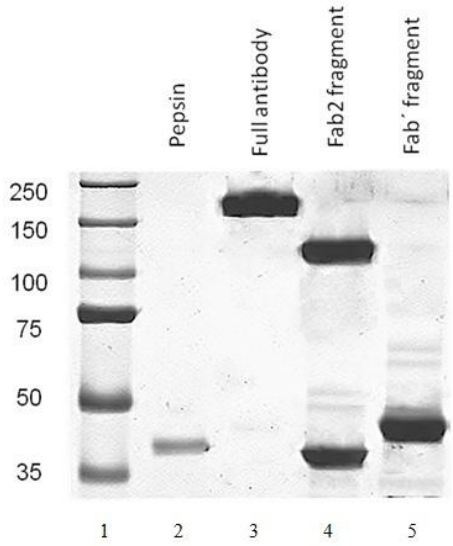

Figure 1. *SDS page with a 8% gel to control the molecules obtained during CTX fragmentation. Lines represent: 1, molecular weight size marker; 2, pepsin; 3, monoclonal antibody, CTX; 4, Fab2 fragments; 5, individual Fab' fragment.*

3.2. - Physicochemical characterization of the liposomes

Table 1 lists the results corresponding to the particle size, the polidispersity index and the Zeta potential of the three types of liposomes developed in this study.

Table 1. *Characterization of the three types of liposomes developed. Data represent the average of three independent studies and the standard deviation.*

	LP-N	***LP-Fab'***	***LP-CTX***
Size	*119.6 (0.36)*	*123.0 (1.59)*	*120.0 (1.19)*
PDI	*0.065 (0.018)*	*0. 057 (0.022)*	*0.053 (0.024)*
Zeta potential	*-22.4 (1.08)*	*-20.8 (2.19)*	*-24.4 (1.45)*

PDI, polydispersity index.

Any statistical difference for the three assayed parameters was observed among the three formulations. The particle size was aproximately 120 nm, associated with a very low PDI, suggesting a homogenous morphology in the liposomes. These formulations were stable at 4ºC for, at least, one month.

3.3. - Evaluation of the different ligand/lipid ratios

The relationship between the final amount of the ligands coupled to liposomes and the initial amount used for the conjugation is represented in Figure 2. No significant differences were found in the relation between Fab' and CTX. The coupling efficiency of the ligands to the surface of liposomes, showed a linear increase in the range between 5 and 30 µg of protein per mmol of lipid. The highest efficiency was found at the lower concentration, 94% for 5 µgof ligand/mmol of lipid, while for higher concentrations, 30 µg of protein/mmol of lipid, that percentage decreased until 66.66 ± 2.85% for Fab' and 63.40 ± 3.94% for CTX ($p<0.05$). Based on the results, 30 µg of protein/mmol of lipid was the selected ratio, due to that process reaches a plateau between 30 and 40 µg protein/mmol of lipid.

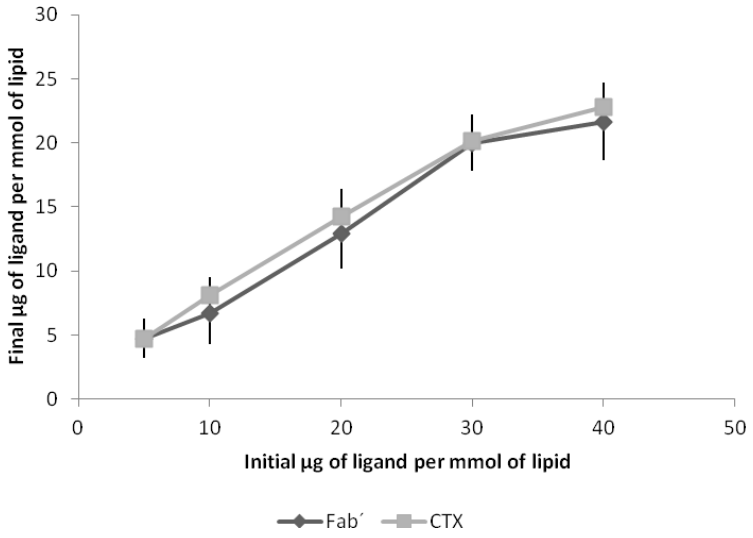

***Figure 2**. Relationship between the initial and the final amount of ligand per mmol of lipid in the developed formulations. Symbols represent the average and vertical lines the corresponding standard deviations of 3 independent studies. Lines represent the interpolation of the experimental data.*

Although the characterization shown in Table 1 corresponds to 30 µg of ligand/mmol of lipid, the size, polydispersity index and Zeta potential did not change with the other tested ratios (data not shown).

The results presented in Figure 2 could also be supported by the results found in the cell uptake study. The targeted-formulations developed with the different ligand/lipid ratios including 10, 20, 30, 40 and 50 µg of protein/mmol of lipid, were assayed in HCT-116 cells. After 24 h of cells incubation with the different liposomes, the RLU (relative light units) signal increased, as it is represented in Figure 3. Statistical significance ($p<0.05$) was found between 10 and 30 µg protein/mmol lipid. However no differences were observed between 30 and 50 µg protein/mmol lipid. Similar results were also found between 30 and 40 µg protein/mmol lipid (data not shown).

In addition, a tendency to have a higher uptake of LP-Fab' compared to LP-CTX was observed. Despite the higher affinity of CTX due to the presence of two sites of binding per molecule, the coupling of the mAb to the liposomes is randomly, representing a limitation for its binding to EGFr. However, the Fab' fragment shows always the same direction for the coupling, providing to a higher ability to receptor binding. This could explain the difference in the uptake of the two ligands.

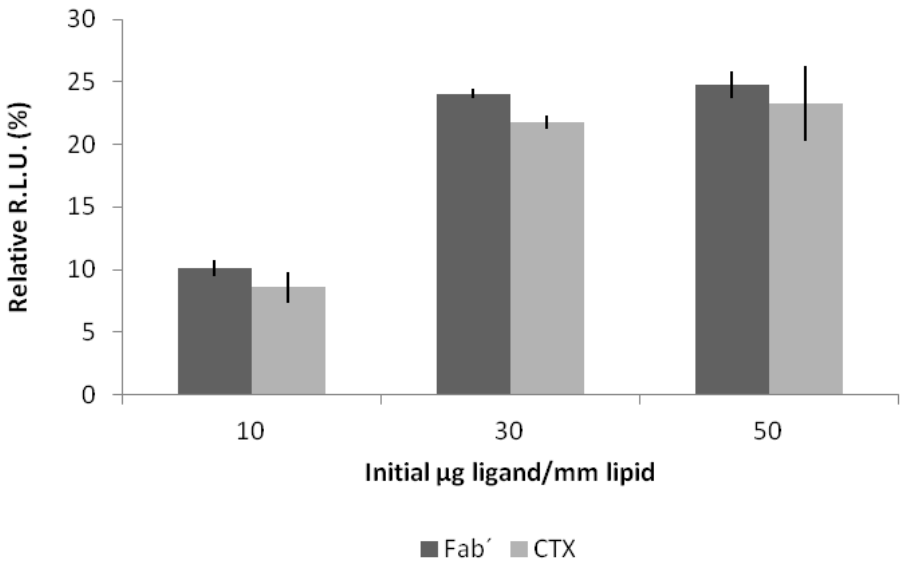

Figure 3*. Relative internalization rate, expressed as percentage of the RLU (relative light units), of the fluorescent liposomes in the HCT-116 cell line, to evaluate the three ligand/lipid ratios 10, 30 and 50 µg, of ligand/mmol of lipid. The results correspond to data from three independent studies with their standard deviation.*

3.4. - Oxaliplatin release

Figure 4 shows that the time profile of oxaliplatin released from the liposomes was maintained, slower and lower than the diffusion profile of the free oxaliplatin used as control. This release, expressed as the percentage of the initial amount, was 80% at 24 h for free solution, while for the formulations, were between 25 and 30%. To study the impact of proteins in the stability of the liposomes, FBS was added 50% (v/v) to the liposome solutions for the dyalisis. This percentage of serum led to an increase in the release between 8 and 12% in the case of liposomes. However, it did not affect the profile of free oxaliplatin. This suggests that a similar behavior could occur in an *in-vivo* system.

The time release accumulative profile for targeted liposomes was similar to the profile for non-targeted. Note that the oxaliplatin was released slower from LP-Fab' than from LP-N and LP-CTX, especially during the first 3 h. In the presence of FBS, the

platinum release from LP-Fab' after 24 h reached similar level than those observed for the other formulations release.

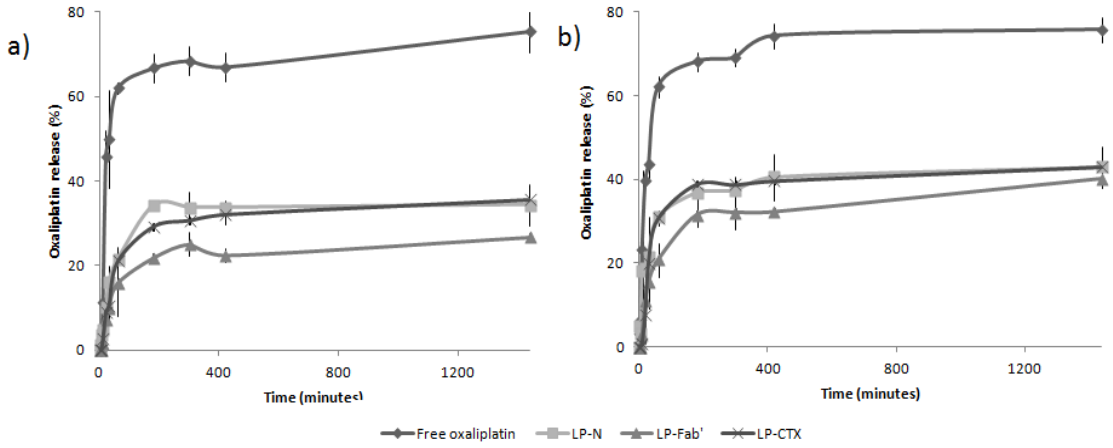

Figure 4. *Accumulative oxaliplatin release percentage from free solution, from non-targeted liposomes [LP-N] and targeted liposomes [LP-Fab' and LP-CTX] in two different conditions, a) in absence of FBS and b) with 50% of FBS. Each point represents the average of three independent studies and their corresponding standard deviation.*

3.5. - *In-vitro* quantification of cellular uptake

The internalization of the different liposome formulations assayed in four human colorectal cancer cell lines, characterized by differences in the EGFr expression levels, is represented in Figure 5. SW-620 is a cell line used as a negative control for this receptor, due to its extremely low EGFr expression. This characteristic is observed in Figure 5, where SW-620 did not show differences in the uptake of liposomes with and without ligands.

However, in the case of the HCT-116 and SW-480 the uptake at 37ºC for targeted liposomes LP-Fab' and LP-CTX, was higher and faster than in HT-29. This might due to HT-29 presents a moderate EGFr levels in comparison with the other two cell lines. In addition, when the cell lines were pre-treated with CTX blocking the EGFr, the uptake of

targeted-liposomes was dramatically reduced reaching similar levels to those observed for non-targeted.

On the other hand, at 4ºC the signal found inside the cells may be explained by a non-specific binding of the formulations to the surface of the cells. The relative values of the fluorescence were very similar among the four cell lines and for the three formulations. This result suggests that the uptake of liposomes represents an active process, which is not available at this temperature.

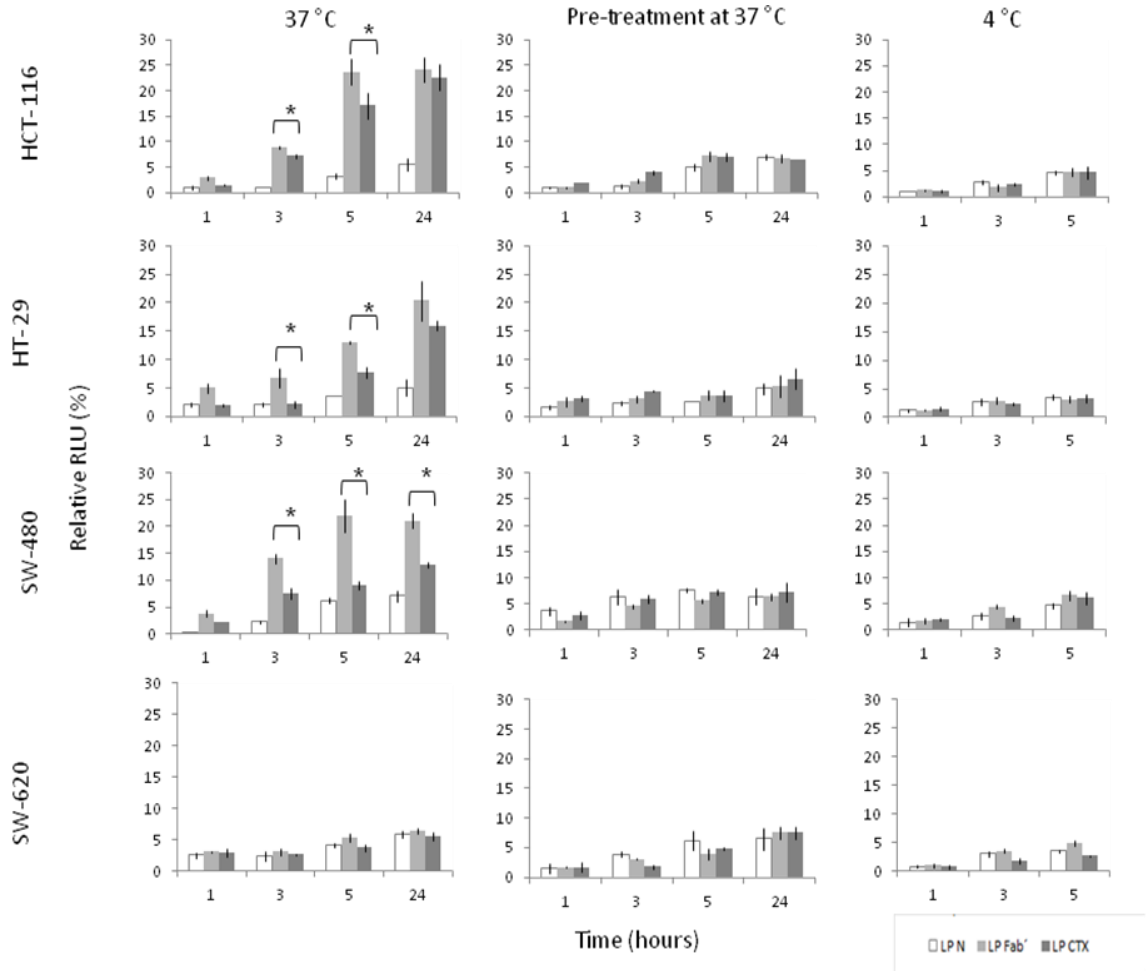

Figure 5. *Relative internalization rate, expressed as percentage of RLU,obtained at several time points during the first 24 h of treatments with targeted and non-targeted liposomes under different conditions: at 37ºC (right panels), with a pretreatment with free CTX (middle panels) and at 4ºC (left panels). Bars represent the average of three replicates with their standard deviation.*

At physiological conditions, the highest uptake was observed for LP-Fab' in SW-480, HT-29 and HCT-116, followed by LP-CTX and the lowest was for LP-N, as is observed in Figure 5, panel A.

Regarding the conventional liposomes, LP-N, their behaviour was very similar among the four cell lines and the CTX pretreatment did not influence the uptake of the liposomes.

The cell uptake for liposomes at 37ºC was also assayed by confocal microscopy. Figure 6 shows two examples corresponding to HCT-116, a cell line able to internalize all the formulations especially targeted-liposomes, and SW-620, a cell line used as a negative control for EGFr. Then, for HCT-116 the uptake was a time dependent process, because the fluorescent signal was higher after 24 h incubation than after 4 h, as is observed in Figure 6, panels a and b. In addition, that uptake was also higher for targeted liposomes than conventional liposomes.

This process was dramatically reduced again, when the cells were pre-treated with CTX, suggesting a certain binding of mAb to the EGFr. In the case of HT-29 and SW-480, similar behaviour was also found as can be observed in Figure 5 (data not shown).

SW-620 cell line showed a different behavior in comparison with the other three cell lines. In this case, the uptake was low and similar for all formulations; and the CTX pretreatment did not influence the previous results, as was expected on the basis of the previous results in Figure 5.

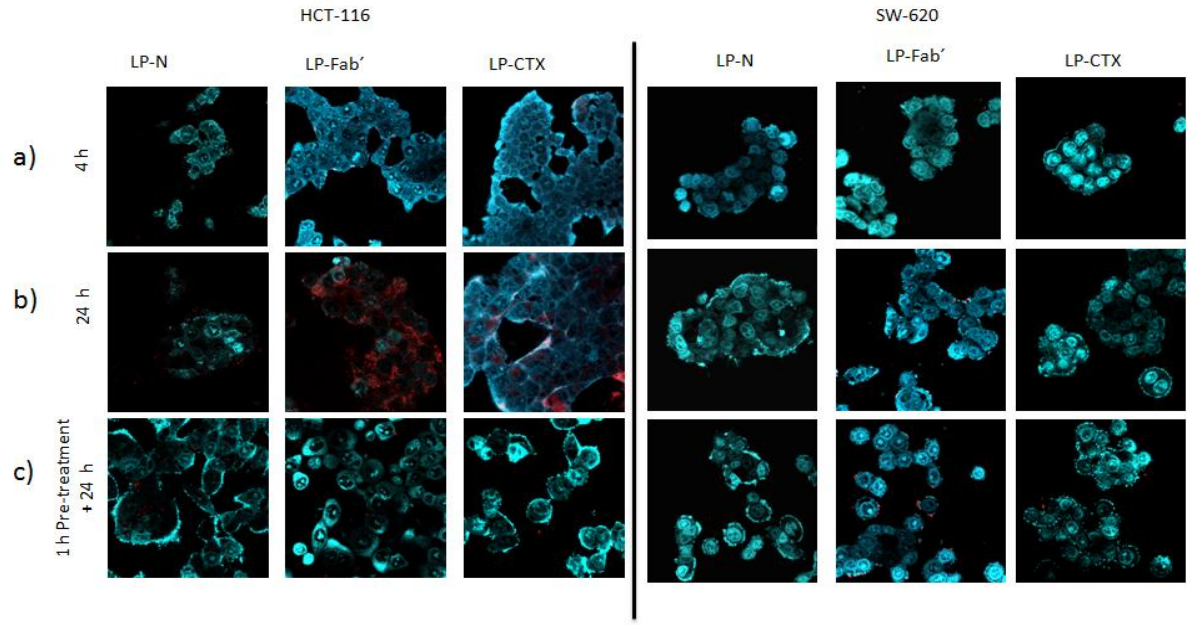

***Figure 6**. Images (63x) represent the internalization of fluorescent LP-N, LP-Fab' and LP-CTX at a) 4 hours of incubation, b) 24 hours of incubation and c) after a pretreatment with CTX and 24 h of incubation, in HCT-116 and SW-620 colorectal cancer cell lines.*

3.6. - Cytotoxicity assay

The assayed cell lines displayed different sensitivity to oxaliplatin, as is shown in Table 2. The free drug displayed a higher IC_{50} value than the formulations. In fact, these values were very different depending on the cell line. Thus, the lower IC_{50} was found for HCT-116 while the higher was for SW-480. Therefore, the order of these cell lines regarding the oxaliplatin sensitivity was as follows: HCT-116 > SW-620 > HT-29 > SW-480.

However, after the same treatment with liposomes, these values were reduced by almost one half. In addition, targeted-liposomes were even more efficient than conventional liposomes, as is observed in Table 2, where no statistically differences were found between LP-CTX and LP-Fab'. Non-targeted liposomes present values statistically higher than the value for targeted-liposomes, but statistically lower than for the free drug.

No differences were found among the three formulations in SW-620, demonstrating the influence in the uptake process of the EGFr.

The blank liposomes did not display any cytotoxic effect.

When the cytotoxic effect was studied after 1 h of drug exposure, the IC_{50} for the free drug was higher than the highest tested concentration, 100 µM (data not shown).

Table 2. *IC_{50} (µM) values measured at 72 h after 4 h of drug exposition. Data represent the average and standard deviation of 3 independent studies. *P<0.05; a, difference among cell lines; b, difference between free and non-targeted; c, difference between free and targeted; d, difference between non-targeted and targeted.*

	HCT-116	*HT-29*	*SW-480*	*SW-620*
OX	*28.67 (5.17)*^{a,b,c}	*57.87 (7.16) *^{a,b,c}	*79.22 (8.11) *^{a,b,c}	*40.49 (0.28) *^{a,b,c}
LP-N	*22.20 (1.73)*^{a,d}	*26.00 (3.91) *^{a,d}	*39.30 (4.46) *^{a,d}	*31.99 (2.12) *^{a}
LP-CTX	*16.64 (2.71)*^{a}	*21.26 (1.26) *^{a}	*26.01 (2.61) *^{a}	*30.65 (2.69) *^{a}
LP-Fab'	*17.19 (1.12) *^{a}	*23.82 (0.89) *^{a}	*28.12 (0.40) *^{a}	*35.86 (3.51) *^{a}

3.7. - *In-vivo* efficacy

SW-480 xenografts were grown as subcutaneous tumors in the right flank of the mice. When the tumor reached 200-300 mm^3, mice received their corresponding treatments in two cycles of administration.

Figure 7 shows the time evolution of the tumor growth, which was not statically different among groups after 14 days post cells implantation. However, after first drug administration, the control group showed a statistical increase ($p<0.01$) compared to all the treatments. In addition, the effect of the free drug was also statistically lower ($p<0.01$) than the effect for the formulations. It was also statistically lower than the CTX effect ($p<0.05$), only after the second administration.

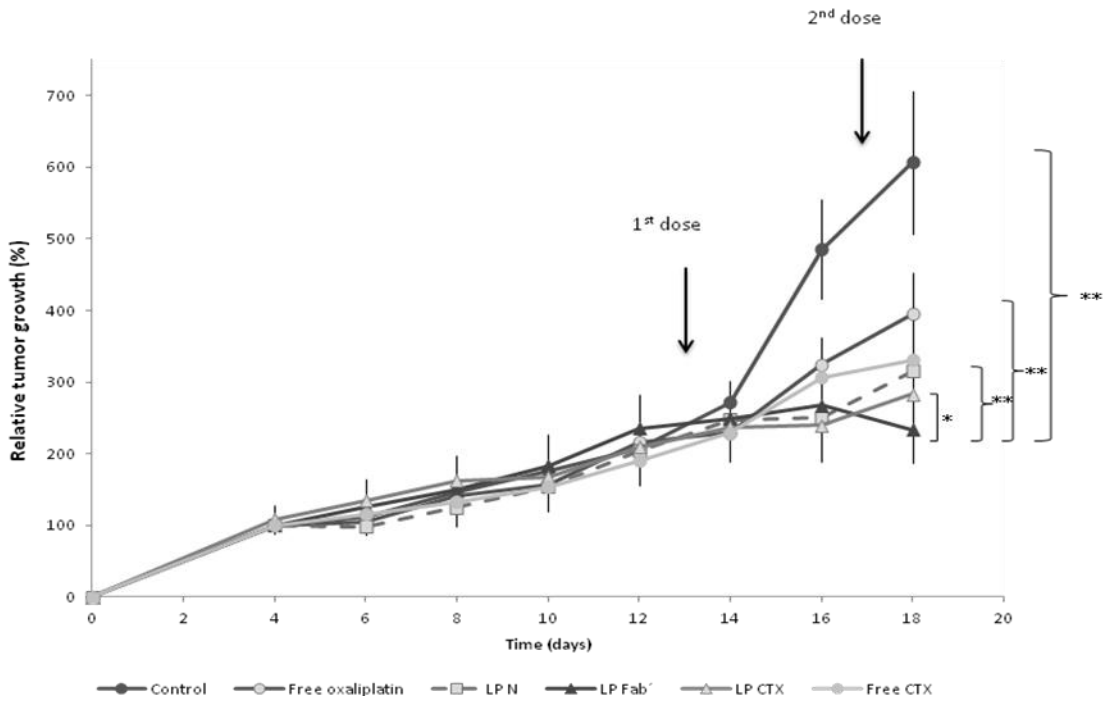

Figure 7*. Time profile of the relative tumor growth for the different treatments. Each point represents the average of six mice and the bars are the standard deviation. Arrows represent the administration of the dose.*

Therefore, all formulations were able to induce an efficient delay in the tumor growth. Although the differences among formulations were not observed after the first administration, that difference was more evident after the second treatment, as is shown in Figure 7. Thus, 24 h later a statistical difference ($p<0.05$) was found between non-targeted liposomes and LP-Fab', and between LP-Fab' and LP-CTX ($p<0.05$). The administration of the blank liposomes did not show any influence in the growth (data not shown).

Therefore, the LP-Fab' seemed to be the most efficient formulation in this *in-vivo* model.

3.8. - Biodistribution study

To explore the *in-vivo* results regarding the tumor uptake, a biodistribution study was carried out using 4 and 24 h set points after the second drug exposure or

administration. Thus, mice were sacrificed at the selected times. Figure 8 summarizes the oxaliplatin levels found in the more representative organs after second drug administration.

The accumulation of the liposomes was found in the two main irrigated organs, liver and spleen, while for the free drug this effect is not observed. However, the pattern of that accumulation is different for both organs. The drug concentration decreased in time in the liver. However, this behaviour was opposite in the spleen, increasing between 4 and 24 h.

LP-Fab' was the formulation with higher accumulation in both organs, being especially important at 24 h in the spleen. A similar result was observed for this formulation in the tumor. In this tissue, at 4 h the free drug reached an accumulation value in the range as the values for liposomes. However, at 24 h the accumulation of the free drug was three times lower than LP-N, eight times than LP-Fab' and six times than LP-CTX, being the LP-Fab' the formulation with the highest accumulative effect after two administrations. This result might explain the profile of the tumor growth in mice especially the tendency observed at day 18, one day after the second drug administration.

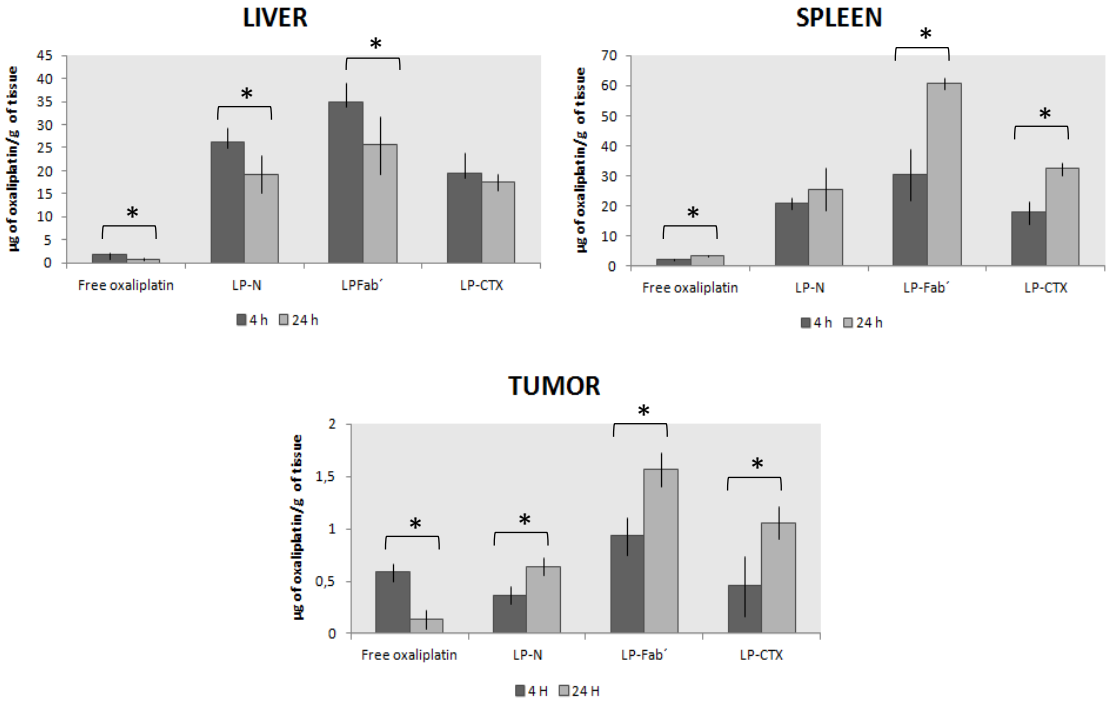

Figure 8. *Oxaliplatin levels in liver, spleen and tumor after free and encapsulated drug administration. Bars represent the average of 3 mice, and the vertical line to the corresponding standard deviation.*

4. - Discussion

In this work, two types of immunoliposomes, using as ligands CTX and its variable fragment, Fab', have been developed and assayed in *in-vitro* and *in-vivo* studies.

In a previous work, non targeted pegylated liposomes of oxaliplatin were formulated [6]. These liposomes, LP-N, showed an efficient antitumor effect in a xenograft model using the HT-29, a human colorectal cancer cell line.

Based on this formulation and taking into account the fact that solid tumors like CRC over-express EGFr [9], targeted liposomes were developed selecting the whole mAb against this receptor and its Fab' fragment, as ligands. They were conjugated with the pegylated oxaliplatin liposomes.

In general, the coupling methods do not alter the main characteristics of the liposomes such as drug loading efficiency and drug release [16]. In this case, after several steps, the SH- group of the mAb and its Fab' was coupled to the maleimid molecule of liposomes through a thioether linkage. This method has been previously applied for other formulations resulting in a stable bond [18].

Previously to the coupling, the Fab' fragment was purified by an enzymatic digestion of the CTX with pepsin, followed by a reduction reaction with β-Mercaptoethanol. This method is commonly used by other authors in order to eliminate the Fc' portion of immunoglobulins and obtain the single Fab' fragment [19]. In fact, Figure 1 confirms the efficiency of this methodology, which was used in this work to obtain the single Fab' fragments. These antibody fragments allow better moieties for the conjugation with functionalized PEG derivatives of liposomes [20].

The efficiency of conjugation is not a linear process, thus to optimize the relationship between ligand and lipid concentration, several amounts of ligands were used. That efficiency decreased from 94% for lower ligand concentration to 65% for higher, reaching a plateau between 30 and 40 µg of ligand per mmol of lipid, as was observed in Figure 2. This result was also supported by the cell uptake observed in HCT-116 cells treated with the targeted-liposomes prepared with the different ligand/lipid ratios (Figure 3). A remarkable plateau was observed in the intracellular accumulation of liposomes between 30 and 50 µg of ligand per mmol of lipid.

Moreover, liposomes coupled to the single Fab' fragment showed a higher tendency ($p>0.05$) to be incorporated in the cells than liposomes conjugated with the whole antibody. This effect might be associated with the random orientation of the antibody molecule within the conjugated structure, providing a limited receptor binding. On the contrary, the molecule of the Fab' fragment presents always the same orientation in the

receptor binding. This, would compensate the monovalent binding site of Fab' fragment opposite to the antibody bivalent binding sites [21].

This observation was also found in the cell uptake of targeted and non-targeted liposomes assayed in the four human cell lines which express different EGFr levels. At 37ºC, the cell uptake of liposomes in HCT-116, SW-480 and HT-29 was time dependent, although between 5 and 24 h the difference was not very significant. Note that in this study the uptake of LP-Fab' was also higher than for LP-CTX, supporting the previous observations. HT-29 did not show significant differences between both targeted-liposomes. This might be due to for this cell line the EGFr expression is weak, while for HCT-116 and SW-480, it is high. In this line, the pre-treatment with CTX, led to a reduction in the uptake of the targeted-liposomes but not of the non-targeted formulations. These results were expected because the mechanism for the uptake of both types of formulations is different, non-especific endocytosis for non-targeted liposomes, while for targeted it is a receptor-mediated endocytosis. In addition, the blocking of EGFr by the free mAb had a higher impact in the uptake of LP-Fab' than LP-CTX. In this case, the affinity of the free and coupled mAb by EGFr is the same. Nevertheless, for Fab' it is lower, representing a limitation to remove the mAb bound to the receptor.

Therefore, Fab' and CTX represent two selective EGFr ligands. In case of SW-620 the uptake of all formulations was very similar, supporting the low levels of EGFr as is described in the literature. These results were also supported by the image provided by confocal microscopy (Figure 6).

At 4ºC any significant differences in the cell uptake of the three formulations among cell lines was found. This suggests that the uptake is trough an active endocytosis, which needs energy to take place.

On the other hand, these formulations show a different behaviour compared to the free drug, as is observed in Table 2, where the IC_{50} for liposomes was lower than for the free drug.

The mechanism for the cell uptake of the free drug is described to be transporter-mediated, specifically by the copper transporters. Then, its uptake depends on the number of transporters free to bind to oxaliplatin or their turnover. However, that mechanism is completely different for the liposomes, as was commented above. Therefore, the difference observed in Table 2 might be explained by this difference in the cell uptake. Thus, and despite the sensitivity to oxaliplatin displayed by the cell lines according to data from the literature, the encapsulation of oxaliplatin in liposomes represents a strategy to increase its efficacy. This approach has been also commented by other authors [22]. In addition, the IC_{50} for targeted liposomes is in all cases lower than the non-targeted, except for SW-620, the negative control for EGFr. Based on these results, the EGFr targeting plays an important role in the selectivity of the formulations, and supports its target.

The intravenous administration of oxaliplatin liposomes into SW-480 tumor-bearing mice demonstrated a greater efficacy than the free drug, especially after the second dose. For this second treatment, the effect of LP-Fab' was more evident than for LP-CTX. The dose of oxaliplatin used in this study was lower than the doses used by other authors. The idea was to evaluate the impact of the targeting strategy when the effect induced by the free drug was not very relevant compared to the control group. Under these conditions, the efficacy could be more evident. After the first dose administration, a delay on the tumor growth was observed for the three formulations, which was not observed for the free drug. This result is in line with the results found by Suzuki and coworkers (2008) [23]. They found that the suppression of the tumor growth by transferrin-pegylated-liposomes of oxaliplatin was higher than the suppression for the free drug and bare or non-targeted

liposomes. In addition, the transferrin-PEG-liposomes, with an average diameter of 100-200 nm, showed a prolonged circulation and a higher tumor accumulation. This result has been also found in the *in-vivo* model assayed in this work. Thus, the tumor accumulation of oxaliplatin from targeted liposomes was higher in comparison with the other treatments, the free drug and non-targeted. In this way, the time profile of oxaliplatin in the different organs suggests that the free drug is rapidly cleared from the circulation. This is supported by the amount of the drug found in the liver and spleen, which decreased very rapidly, representing the main limitation of oxaliplatin to reach a target tissue [3]. However, pegylated liposomes showed a long circulation that together with the EPR effect, allowing the delivering of oxaliplatin to tumor tissue [24]. In the case of the LP-N the efficiency is lower than for the targeted liposomes. This might be due to although the non-targeted liposomes are able to deliver the drug to tumor tissue this drug cannot be effectively internalized or released into the tumor cells to form the DNA-adducts. On the contrary, the targeted formulations are able to reach the tumor tissue as the LP-N but they use the receptor-mediated endocytosis to provide a more efficient drug delivery to the cells [23].

On the other hand, the difference in drug levels found in the three organs for LP-CTX, might be explained by the clearance of these liposomes due to the recognition of the Fc' fraction of the antibody by phagocytic cells which accumulate the liposome in liver, spleen and in blood [20]. In addition, the recognition of the EGFr might be reduced as was observed in the results found in *in-vitro* study (Figures 5 and 6). This result would support by the random orientation of the CTX in the liposome surface.

Finally, the stability of the formulations did not represent any limitation *in-vivo*. Although the release profile in presence of 50% FBS (Figure 4) was modified for the three types of liposomes, the main effect of the formulations was not due to controlled release of the drug but to the presence or absence of ligands. This fact was also supported by the

treatment with the free CTX. Thus, after the second dose of CTX, the behaviour was similar to the LP-N, suggesting that is the receptor target the main responsible of the effect observed for targeted liposomes and especially for LP-Fab'. Due to the dose administered to mice is very low, none of the animals showed significant side effects. In fact, the body weight did not change among groups.

In conclusion, EGFr targeted liposomes might be a promising carrier for oxaliplatin treatment in CRC that over-express this receptor. Although there are no significant differences in the *in-vitro* studies for both types of immunoliposomes, the LP-Fab' provided a better efficiency *in-vivo* due to the use of both passive and active targeting to deliver the oxaliplatin to the tumoral cells.

5. - Acknowledgments

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GENERAL DISCUSSION

GENERAL DISCUSSION

The work presented in this thesis details the relevance of new strategies to increase the therapeutic efficacy of oxaliplatin. The platinum derivatives are widely used in the treatment of cancer [1]. Thus, several types of solid tumors show high sensitivity to this family of drugs. However, the presence of side effects such as nephrotoxicity, hematotoxicity or neurotoxicity associated mainly with cisplatin, carboplatin and oxaliplatin, respectively, represents a serious dose-limitation during their treatments.

The application of nanotechnology has been one of the approaches to overcome these problems. In this way, liposomes are considered the most popular delivery systems used to encapsulate cisplatin, oxaliplatin and carboplatin.

The therapeutic interest about this strategy, and the preclinical and clinical results reported by several authors in the literature, are extensively presented in Chapter 1. This Chapter provides an overview regarding the relationship between therapeutic response and side effects for these drugs and includes how this new strategy is able to modify that relationship. In addition, the incorporation of new platinum-based molecules together with their corresponding clinical trials is summarized in this Chapter.

For cisplatin, the first liposomal formulation assayed in phase II was SPI-077. In these clinical studies, this formulation fell because its drug release profile was very slow, leading to reach sub-therapeutic levels in the tumor [2, 3].

Although, the improvement in the incidence of side effects, especially renal toxicity, was evident, its antitumor efficacy was extremely low compared to the free drug. Note that, the accumulation of liposomes in tumor tissue does not represent the availability of adequate drug levels, which is the case of SPI-077 and other formulations. Therefore, to modify the limitation in the SPI-077 drug release, a new lipid composition was used to develop Lipoplatin. This is the most promising formulation for cisplatin, which is actually being assayed in several phase III studies [4, 5].

A similar strategy was followed by oxaliplatin encapsulation. But in this case, its good tolerability in patients is associated with a low therapeutic efficacy [6]. The problem is principally due to its high partitioning coefficient to erythrocytes and plasma protein binding, after intravenous administration. This leads to a rapid clearance of the free drug from the circulation, providing very low levels in tumor tissue [7].

In order to improve its therapeutic application, Lipoxal, a liposomal formulation, was developed. This formulation is currently in phase I of clinical trials [8]. There is little data in the literature regarding its therapeutic properties and more studies would be required to demonstrate the real benefits compared to oxaliplatin.

On the other hand, several new liposomal formulations which try to overcome its low efficacy in monotherapy have also been described in the literature [9-13].

Based on this, Abu-Lila and coworkers [9-11] have developed different types of formulations, with cationic and anionic lipids. They found that these formulations were able to display different properties. For example, cationic

liposomes exhibited antiangiogenic property, since they were able to inhibit the formation of new vessels in the tumor area. On the other hand, anionic liposomes showed a higher antitumor efficacy than oxaliplatin. This was compatible with higher tumor drug-levels [9-11]. Following these articles, Zalba et al. (2012) have reported the application of three different methods to develop anionic and cationic liposomes for oxaliplatin. The methodology and results of this article are described in detail in Chapter 2. The authors proved that anionic liposomes formulated by film method, showed good stability in *in-vitro* studies and *in-vivo* using a xenograft model. In addition, the antitumor effect was greater than for the free drug [13].

All the described formulations correspond to pegylated liposomes, also known as sthealth liposomes. These liposomes are coated with poliethylenglycol, a polymer which is able to reduce the caption by the reticuloendothelial system of liposomes. As a consequence their half-life in circulation increases [14]. Thus, the sthealth liposomes have a significant impact on the pharmacokinetics of the encapsulated drug, providing a certain increase in therapeutic activity and reducing side effects. That modification together with the EPR (Enhanced Permeability and Retention) effect presented in all solid tumors, allows a higher accumulation by passive transport of these liposomes compared to conventional or bare liposomes [15]. Nevertheless, the passive effect, a heterogeneous process with high variability between tumors, provides the accumulation of the liposomes in the tumor interstitium [16]. But, this does not guarantee the uptake in the cell. Therefore, the last strategy to improve the tumor target is the development of "targeted liposomes". They are based on the conjugation of molecules in the termini of PEG, which are able to bind to receptors over- or specifically expressed in cancer cells. These new formulations allow a change in the mechanism of transport of the

liposomes to the tumor, from passive to active transport. The coupling in the surface of liposomes of specific ligands such as proteins, peptides, hormones, etc, provides the internalization of liposomes in tumor cells [17, 18].

Thus, in oncology the over-expression of certain types of receptors implicated in the cell proliferation process represents a challenge for developing specific and selective treatments.

The epidermal growth factor receptor (EGFr) is one of those receptors over-expressed in many types of solid tumors, including CRC (colorectal cancer) [19]. New biomolecules like monoclonal antibodies (mAb), which are able to recognize with great affinity this receptor, have been incorporated in the oncology therapy. Their binding leads to inhibit the activity of this receptor. Currently, these biomolecules are combined with conventional antitumor drugs displaying a significant improvement in the therapeutic responses. For example, oxaliplatin is administered with cetuximab, one of the mAb against EGFr. This combination shows an improvement in the efficacy of oxaliplatin compared to its monotherapy [20].

Therefore, the development of a pegyleted liposomal formulation of oxaliplatin conjugated with CTX would be a possible strategy to combine the advantages of both treatments [21]. Thus, in Chapter 3 this strategy is described in detail. In this paper, the establishment of coupling conditions together with the in *in-vitro* and *in-vivo* studies, carried out to compare the efficacy of targeted liposomes to non-targeted liposomes or the free drug, are extensively discussed.

In addition, the behavior of two types of immunoliposomes, were compared. One of then was obtained by the coupling to whole CTX and the other one to the monovalent fragment Fab' of CTX. This second approach has been previously

proposed by other authors. They suggest that the coupling to small molecules like Fab' may increase the efficacy in the internalization of nanocarriers. This causes a reduction of side effects like immunogenicity induced by the whole mAb [21].

The cell uptake of both types of targeted liposomes in four human CRC cell lines, expressing different levels of EGFr, resulted in a higher accumulation of Fab' liposomes in comparison with those bound to CTX. The role of the EGFr in this uptake was demonstrated by the pretreatment with the free cetuximab. This assay led to a dramatic reduction of the internalization.for targeted liposomes.

On the other hand, in a SW-480 tumor cell bearing mouse model the formulation conjugated with Fab' was able to induce higher drug accumulation in tumor compared to the formulation coupled to CTX or non-targeted liposomes. This result, was also associated with a higher antitumor efficacy of the Fab' formulation. The clearance from the circulation of CTX-liposomes could be a limitation for this type of formulation. This phenomenon, previously described by several authors for these types of immunoliposomes, has been explained by the presence of Fc' in the whole antibody. This fragment induces the activation of the phagocitic cells, leading to a lower extravasation of the formulation to the tumor area [22, 23].

Therefore, this strategy based on the encapsulation of oxaliplatin in pegylated liposomes represents a significant improvement in the efficacy of the free drug. Moreover, the coupling to specific ligands in the surface allowed an increase in the antitumor effect of the targeted liposomes, due to a greater drug accumulation in this tissue compared to other treatments. Finally, Fab' seems the most promising alternative regarding the antitumor efficacy and drug tumor accumulation.

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CONCLUSIONS

CONCLUSIONES

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The work presented has focused on the development of EGFr targeted oxaliplatin liposomes for the treatment of CRC. The results obtained have allowed us to draw the following conclusions:

1. The encapsulation of oxaliplatin into pegylated liposomes was successfully obtained using the Film method. The particle size of the liposomes was 120 nm achieving an efficiency of encapsulation of 30%. These formulations presented a good stability in different biological mediums, and also after their lyophilization using several cryoprotectants.

2. The antitumor efficacy assayed in HT-29 cell bearing mice was higher for encapsulated drug than for the free drug.

3. The conjugation of liposomes with CTX and Fab' ligands at their surface did not change the physicochemical characteristics regarding the non-conjugated oxaliplatin liposomes.

4. The presence of the ligands improved the accumulation and internalization of the liposomes into the human colocarcinoma cell lines positives for EGFr. This cell uptake could be classified as an active process dependent on the presence of the EGFr at the surface of these cells. It was proved by the reduction of the uptake after the inhibition of this receptor by Cetuximab, and also with the EGFr-negative control corresponding to the SW-620 cell line.

5. The active targeting of both types of targeted liposomes, with CTX and Fab' fragment seemed to be a good strategy for inducing a delay in the SW-480 colorectal cancer tumor growth rather than the non-targeted liposomes.

6. The targeted formulations showed a different behaviour depending on the ligand. The LP-Fab' induced a higher oxaliplatin accumulation in SW-480 tumor tissue and a higher antitumor efficacy than LP-CTX.

CONCLUSIONES

El trabajo presentado se ha centrado en el desarrollo de liposomas de oxaliplatino dirigidos frente al EGFr para el tratamiento del cáncer colorectal. Los resultados obtenidos nos permiten extraer las siguientes conclusiones:

1. El método del Film permitió la obtención de liposomas pegilados de oxaliplatino con un tamaño de partícula de 120 nm y con una eficacia de encapsulación del 30%. Estas formulaciones mostraron gran estabilidad en diferentes medios biológicos, y después de su liofilización con diversos crioprotectores.

2. La eficacia antitumoral ensayada en un modelo animal de cáncer colorrectal con la línea humana HT-29, fue superior tras el tratamiento con los liposomas comparada con el tratamiento del fármaco libre.

3. La conjugación en la superficie de los liposomas con los ligandos, Cetuximab y Fab, no modificó las características fisicoquímicas de los liposomas respecto a los no dirigidos previamente desarrollados.

4. La presencia de estos ligandos permitió un aumento de la internalización y por lo tanto, acumulación de las formulaciones en las células tumorales de carcinoma colorectal humanas positivas para el EGFr. Dicha internalización se asoció a un proceso activo dependiente de la expresión del EGFr en dichas células. Esta característica se demostró mediante la inhibición del receptor usando el Cetuximab libre como pretratamiento en estas células, lo que indujo una drástica reducción del grado de internalización de las formulaciones dirigidas. Así mismo, la internalización con la línea celular SW-620 utilizada como control negativo, no presentó diferencias entre formulaciones con ligandos y sin ellos.

5. La direccionalización activa con los dos tipos de liposomas dirigidos, con CTX y Fab', ha permitido un retraso en el crecimiento del tumor inducido por la línea SW-480 de cáncer colorectal, superior al obtenido por liposomas no dirigidos.

6. Las formulaciones dirigidas tuvieron un comportamiento diferente dependiendo del ligando. Así, los liposomas LP-Fab' presentaron una mayor acumulación de oxaliplatino en el tumor asociado a una mejor eficacia antitumoral, que LP-CTX.

APPENDIX

APÉNDICE

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