Enhanced cell uptake of cell penetrating peptide modified liposomes
diploma thesis

Author(s):
Meylan-Gonin, Carole

Publication Date:
2003

Permanent Link:
https://doi.org/10.3929/ethz-a-004596555

Rights / License:
In Copyright - Non-Commercial Use Permitted
Enhanced cell uptake of cell penetrating peptide modified liposomes

Diploma Thesis

by

Carole Meylan-Gonin

Citizen of Le Chenit, VD

Direction: Prof. Dr. P.A. Schubiger
Supervision: Prof. Dr. K. Ballmer-Hofer
Prof. Dr. R. Schwendener
Dr. C. Marty

March 3rd to July 25th 2003
ACKNOWLEDGEMENTS

I would like to thank Prof. Dr. P.A. Schubiger who allowed me to realise this diploma thesis at the Paul Scherrer Institut (PSI).

I am grateful to Prof. Dr. K. Ballmer-Hofer for giving me the great opportunity to work in his group of Molecular Cell Biology on such an interesting project and also for his precious advice about fluorescence microscopy.

I wish to express my gratitude to Prof. Dr. R. Schwendener for what he taught me during the time of this research: he generously shared with me his large experience about liposomes and various topics raised by this diploma thesis.
A special thank goes to Dr. C. Marty for her constant support and encouragements, and also for her patient initiation to the discovery of the scientific world.
I much appreciated the disponibility of Prof. Dr. R. Schwendener and Dr. C. Marty, their helpful recommendation and their answers when fundamental questions appeared.

Thanks to each person of the group for the pleasant working atmosphere and for their assistance.

I would also like to thank Grégoire for his precious support during all this time.
TABLE OF CONTENTS

1 ABSTRACT ........................................................................................................................................................................... 1

2 INTRODUCTION..................................................................................................................................................................... 2
   2.1 CELL PENETRATING PEPTIDES................................................................................................................................. 2
   2.2 LIPOSOMES................................................................................................................................................................. 4
   2.3 COUPLING STRATEGIES TO LINK LIPOSOMES TO CELL PENETRATING PEPTIDES .................................... 5
   2.4 CYTOTOXIC SUBSTANCES........................................................................................................................................... 6
   2.5 AIM OF THIS STUDY.................................................................................................................................................... 7

3 MATERIALS AND METHODS .............................................................................................................................................. 8
   3.1 CHEMICALS AND SOLUTIONS................................................................................................................................... 8
       3.1.1 Liposomes ............................................................................................................................................................. 8
       3.1.2 Peptides ................................................................................................................................................................ 9
       3.1.3 Cell culture .......................................................................................................................................................... 9
       3.1.4 Cytototoxic compounds .................................................................................................................................... 9
       3.1.5 Plasmid ............................................................................................................................................................... 9
   3.2 CELL CULTURE.......................................................................................................................................................... 10
   3.3 LIPOSOMES.............................................................................................................................................................. 10
       3.3.1 Preparation of liposomes by extrusion .............................................................................................................. 10
       3.3.2 Drug quantification ............................................................................................................................................ 12
       3.3.3 Modification of liposomes with sulfo-SMCC .................................................................................................... 12
       3.3.4 Attachment of Antp- or TAT- peptides to liposomes .................................................................................... 12
   3.4 CELLULAR ASSOCIATION OF ANT- OR TAT- LIPOSOMES ............................................................................... 12
       3.4.1 Flow Cytometry ............................................................................................................................................... 13
       3.4.2 Fluorescence microscopy ................................................................................................................................ 13
   3.5 DETERMINATION OF THE NUMBER OF ANT- PEPTIDES PER LIPOSOME ......................................................... 13
   3.6 CYTOTOXICITY TESTS.............................................................................................................................................. 14
   3.7 DNA-LOADED LIPOSOMES ........................................................................................................................................ 14
       3.7.1 Liposome preparation ......................................................................................................................................... 14
       3.7.2 Digestion of non encapsulated DNA ................................................................................................................ 15
       3.7.3 Quantification of DNA loading to liposomes: Pico Green Test ..................................................................... 16
       3.7.4 Modification of DNA-liposomes with Antp peptide ....................................................................................... 16
       3.7.5 Transfection of porcine arterial endothelial cells (PAE) with DNA ............................................................. 16

4 RESULTS ........................................................................................................................................................................... 17
   4.1 LIPOSOME CHARACTERISATION ............................................................................................................................. 17
   4.2 RELATIONSHIP BETWEEN PEPTIDE NUMBERS AND LIPOSOME UPTAKE EFFICIENCY ......................... 17
   4.3 DETERMINATION OF THE NUMBER OF ANT- PEPTIDES PER LIPOSOME ....................................................... 19
   4.4 ASSOCIATION OF PEPTIDE MODIFIED LIPOSOMES WITH VARIOUS CELL LINES.................................... 20
   4.5 INHIBITION OF PEPTIDE MODIFIED LIPOSOME ASSOCIATION ................................................................ 21
   4.6 CYTOTOXICITY TESTS .............................................................................................................................................. 22
       4.6.1 Quantification of the cytotoxic effects incorporated into liposomes .............................................................. 22
       4.6.2 EPO-B: comparison of cytotoxic effect between EPO-B in solution and EPO-B in liposomes ................ 22
       4.6.3 Comparison of IC50 of Antp modified liposomes and Antp free-liposomes .............................................. 23
   4.7 DNA-LOADED LIPOSOMES .................................................................................................................................... 24
       4.7.1 Determination of DNA concentration in liposomes ....................................................................................... 24
       4.7.2 Expression of plasmid delivered by liposomes into PAE cells ........................................................................ 25
5 DISCUSSION AND PERSPECTIVES ........................................................................................................................................................................26
  5.1 LABELLING OF PEPTIDE MODIFIED LIPOSOMES ........................................................................................................................................26
  5.2 CELLULAR ASSOCIATION OF ANTP- OR TAT- LIPOSOMES ..................................................................................................................26
    5.2.1 Effect of peptide number per liposome on the association efficiency of liposomes ........................................................26
    5.2.2 Mechanism of cellular association of Antp- or TAT- liposomes ..........................................................................................27
  5.3 CYTOTOXICITY OF ANTITUMOR DRUGS ........................................................................................................................................27
  5.4 DNA-LOADED LIPOSOMES ..........................................................................................................................................................28
  5.5 PERSPECTIVES ..................................................................................................................................................................................29

6 ABBREVIATIONS ......................................................................................................................................................................................30

7 REFERENCES .........................................................................................................................................................................................................31

8 APPENDIX ........................................................................................................................................................................................................34
1 ABSTRACT

One of the major problems of drug delivery is that the biologically active compounds can not freely pass through the lipid membrane of cells. That reduces significantly their therapeutic efficiency.

Cell penetrating peptides were recently discovered which rapidly translocate across the plasma membrane of various cells types both in vitro and in vivo. These peptides also promote the cellular uptake of different cargo molecules. In this project, two of these peptides, the Antennapedia (Antp) and the TAT peptides were used to improve the transmembrane transport of particular delivery vehicles such as liposomes.

The results obtained demonstrate that in comparison with control liposomes, both Antp and TAT peptides significantly enhance association of liposomes with various cells, provided that a sufficient number of peptide molecules is attached to the liposome surface. Furthermore, cellular association is peptide- and cell-type dependent. Although the mechanism of cellular uptake is far from being identified, a possible interaction with cell surface-expressed glycosaminoglycans is demonstrated for both Antp and TAT peptide modified liposomes.

The presence of Antp peptide molecules on liposomes increases the cytotoxicity of the new antitumor nucleoside dimer drug NOAC-ETC encapsulated in liposomes by 2- and 3-fold after a 2 h and a 4 h incubation, respectively. Another new cytotoxic drug, Epothilone B (EPO-B) was encapsulated in liposomes for the first time and IC$_{50}$ values up to 150-fold lower than those observed with nucleoside dimer analogs were obtained. Thus, NOAC-ETC and EPO-B carrying Antp liposomes may be a new kind of anticancer drugs useful for clinical applications.

The results obtained in this research encourage the use of cell penetrating peptides attached to liposomes for many applications in basic research or for improved drug delivery in therapeutic applications.
2 INTRODUCTION

The inefficiency of uptake of pharmaceutically active molecules, such as drugs, peptides or plasmids into the cells of a target tissue, is a severe limitation in the treatment of many diseases. The hydrophobic cellular membrane represents an impermeable barrier for such hydrophilic molecules and prevents them from translocating into cells by passive diffusion. For many years improving membrane permeability for pharmaceutically active molecules has been a challenge for the pharmaceutical industry. The recent discovery of natural and synthetic peptides, which can translocate across cell membranes, has become an increasingly attractive solution for this problem, opening new possibilities in biomedical research.

2.1 Cell penetrating peptides

During the last decade, several natural proteins were found to penetrate cell membranes directly. Examples include the TAT-protein from the HIV-1 virus, the homeodomain of the Antennapedia transcription factor from Drosophila (penetratin), the herpes simplex virus type 1 (HSV-1) VP22 transcription factor and others. Small sections of these proteins (sequences of less than 20 amino acids) are responsible for their transduction across the membrane. Collectively they are now called "cell penetrating peptides" (CPPs) or "protein transduction domains" (PTDs). The present work will focus on two of these cell penetrating peptides called the TAT and the Antp peptides.

The first cell penetrating peptide is a peptide derived from the TAT (transactivator of transcription) protein from Human Immunodeficiency Virus (HIV-1). This 86-residue TAT protein trans-activates certain viral genes and is essential for viral replication. The ability to cross membranes was attributed to its C-terminus (residues 37-72) [1]. Specifically, the minimal sequence sufficient for membrane translocation is TAT (48-57), as represented in Figure 1 [2]. Circular dichroism and nuclear magnetic resonance studies strongly suggest that TAT peptide can adopt an alpha helix conformation [3].

The second cell penetrating peptide is derived from Antennapedia, a transcription factor of Drosophila. This transcription factor binds to DNA through a specific sequence of 60 amino acids, called the homeodomain. This homeodomain is composed of three alpha-helices and is internalised by cells in culture. In the past few years, this third alpha-helix of the Antp homeodomain was found to be responsible for the translocation of the entire protein. Therefore, a peptide of 16 residues, corresponding to amino acids 43-58 of the third alpha helix of the homeodomain was proposed as a universal intercellular delivery vector (Figure 2) [4].

![Figure 1: (A) Helical plot and (B) linear sequence of the TAT peptide. Arginine and lysine are represented with the filled circles and hydrophobic amino acids with the open squares [5].](image-url)
The two peptides were biotinylated at the N-terminus for studies analysing the internalisation of PTD conjugated with fluorescence labelled streptavidin [6]. Some experiments showed that this biotinylation has no influence on the cellular uptake of these peptides. The peptides were further modified at their C-terminus with one cysteine used for the covalent attachment to liposomes (see Figure 4).

A characteristic feature of these peptides is the high proportion of basic, strong positively charged amino acids arginine (R) and lysine (K). It was found that such amino acids are required for plasma membrane translocation by interaction with the negatively charged components of the membrane [7]-[9].

Nevertheless, the complete mechanism of internalisation of PTDs is not well understood and is still the subject of controversy. Moreover, each PTD might transduce into cells by a different mechanism. In many reports, it was described that internalisation is not significantly inhibited by incubation at low temperature (4°C) or by inhibitors of endocytosis. These data dismiss the classical endocytosis pathway as a possible mechanism of translocation, because of its energy-dependence [2].

Therefore, a direct transport through the lipid bilayer of cell membranes was proposed for the TAT peptide [10]. In contrast, a model of translocation across the cell membrane that involves the formation of inverted micelles with the internal cavity was suggested for the Antp peptide [4].

More recent data reestablish the role of endocytosis in the translocation and suggest that, at least for the TAT peptide, membrane translocation is mediated by glycosaminoglycans of the heparin family, which are expressed on the surface of almost all cell types [11]-[13].

Despite their unknown uptake mechanism(s), PTDs allow the non-specific cellular delivery of various types of molecules with different sizes, such as antigenic peptides, peptide nucleic acids, antisense oligonucleotides, full-length proteins (e.g. the enzyme β-galactosidase [14]) or even nanoparticles [15] and liposomes [16], [17].

In this diploma work, the TAT-derived basic peptide and the Antp peptide were used to improve the delivery of liposomes to cells.
2.2 Liposomes

Liposomes are vesicles containing lipid bilayers surrounding aqueous compartments, whose diameters can vary from 20 nm to 1 µm. They have been used for more than 30 years as vehicles to improve the delivery of various drugs, such as anticancer drugs (doxorubicin), antibiotics (anthracycline, amphotericin B) or vaccines [18]. Lipophilic molecules can be incorporated into the lipid bilayer of liposomes, while hydrophilic components can be entrapped in the inner aqueous phase as shown in Figure 3. Liposomes have been used as drug delivery system in various therapeutic applications such as gene therapy, drug-targeting and many others, mainly because of the following advantages [19]:

1. They are biodegradable and non-toxic.
2. Most molecules can be incorporated into liposomes without modification at a very high concentration.
3. Biologically active molecules are protected by the lipid bilayer of liposome from damage by chemicals and enzymes after injection into the blood stream of an organism.
4. The host cells are also protected from the toxicity of the entrapped molecules.

During these last 30 years, different liposomes have been developed in order to improve their stability in vivo. Conventional liposomes are the original ones, composed of phospholipids and cholesterol and are stable enough for in vitro experiments, but in vivo they are rapidly taken up either by the organs of the reticuloendothelial system such as the liver and spleen or by phagocytic cells such as macrophages. To prolong the liposome half-life in the circulation, liposomes are coated with polyethyleneglycol (PEG), reducing interactions with plasma proteins or receptors and these liposomes are called "stealth liposomes". The most recently developed type of liposomes are the cationic liposomes, used for the gene therapy. Their cationic lipids can interact with a negatively charged DNA to form a neutral noncovalent complex (Figure 3) [20].

![Figure 3: Schematic representation of different liposomes: (A) conventional liposomes neutral or negatively charged, (B) sterically stabilised ("stealth") liposomes coated with PEG, (C) immunoliposomes and (D) cationic and glycosylated liposomes[20].]
The major component of liposomes is phosphatidylcholine (PC), which can be extracted from natural sources such as egg yolk, brain tissues or Soya beans or can be prepared synthetically. Charged lipids such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) or dioleoyltrimethylammonium propane (DOTAP) are added to introduce charges. Cholesterol is ordinarily included to further improve mechanical stability and to decrease leakage of the encapsulated contents. To prevent the oxidation of the lipids, antioxidants such as α-tocopherol or ascorbic acid are often added [21], [22].

The preparation of liposomes is very simple. The lipid components are mixed in an organic solvent, which is then evaporated. A thin lipid film is formed. Hydration and agitation in an aqueous buffer produces a suspension of large multilamellar vesicles (MLV, 1 µm). These vesicles are too large, too heterogeneous and their capacity to carry drugs is too small to be useful. Therefore, to prepare small unilamellar vesicles (SUV) of sizes ranging from 50 to 200 nm, sonication or filter extrusion through well defined filters was established of which the second method was used in this project.

The lipid components of conventional liposomes used in this study are soy PC (SPC), cholesterol, DPPE and those of the cationic liposomes are oleoylpalmitoylglycerophosphocholine (OPPC) in combination with the cationic component didodecyldimethylammonium bromide (DDAB).

2.3 Coupling strategies to link liposomes to cell penetrating peptides

Different strategies were developed to attach peptides to the surface of liposomes, requiring efficient linkage by the inclusion of reactive residues in either the PTD or liposomes. Cysteines are often added to the C- or N- termini of the peptides and of cargoes, resulting in a very efficient coupling through disulfide bonds [15]. Another strategy is the use of a spacer between the peptides and the liposomes [5].

In this study, conventional liposomes containing amino groups were prepared. Liposomes were modified with a bifunctional molecule called sulfo-SMCC, in which its succinimide group can react with amino groups of liposomes and its maleimide groups can be attached to the cysteine of the C-terminus of a peptide. These covalent thioether bonds (-S-C-) are more stable in vivo than the disulfide bonds (-S-S-) and are very often used as coupling strategies (Figure 4) [23].

The coupling reaction was done in presence of a reducing agent, tributylphosphine, avoiding formation of peptide dimers.

In control liposomes without peptides, the maleimide groups were blocked with cysteine.

Figure 4: Peptide-liposome coupling reaction (A) Introduction of maleimide groups (red) to liposomes by NH₂-modification with sulfo-SMCC. In blue: succinimide group of sulfo-SMCC; (B) Attachement of cysteine modified peptide to liposomes.
2.4 Cytotoxic substances

The cytotoxic effects of a large number of antitumor drugs (cisplatin, doxorubicin, mitoxanthrone, cyclophosphamide) used in chemotherapy on solid tumors are generally unsatisfactory. Consequently, evaluation of the effect of new drugs or new combinations with already known ones is required. In the last years, a new class of anticancer drugs, the amphiphilic nucleoside duplex drugs, were developed. They are composed of two nucleosides, whereby their combination leads to an improvement of the cytotoxicity of each monomer. Among them, 5-FdU-NOAC (Figure 5) is composed of 5-FdU (floxuridine) and NOAC, which is a lipophilic derivative of cytosine arabinoside (ara-C), one of the most widely used agents for the treatment of acute myelogenous leukaemia. This cytotoxic drug inhibits DNA synthesis in S phase of the cell cycle [24], [25].

NOAC-ETC, a second kind of antitumor nucleoside analogue, represents a very attractive drug combination, because of the mechanism of ETC (Figure 6)[26]. ETC is very rapidly phosphorylated into a metabolite, which inhibits RNA-synthesis [27].

![Figure 5: 5-FdU-NOAC](image1)

![Figure 6: NOAC-ETC](image2)

Recently, another class of natural product chemotherapeutic agents evoked much attention among scientists because of their remarkable antitumor activities. Epothilone B, the third cytotoxic agent used in this project, belongs to this new class (Figure 7). Epothilones are macrolides isolated from the cellulose degrading myxobacterium Sorangium cellulosum and were originally described as antifungal agents. The mechanism of action is similar to that of paclitaxel (Taxol) although their chemical structure and pharmacological profiles are drastically different. Both drugs bind to tubulin and cause the hyperstabilisation of microtubules with subsequent mitotic arrest and apoptic cell death [28], [29].

![Figure 7: Epothilone B (EPO-B)](image3)

In this project, these different lipophilic cytotoxic drugs were added to the organic solvent during liposome preparation. Their cytotoxicity was analysed using a WST-1 cell proliferation assay. This colorimetric test is based on the intracellular reduction of WST-1, a tetrazolium salt, to a strong red dye (formazan) requiring cellular mitochondrial dehydrogenases. Thus, a direct correlation between dye quantity and living cells can be established.
2.5 Aim of this study

This project was done in the context of an extensive study by Dr. C. Marty with the goal to improve the cellular transmembrane transport of high M₉ compounds such as liposomes. The specific purpose of this project was to further improve the uptake of liposomes by modification with TAT and Antp peptides which are specifically known as cell penetrating peptides.

The first part of this study analysed the relationship between the number of peptides attached to the surface of liposomes and their cellular association with various cells such as tumor cells (B16F1, F9), dendritic cells (DC) and fibroblasts (W38). The number of Antp peptides attached to one liposome was determined using fluorescence labelled Antp (FITC-Antp). In order to investigate the mechanisms of translocation of these peptides and to compare TAT- with Antp- modified liposomes, their cellular association was analysed using various cells including two CHO cell mutants, deficient for glycosaminoglycan synthesis.

In the second part of this study, the cytotoxicity of different Antp modified liposomes containing antitumor agents such as 5-FdU-NOAC, NOAC-ETC and EPO-B was analysed.

Finally, in the third part the cellular expression of a plasmid encoding the Green Fluorescent Protein (GFP) encapsulated in positively charged Antp modified liposomes was investigated.
3 MATERIALS AND METHODS

3.1 Chemicals and solutions

All solvents and buffer components were of analytical grade and were obtained from Sigma or Fluka (Bruchs, CH).

3.1.1 Liposomes

<table>
<thead>
<tr>
<th>Component</th>
<th>Supplier Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>Soy phosphatidylcholine 770 g/mol Lucas Meyer, Sugro AG, FR</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>387 g/mol Fluka, Buchs, CH</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>371 g/mol Merck, Darmstadt, DE</td>
</tr>
<tr>
<td>OPPC</td>
<td>1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine 760 g/mol Avanti polar-Lipids, USA</td>
</tr>
<tr>
<td>DDAB</td>
<td>Didodecyltrimethylammonium bromide 462.6 g/mol Fluka, Buchs, CH</td>
</tr>
<tr>
<td>DPPE</td>
<td>2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine 692 g/mol Sygena AG, Liestal, CH</td>
</tr>
<tr>
<td>PE-PEG-Mal</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)2000] 2819.4 g/mol Avanti polar-Lipids, USA</td>
</tr>
<tr>
<td>Sulfo-SMCC</td>
<td>Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate 436.4 g/mol Socochim, Lausanne, CH</td>
</tr>
<tr>
<td>Cysteine</td>
<td>175.6 g/mol Sigma, Buchs, CH</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3’-dioctadecyloxacarbocyanine perchlorate 882 g/mol Molecular Probes, OR, USA</td>
</tr>
<tr>
<td>Texas-red</td>
<td>N-(Texas Red sulfonyl)-1,2-dihexadecanoyl-sn-glycer-3-phosphoethanolamine, triethylammonium salt 1392 g/mol Molecular Probes, OR, USA</td>
</tr>
<tr>
<td>TBP</td>
<td>Tributylphosphine, 3.85 M 202.32 g/mol Fluka, Buchs, CH</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate isomer I 389.39 g/mol Fluka, Buchs, CH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB Buffer, 67mM, pH 7.4</td>
<td>1.77 g KH₂PO₄, 9.6 g Na₂HPO₄ * 2H₂O ad 1 liter aqua dest.</td>
</tr>
<tr>
<td>TE Buffer</td>
<td>0.01 M Tris, 0.01 M EDTA</td>
</tr>
<tr>
<td>Tris Buffer</td>
<td>50 mM, pH 8</td>
</tr>
</tbody>
</table>
3.1.2 Peptides

Antp peptide \((\text{biotin-S-G-R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-C-SH})\), MW = 3517.51 g/mol, Code SP030235, Lot LD03443) and FITC-Antp (Fluorescein-BA-20AA-C, MW = 3938.9 g/mol) were prepared by Neosystems (Strasbourg, FR) and TAT peptide \((\text{biotin-S-G-Y-G-R-K-K-R-R-Q-R-R-R-C-SH})\), MW = 2830.31 g/mol, Code NVP-AFQ 535-AI-1) was synthesised by C. García-Echeverriá (Novartis, CH).

3.1.3 Cell culture

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s modified eagle medium (DMEM)</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>OptiMem I medium</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>RPMI 1640 medium</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>Ham’s F12 medium</td>
<td>Bioconcept, Allschwil, CH</td>
</tr>
<tr>
<td>Fetel bovine serum (FBS)</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Gibco BRL, Basel, CH</td>
</tr>
<tr>
<td>Formaldehide 3.7%</td>
<td>10 g formaldehide 37% ad 100 ml PBS</td>
</tr>
<tr>
<td>PBS pH 7.4</td>
<td>0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄</td>
</tr>
<tr>
<td>Fibronectin solution</td>
<td>100 mg/ml</td>
</tr>
<tr>
<td>Metrizamide</td>
<td>Fluka, Bruchs, CH</td>
</tr>
</tbody>
</table>

3.1.4 Cytotoxic compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FdU-NOAC</td>
<td>804 g/mol</td>
<td>Prof. Dr. H. Schott, Institut of Organic Chemistry, Tübingen, DE</td>
</tr>
<tr>
<td>NOAC-ETC</td>
<td>825 g/mol</td>
<td></td>
</tr>
<tr>
<td>EPO-B Epothilone B, C₇₁H₄₁NO₆S (ED 906-1αxΑ)</td>
<td>507.68 g/mol</td>
<td>Novartis, CH</td>
</tr>
<tr>
<td>WST-1 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazol)1,2-benzol disulfonate</td>
<td></td>
<td>Roche Diagnostics GmbH, Mannheim, DE</td>
</tr>
</tbody>
</table>

3.1.5 Plasmid

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid, enhanced Green Fluorescent Protein (GFP)</td>
<td>pEGFP-NH₂, 4.7 kb, Clontech, USA</td>
</tr>
<tr>
<td>DNAase I from bovine pancreas</td>
<td>Boehringer, Mannheim, DE</td>
</tr>
<tr>
<td>Triton X-100 10%</td>
<td>10 g Triton X-100 ad 100 ml aqua dest.</td>
</tr>
<tr>
<td>TAE Buffer</td>
<td>0.04 M Tris-acetate, 2 mM EDTA</td>
</tr>
<tr>
<td>PicoGreen ds DNA Quantitation Kit:</td>
<td>Molecular Probes, OR, USA</td>
</tr>
<tr>
<td>- PicoGreen dsDNA quantitation reagent, negative control TE 20x: 25 ml of 200 mM Tris-HCl, 20 mM EDTA, pH 7.5</td>
<td></td>
</tr>
<tr>
<td>- Lambda DNA standard, 1 ml of 100 µg/ml in TE</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Cell culture

Mouse melanoma cells B16F1, murine F9 teratocarcinoma cells, fibroblasts W38 and porcine aortic endothelium PAE cells were maintained in Dulbecco’s modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 10 µg/ml streptomycin, 50 U/ml nystatin). Wild type CHO K1, CHO pgs A-677 and CHO pgs A-745 cells were maintained in Ham’s F12 medium containing 5% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 10 µg/ml streptomycin, 50 U/ml nystatin). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Every 3 - 4 days they were splitted into new plates. The medium was removed and 0.05% Trypsin/EDTA (0.025% EDTA in PBS) was added twice; first to wash cells and the second time to detach cells. After a short incubation of 1-2 min, the growth medium was added to stop the trypsinisation and cells were maintained in growth medium with antibiotics.

Dendritic cells (DC) were maintained in RPMI medium containing 5% fetal bovine serum (FBS), 10 mM HEPES pH 7.4, 2 mM glutamine with antibiotics (100U/ml penicillin, 100 µg/ml streptomycin). Cells were grown in a 6 well plate (3ml/well) and incubated overnight at 37°C. They were separated from the macrophages and monocytes, using a metrizamide gradient. Cells were transferred into a 50 ml falcon tube. After centrifugation (5 min, 600 g), they were resuspended in 5 ml growth medium and were layered carefully onto a 0.2 M metrizamide solution. After centrifugation (10 min, 1500 g), the supernatant was removed by aspiration to about 1 ml over the interphase. The interphase containing the DC was washed twice with the growth medium and cells were counted and resuspended at a density of $10^6$ cells/ml.

3.3 Liposomes

3.3.1 Preparation of liposomes by extrusion

The liposome components (see Table 1) were dissolved in methanol/methylenchloride (1:1 v/v), followed by evaporation on a Rotavap instrument (Büchi, Flawil, CH) at 40°C, resulting in a dry lipid film. The lipid film was dispersed in PB by agitation to get large multilamellar vesicles. As exception, liposomes loaded with EPO-B were frozen 4 times in liquid nitrogen and thawed at RT before extrusion. Size reduction to unilamellar liposomes (size < 100 nm) was performed by repeated extrusions in a Lipex™ extruder (Lipex Biomembranes, Vancouver, Canada) using Nucleopore membranes (Sterico, Dietikon, CH) of 0.4, 0.2 and 0.1 µm pore sizes.

To measure size and stability of liposomes, 1 to 5 µl liposomes were diluted in 500 µl PB and analysed by dynamic laser scattering with a particle sizer (Submicron Particle Sizer, Nicomp Model 370, Santa Barbara, USA).

Table 1: Basic lipid composition for the preparation of 1.0 ml liposomes

<table>
<thead>
<tr>
<th>Substances</th>
<th>MW g/mol</th>
<th>mol %</th>
<th>mol/ml</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>770</td>
<td>100</td>
<td>$1.04 \times 10^4$</td>
<td>80</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>387</td>
<td>20</td>
<td>$2.08 \times 10^3$</td>
<td>8</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>431</td>
<td>1</td>
<td>$1.04 \times 10^6$</td>
<td>0.48</td>
</tr>
<tr>
<td>DPPE</td>
<td>692</td>
<td>3.5</td>
<td>$3.6 \times 10^6$</td>
<td>2.5</td>
</tr>
</tbody>
</table>
As exception, the EPO-liposomes were composed of 40 mg/ml SPC as reported in Table 3. All liposomes prepared are listed in Table 3. The composition of liposomes loaded with DNA is shown in Table 5, chapter 3.8.

All the substances listed in Table 2 are lipophilic and added to the organic solvent during the liposome preparation.

### Table 2: Overview of fluorescent markers and cytotoxic drugs added to the basic liposome composition.

<table>
<thead>
<tr>
<th>Fluorescent dyes</th>
<th>MW g/mol</th>
<th>mol %</th>
<th>mol/ml</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiO</td>
<td>882</td>
<td>0.44</td>
<td>4.54 * 10^{-7}</td>
<td>0.4</td>
</tr>
<tr>
<td>Texas-red</td>
<td>1392</td>
<td>0.03</td>
<td>2.9 * 10^{-8}</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytotoxic drugs</th>
<th>MW g/mol</th>
<th>mol %</th>
<th>mol/ml</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FdU-NOAC</td>
<td>804</td>
<td>6</td>
<td>6.22 * 10^{-6}</td>
<td>5</td>
</tr>
<tr>
<td>NOAC-ETC</td>
<td>825</td>
<td>5.8</td>
<td>6.06 * 10^{-6}</td>
<td>5</td>
</tr>
<tr>
<td>EPO-B *</td>
<td>493.25</td>
<td>3.6</td>
<td>2.03 * 10^{-6}</td>
<td>1</td>
</tr>
</tbody>
</table>

* = EPO-B : 10.0 mg EPO-B were dissolved in 10.0 ml MeOH. Aliquots from this solution were added to the organic solvent drug liposome preparation.

### Table 3: Overview of the prepared liposomes.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic lipid composition</th>
<th>Fluorescent dyes</th>
<th>Maleimide : Peptides (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lipo-A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control lipo-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antp-lipo-C, -D, -E, -F</td>
<td>SPC 80 mg/ml 100 mol %</td>
<td>DiO 0.4 mg/ml 0.44 mol %</td>
<td>C: Mal : Antp = 1:1.2 ²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D: Mal : Antp = 1:0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E: Mal : Antp = 1:0.24 ²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F: Mal : Antp = 1:0.12 ²</td>
</tr>
<tr>
<td>TAT-lipo-G, -H, -I, -J</td>
<td>Cholesterol 8 mg/ml 20 mol %</td>
<td>Texas-red 0.04 mg/ml 0.03 mol %</td>
<td>G: Mal : TAT = 1:1.2 ²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H: Mal : TAT = 1:0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I: Mal : TAT = 1:0.24 ²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>J: Mal : TAT = 1:0.12 ²</td>
</tr>
<tr>
<td>Control lipo-K</td>
<td>α-tocopherol 0.48 mg/ml 1 mol %</td>
<td></td>
<td>L: Mal : Antp = 1:1.2 ²</td>
</tr>
<tr>
<td></td>
<td>DPPE 2.5 mg/ml 3.5 mol %</td>
<td></td>
<td>M: Mal : Antp = 1:0.6 ²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N: Mal : Antp = 1:0.24 ²</td>
</tr>
<tr>
<td>5-FdU-NOAC lipo-O</td>
<td>DPPE</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5-FdU-NOAC + Antp lipo-P</td>
<td></td>
<td></td>
<td>Mal : Antp = 1:1.2 ²</td>
</tr>
<tr>
<td>NOAC-ETC lipo-Q</td>
<td>DPPE</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>NOAC-ETC + Antp lipo-R</td>
<td></td>
<td></td>
<td>Mal : Antp = 1:1.2 ²</td>
</tr>
<tr>
<td>EPO-B lipo-S</td>
<td>DPPE</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>EPO-B + Antp lipo-T</td>
<td>DPPE</td>
<td></td>
<td>Mal : Antp = 1:1.2 ²</td>
</tr>
</tbody>
</table>

¹ = lipid composition 40 mg SPC, 4 mg cholesterol, 0.24 mg α-tocopherol, PE-PEG-Mal 5.2 mg
² = corresponding to molar ratio of maleimide groups to peptide
Mal = maleimide groups
3.3.2 Drug quantification

First, a standard curve for each cytotoxic drug was established using the Lambert-Beer equation (see below), which correlates the extinction coefficient (e) of a substance with the absorbance (A):

\[ A = \varepsilon \cdot c \cdot b \]

\( A \) = absorbance
\( \varepsilon \) = extinction coefficient
\( c \) = concentration of solution [mol/l]
\( b \) = thickness [cm]

Four mg 5-FdU-NOAC (\( \varepsilon = 7570 \) in MeOH) and NOAC-ETC (\( \varepsilon = 6100 \) in MeOH) were dissolved in 50 ml MeOH and analysed at 270 nm (maximal absorbance of 5-FdU-NOAC) and at 273 nm (maximal absorbance of NOAC-ETC), respectively, using an UV-spectrophotometer (UV-1601, Shimadzu, CH). A standard curve from 0.8 mg/ml to 0.0025 mg/ml cytotoxic drug in MeOH was established for both compounds (see Figure 17 and Figure 18 in Appendix I). Liposomes containing 5-FdU-NOAC or NOAC-ETC (50 µl) were dissolved in 5 ml MeOH and analysed at 270 nm or 273 nm wavelength, respectively, to determine the quantity of cytotoxic drug in liposomes.

For EPO-B, the initial EPO-B concentration in liposomes after extrusion was taken as 100%. One hundred µl liposomes were dissolved in 5 ml MeOH and measured at 236 nm wavelength (maximal absorbance of EPO-B) against an equal concentration of empty liposomes dissolved in MeOH as reference.

3.3.3 Modification of liposomes with sulfo-SMCC

One ml liposomes containing an average of \( 10^{18} \) amino groups on the surface were incubated with 3.6 mg sulfo-SMCC (20% excess) added as crystals at 37°C for 2 h under agitation. Excess sulfo-SMCC was removed by dialysis with a dialysis tube of 6-8000 MW cut-off overnight at 4°C against PB (1:1000, v/v).

3.3.4 Attachment of Antp- or TAT- peptides to liposomes

For control liposomes without peptide (liposomes B), the maleimide groups were blocked with a cysteine solution at a final concentration of 5.3 mM (10 x excess cysteine). Antp or TAT (3.2 mg corresponding to a molar ratio of maleimide groups to peptides of 1:1.2) were incubated with 1 ml of sulfo-SMCC modified liposomes for 48 h at RT under agitation in presence of tributylphosphine in a final concentration of 7.7 mM. The molar ratios of maleimide groups to Antp or TAT were 1:1.2, 1:0.6, 1:0.24, 1:0.12 (see Table 3). Non-reacted peptide was removed by dialysis with a dialysis tube of 12-14000 MW cut-off overnight at 4°C against PB (1:1000, v/v). All liposomes were kept at 4°C.

3.4 Cellular association of Antp- or TAT- liposomes

Control liposomes and liposomes conjugated with various amounts of peptides (Table 3: liposomes A-J) were labelled with the fluorescent dye DiO and used for association experiments with B16F1, F9, W38, CHO and DC cells. Before their application on cells, the absorbance of DiO of each lipidosome preparation was measured with an UV-spectrophotometer (UV-1601, Shimadzu, CH) to adjust the liposome concentrations. Five µl of liposomes were dissolved in 500 µl PB and analysed at 484 nm wavelength.
3.4.1 Flow Cytometry

B16F1, F9, W38 and CHO cells (2 x 10^5 cells/well in a 12 well plate) were seeded in growth medium for 24 h at 37°C. The medium was removed and cells were incubated in serum free OptiMEM 1 medium for 30 min at 37°C (1 ml/well). During this time, liposomes were diluted in serum free OptiMEM 1 medium as summarised in Table 4. After removing the medium, cells were incubated with 300 µl liposomes/well for 1 h at 37°C. Liposomes were removed and cells were cultured in growth medium for another 30 min at 37°C. Cells were washed with PBS, detached with 10 mM EDTA in PBS, resuspended and transferred into FACS-tubes. After centrifugation (5 min, 600 g), cells were fixed in 3.7% formaldehyde in PBS. Binding of liposomes was measured using a FACScan flow cytometer (FACScan, Becton Dickinson). Ten thousand cells were analysed per data point and all experiments were carried out in triplicates.

Murine dendritic cells (DC) (10^6 cells/tube) were suspended in growth medium in FACS-tubes. After centrifugation (5 min, 600 g) the medium was removed and cells were incubated with the diluted liposomes as described above for 1 h at 37°C. Cells were washed with PBS as described above and analysed directly in 1 ml growth medium.

Table 4: Amounts of liposomes diluted in 1 ml serum free OptiMEM 1 medium.

<table>
<thead>
<tr>
<th>Experiment N°</th>
<th>Control lipo A, B</th>
<th>Antp lipo-C TAT lipo-G</th>
<th>Antp lipo-D TAT lipo-H</th>
<th>Antp lipo-E TAT lipo-I</th>
<th>Antp lipo-F TAT lipo-J</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.6 * 10^-7 mol lipids</td>
<td>2.5 µl</td>
<td>2.5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.6 * 10^-7 mol lipids</td>
<td>2.5 µl</td>
<td>2.5 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.4.2 Fluorescence microscopy

Coverslips (diameter: 12-14 mm) were coated with fibronectin for 1 h to obtain better cell adhesion. After removing fibronectin, B16F1 cells were cultured on the coverslips in growth medium to a density of 80%. Different Antp or Antp-free liposomes (liposomes A, C, E and F, 3 µl each) were mixed in 100 µl OptiMEM 1 medium. The coverslips were put into a Plexiglas box and 30 µl of the diluted liposomes were added. Cells were incubated for 1 h at 37°C with 5% CO₂.

After washing in PBS, cells on coverslips were fixed in 3.7% formaldehyde in PBS for 30 min and washed again with PBS. Cells were embedded in Gelvatol and analysed with a fluorescence microscope Type Axiophot (Zeiss). Photos were taken with a Kappa CF 8/1 CCD camera, exposure time was 80 ms for all pictures in the series.

3.5 Determination of the number of Antp peptides per liposome

One mg FITC-Antp was dissolved in 10 ml PB to obtain a saturated solution of FITC-labelled-Antp. To optimise the detection method, methanol was added in a ratio of FITC-labelled-Antp solution to methanol of 1:1 to 1:10. Liposomes before and after treatment with 10% Triton X-100 were also added to observe variations of the fluorescence of the FITC-labelled-Antp solution. The fluorescence of these solutions was analysed on a 96-well plate with a spectrofluorometer (TECAN Ultra Evolution; filters: excitation 485 nm, emission 535 nm, Software Magellan Vo 4.0).
To quantify the coupling efficiency of Antp to liposomes, 2 mg Antp peptide were dissolved in 200 µl saturated FITC-labelled-Antp solution. Different volumes of this solution (100 µl, 50 µl and 20 µl respectively) were added to three Eppendorf tubes, containing each 300 µl of sulfo-SMCC-liposomes, in order to prepare Antp-lipo -L, -M, -N. The Antp-coupling was performed as described in chapter 3.3.3. The fluorescence of these FITC-Antp-liposomes dissolved in methanol (2:3, v/v) was measured both before and after the coupling and the dialysis with the TECAN instrument in a 96 well plate in triplicates. The fluorescence value of FITC-Antp of each Antp-liposome-solution before the coupling was taken as 100% liposome fluorescence.

The fluorescent dye, Texas-red-DPPE in the lipid bilayer of liposomes was analysed with a UV-spectrophotometer (UV-1601, Shimadzu, CH) at 583 nm to determine the concentration of the lipid in liposomes before and after the coupling reaction. Fifty µl of liposomes were added 450 µl MeOH and measured at 583 nm wavelength, where FITC absorption is not present.

3.6 Cytotoxicity tests

B16F1 and F9 cells (10'000 cells/well in 100 µl in 96 well plate for incubation times up to 18 h and 5'000 B16F1 cells/well and 1'000 F9 cells/well in 100 µl for an incubation time of 30 h) were grown in medium for 24 h at 37°C. Sterile filtered (0.2 µm) liposomes containing various cytotoxic agents were diluted in sterile PB to different concentrations (range 1.6 - 100 µM for the liposomes O, P, Q and R and 1.6 - 200 nM for the EPO-B liposomes S, T and free EPO-B). Hundred µl of each concentration were mixed with 100 µl DMEM in quadruplicates. The positive controls were cells without drugs and negative controls were an empty row of wells without cells for determination of the background of the WST-reagent. The medium was removed and diluted liposomes containing the drugs were added to cells for different incubation times (30 min, 1 h, 2 h, 4 h, 18 h and 30 h). After incubation, liposomes were removed and 100 µl growth medium per well were added for about 22 h. Cell viability was determined with the WST-1-test. WST-1 reagent was diluted in 1:10 v/v serum free Optimem 1 medium and 100 µl were added per well. The plate was incubated at 37°C until absorption values of 1.6 – 1.9 were reached and read with a plate-reader (Dynatech MR 4000, Software Biolinx 2.0) at 410 nm. The background of WST-reagent was subtracted and the value of cells without drug treatment was taken as 100% cell viability. The IC_{50}, fifty percent growth-inhibitory concentration, was calculated from interpolations of the graphical data (Figure 14).

3.7 DNA loaded liposomes

3.7.1 Liposome preparation

The plasmid pEGFP-NH\textsubscript{2}, named DNA in the text, was used for the cell transfection assays. Lipid components are listed in Table 5. After the formation of the lipid film, DNA dissolved in a TE-solution (1.3 mg/ml) was added to obtain a total lipid concentration of 120 mM. This liposome-solution was frozen 4 times in liquid nitrogen and thawed at RT, allowing the encapsulation of DNA into liposomes. Then, the liposome-solution was extruded as described in chapter 3.3.1. EDTA was removed by dialysis.
Table 5: Basic lipid composition for DNA loading for the preparation of 1.0 ml liposomes

<table>
<thead>
<tr>
<th>Lipids</th>
<th>MW g/mol</th>
<th>mol %</th>
<th>mol/ml</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPPC</td>
<td>760</td>
<td>100</td>
<td>$1.2 \times 10^{-4}$</td>
<td>91.2</td>
</tr>
<tr>
<td>DDAB</td>
<td>462.65</td>
<td>20</td>
<td>$2.4 \times 10^{-5}$</td>
<td>11.1</td>
</tr>
<tr>
<td>DPPE</td>
<td>692</td>
<td>3.5</td>
<td>$4.2 \times 10^{-6}$</td>
<td>2.9</td>
</tr>
<tr>
<td>PE-PEG-Mal</td>
<td>2819.4</td>
<td>3.5</td>
<td>$4.2 \times 10^{-6}$</td>
<td>11.8</td>
</tr>
<tr>
<td>Texas-red</td>
<td>1392</td>
<td>0.03</td>
<td>$3.6 \times 10^{-8}$</td>
<td>0.05</td>
</tr>
</tbody>
</table>

= PE-PEG-Mal replaces DPPE for the Antp-coupling.

Table 6: Overview of prepared DNA liposomes.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic composition(^{1})</th>
<th>Plasmid mg/ml</th>
<th>Maleimide : Antp molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>Control liposomes</td>
<td>OPPC -</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Plasmid liposomes</td>
<td>DDAB +</td>
<td>1.3</td>
</tr>
<tr>
<td>W</td>
<td>Mal-Plasmid liposomes</td>
<td>Texas-red +</td>
<td>1.3</td>
</tr>
<tr>
<td>X</td>
<td>Plasmid-Antp liposomes</td>
<td></td>
<td>1:1.2</td>
</tr>
</tbody>
</table>

\(^{1}\) = amount corresponding to the Table 5.

3.7.2 Digestion of non encapsulated DNA

Five hundred µl of liposomes (about 0.66 mg DNA) were incubated with 33 µl DNAase I solution (100 mg/ml in 20 mM Tris; 5 µg DNAase I are necessary to digest 1 µg DNA) in presence of 10 mM MgCl\(_2\) for 2.5 h at 37°C under agitation. The reaction was stopped by adding 25 µl 0.5 mM EDTA [30].

- Separation of DNA-fragments with gel electrophoresis

Two µl of each liposome type before and after the digestion were treated with 6 µl 10% Triton X-100 or with 6 µl water. Five µl of these solutions were loaded to a 1% agarose gel in TAE. The gel was run for 30 min at 175 volts.

- Separation of digested DNA from liposomes

DNAase I and digested DNA were removed from liposomes using a Biogel A 15m column. This column was prepared in a plastic syringe of 5 ml, equilibrated with 5 ml 50 mM Tris, pH 8 and centrifuged. After gentle centrifugation of the column (Laborfuge M, Heraeus, Kendro, CH) for 1 min at 100 g, liposomes were added and eluted with 50 mM Tris, pH 8. Fractions were collected after 5 centrifugations and analysed to quantify the DNA with the PicoGreen test (see 3.7.3).

These diluted fractions were concentrated in a 2 ml Vivaspin concentrator (Membrane 10’000 MW cut-off, Vivascience, Sartorius AG, DE) for 24 h at 2500 g and again analysed for their DNA concentration with the Pico Green assay.
3.7.3 Quantification of DNA loading to liposomes: Pico Green Test

A standard curve of Lambda DNA of 10 ng/ml DNA to 1 µg/ml DNA was established according to the protocol from Molecular Probes [31]. To 3 µl of each liposome sample, 7 µl 10% Triton X-100 were added and diluted in TE buffer to a final volume of 1.0 ml. The standard samples (200 µl) or liposome-TE samples (200 µl) were mixed with 200 µl PicoGreen reagent during 2-3 min under light protection. Hundred µl of each sample were pipetted to a 96-well microplate and measured at 485 nm excitation, 535 nm emission using the TECAN instrument. The assay was made in triplicates.

3.7.4 Modification of DNA-liposomes with Antp peptide

Antp was attached to the surface of the DNA-liposomes containing maleimide groups (liposomes W) at a molar ratio of maleimide groups to Antp of 1:1.2 as described in chapter 3.3.3.

3.7.5 Transfection of porcine arterial endothelial cells (PAE) with DNA

PAE cells (2 x 10^5 cells/well in a 12 well plate) were grown in DMEM supplemented with 10% FBS for 24 h at 37°C. The medium was removed and cells were incubated in serum free OptiMEM 1 medium for 30 min at 37°C (1 ml/well). During this time, diluted liposomes were prepared in serum free OptiMEM 1 medium in triplicates (5 µg DNA/300 µl medium according to the theoretical amount of plasmid and to the determination of plasmid with the Pico Green test). As positive control of the transfection efficiency, 3 µg of the plasmid alone were added to cells using a CaCl_2 method (3 µg of plasmid were dissolved in 60 µl of a sterile solution of 250 mM CaCl_2, and 60 µl of a solution of 140 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4 were added. The solution was incubated for 1 min). After removing the medium, cells were incubated with 300 µl liposomes/well for 4 h at 37°C. For the positive control, the medium was not removed and 100 µl plasmid-solution/well were added to cells. Liposomes were removed, cells were washed with PBS (1 ml/well) and cultured in DMEM with antibiotics for 24 h at 37°C and 5% CO_2.

The transfection efficiency was analysed with a FACSscan flow cytometer as described in chapter 3.4.1.

For fluorescence microscope experiments, PAE cells were cultured on coverslips as described in chapter 3.4.2. After incubation for 24 h, the coverslips were washed with PBS and cells were embedded in Gelvatol. Cells were analysed with a fluorescence microscope Type Axiophot (Zeiss).
4 RESULTS

4.1 Liposome characterisation

All liposomes prepared are listed with their measured diameter in Table 10-14, Appendix II. The average size of liposomes varied between 50 and 130 nm depending on the composition of liposomes. A conclusive relation between liposome size and their cellular effect was not found. Contrary to Antp modified liposomes, the diameter of TAT modified liposomes was heterogeneous, reflecting probably the presence of aggregates (Table 10, Appendix II). The mean diameter of liposomes is used as a control of their preparation and of their stability. It allows to calculate the number of liposomes per ml, which is a very important factor for the determination of the number of peptide molecules linked to the surface of liposomes (chapter 4.3). The Antp liposomes C-E, L-M (Abbreviations see Table 3, Materials and Methods) were stable for 4 weeks and the TAT liposomes G-J for 3 weeks, all stored at 4°C. The EPO-B liposomes S were stable for more than 3 months (see Figure 15).

4.2 Relationship between peptide numbers and liposome uptake efficiency

To explore the relationship between peptide density and the efficiency of cellular uptake, a series of Antp- or TAT- modified liposomes (liposomes A-J) were prepared (Table 3, chapter 3.3) and incubated with different cells for 1 h at 37°C. Liposomes were labelled with the fluorescent dye DiO, which was incorporated into the lipid bilayer. This allows to measure the cellular uptake and association using fluorescence microscopy and flow cytometry.

Figure 8: Fluorescence microscopy of B16F1 cells treated with (A) control liposomes A, (B) Antp modified liposomes F with maleimide to Antp 1:0.12, (C) Antp modified liposomes E with maleimide to Antp 1:0.24, (D) Antp modified liposomes C with maleimide to Antp 1:1.2 at 37°C for 1 h. DiO fluorescence was observed with a FITC filter. Exposure time: 80 ms.
As shown in Figure 8, only the cells treated with Antp-liposomes C were fluorescent. No fluorescent cells were detected after incubation with control liposomes A and the Antp modified liposomes E and F, which had low numbers of Antp per liposome (see Table 3).

The association of peptide modified liposomes with a variety of cell lines such as B16F1 melanoma, F9 teratocarcinoma and W38 cells was quantified by flow cytometry (Figure 9).

Figure 9: Effect of peptide number per liposome on association efficiency of liposomes. DiO labelled peptide modified liposomes were incubated with (A) B16F1, (B) W38 and (C) F9 cells. Liposomes (2.6 \times 10^{-7} \text{ mol lipids / 2 \times 10^5 cells}) were incubated with cells for 1 h at 37°C before being analysed by flow cytometry. Each value is the mean ± S.D, n=3.

Only the peptide modified liposomes C, G and H significantly enhanced the cellular association of liposomes on all the cells analysed (Figure 9 A). In comparison to control liposomes, the association of TAT peptide modified liposomes increased 10-fold with B16F1 cells, 15-fold with F9 cells and 8-fold with W38 cells, respectively. In contrast, Antp-derivatised liposomes were taken up 30-times more efficiency into B16F1 cells, 3-times into F9 cells and 11-times into W38 cells compared to control liposomes A and B. In other words, B16F1 and W38 cells were more susceptible to cellular association of Antp modified liposomes than TAT modified liposomes, contrary to F9 cells. TAT modified liposomes H and G showed approximately a comparable cellular association with B16F1 cells, although the number of peptides attached to the surface of liposomes G was 2-fold higher. In contrast, cellular association measured with Antp...
modified liposomes D (maleimide:peptide = 1:0.6) was 6-fold lower than of Antp modified liposome C (Figure 9 A).

Peptide modified liposomes E, F, I and J (maleimide:peptide = 1:0.24 and 1:0.12) showed approximately the same weak association with B16F1 and F9 cells as control liposomes modified with cysteine (control liposomes B). In contrast, TAT modified liposomes I and J were associated with W38 cells in proportion of the number of TAT molecules linked to liposomes (Figure 9 B). Control liposomes A into B16F1 cells showed a weak cellular association, probably resulting from the amino groups of the positively charged DPPE, binding to the negatively charged cell surface (Figure 9 A).

In a second set of experiments, amounts of liposomes containing different numbers of peptides per liposome were adjusted to obtain an absolute identical amount of total peptide molecules per liposome type, as listed in Table 4. Despite increasing the amount of peptide modified liposomes containing a lower quantity of peptide molecules per liposome, only peptide modified liposomes C, D, G and H (maleimide:peptide = 1:1.2 and 1:0.6) enhanced cellular association of liposomes significantly with B16F1 and F9 cells (Figure 10).

TAT modified liposomes H showed a better cellular association with B16F1 cells than TAT modified liposomes G (Figure 10 A). This is caused probably by the tendency of TAT liposomes to form aggregates with increasing numbers of peptide molecules attached per liposome, which results in reduced association with cells. Despite increasing the amount of Antp modified liposomes E and F, their cellular association was not enhanced, contrary to TAT modified liposomes I and J, where cellular binding was slightly increased (Figure 9 A versus Figure 10 A).

Figure 10: Association of different amounts of DiO labelled peptide modified liposomes with (A) B16F1 and (B) F9 cells determined by flow cytometry. Cells were incubated with liposomes for 1 h at 37°C. The correlation between volumes and amounts of liposomes is given in Table 4, Methods. Each value is the mean ± S.D., n=3.

4.3 Determination of the number of Antp peptides per liposome

FITC-labelled-Antp was used for the determination of the coupling efficiency to the surface of liposomes containing a constant amount of maleimide groups. Three types of Antp peptide modified liposomes L, M, and N with initial molar ratios of maleimide to Antp of 1:1.2, 1:0.6 and 1:0.24, respectively, were analysed.

In preliminary tests, the optimal fluorescence of the FITC-labelled-Antp solution was determined in 88% MeOH. The presence of liposomes before or after treatment with 10% Triton X-100 did not influence the fluorescence measurements. Liposomes were labelled
using the lipophilic fluorescent dye Texas-red. This dye would allow to measure the concentration of liposomes after each reaction step. Unfortunately, it was not possible to get absolute values because a fraction of the dye was adsorbed by the extrusion filter during liposome preparation. Therefore yields of liposomes after extrusion and modification were estimated according to the results of C. Marty, [32] with the following modifications:

The preparation of liposomes gave a mean yield of 95% after the extrusion, the modification with sulfo-SMCC a yield of 90% and the attachment with Antp a yield of 90%, resulting in a final yield of $1 \times 10^{15}$ liposomes as reported in Table 14, Appendix III.

The coupling efficiency of Antp to liposomes was determined by measuring the fluorescence of FITC-labelled Antp. As shown in Figure 11 A, using an initial molar ratio of maleimide to Antp of 1:1.2, 1:0.6 and 1:0.24 a coupling efficiency of 24%, 30% and 39% respectively, resulted. Because the quantities of initial Antp peptide and the total number of liposomes were known, as well as the number of phospholipid molecules (SPC) forming a liposome of a given size as described by Huang [33], an approximate number of Antp peptide bound to a single liposome was estimated as illustrated in Figure 11 B. Antp modified liposomes containing 3.5 mol % amino groups (see Table 1) with a mean diameter of 76 nm (liposomes K) carry approximately 770 amino groups on the outer monolayer surface per liposome. According to C. Marty [32], the modification efficiency with sulfo-SMCC is 60%, corresponding to about 466 maleimide groups on the surface of one liposome. The calculations of these active groups are summarised in Table 15, Appendix III. As illustrated in Figure 11 B, Antp modified liposomes L (initial molar ratio maleimide groups to peptides = 1:1.2) carried about 110 Antp peptides on their surface, whereas those with a molar ratio maleimide to peptide of 1:0.6 and 1:0.24 about 69 and 36 Antp peptides, respectively. This distribution was proportional to the initial amount of peptide added.

4.4 Association of peptide modified liposomes with various cell lines

As observed in chapter 4.2, peptides at a molar ratio of 1:1.2 (liposomes C and G) attached to liposomes significantly increase cellular association and uptake in comparison to control liposomes without peptide. To compare their cell association, these liposomes were tested on two other cell types CHO cells and dendritic cells (DC) (method described in chapter 3.4.1).
Cellular association of liposomes depended on the cell type as illustrated in Figure 12. Compared to control liposomes, B16F1, W38 and DC cells were more susceptible to binding of Antp modified liposomes than TAT-derivatised liposomes. The contrary was observed with F9 and CHO cells. Antp peptide showed the highest cellular association of liposomes with B16F1 cells and TAT peptide with CHO cells.

![Figure 12: Association of DiO labelled peptide modified liposomes with various cells. Liposomes (2.6 * 10^{-7} mol lipids / 2 * 10^5 cells) were incubated with cells for 1 h at 37°C before being analysed by flow cytometry. Each value is the mean ± S.D., n=3.](image)

4.5 Inhibition of peptide modified liposome association

To investigate the mechanism responsible for the association of peptide modified liposomes with cells, liposomes (control liposomes A and B, liposomes C and G) were incubated with mutant CHO cells deficient in glycosaminoglycan synthesis. The mutant cell line pgs A-745 lacking xylosyltransferase, an enzyme responsible for glycosaminoglycan synthesis [34] showed a strong reduction in association of Antp and TAT derivatised liposomes. A second mutant of the CHO cells, pgs D-677 deficient in heparin sulfate synthesis showed also a reduction in the uptake of Antp- and in TAT- modified liposomes, which was more marked for TAT modified liposomes (Figure 13).

![Figure 13: Antp and TAT liposomes association with CHO wt and mutant cells. Liposomes (2.6 * 10^{-7} mol lipids / 2 * 10^5 cells) were incubated with cells for 1 h at 37°C before being analysed by flow cytometry. Each value is the mean ± S.D., n=3.](image)
4.6 Cytotoxicity tests

As shown in chapter 4.3, about one hundred Antp peptide molecules per liposome promoted efficient association and delivery of liposomes into various cell types. Thus, this number of Antp peptide molecules corresponding to an initial molar ratio of maleimide to peptide of 1:1.2 was chosen for the cytotoxicity studies.

4.6.1 Quantification of the cytotoxic drugs incorporated into liposomes

To quantify the cytotoxic compounds encapsulated in liposome preparations the absorbance of the anticancer drug was measured after each step. The value measured after extrusion was taken as 100% (Table 7). Fifty-eight % of 5-FdU-NOAC and 53% of NOAC-ETC were still incorporated into the lipid bilayer of liposomes after Antp coupling. Eighty-five % of the initial EPO-B concentration was measured after modification with the Antp peptide. The higher proportion of this cytotoxic drug is probably due to the omission of the sulfo-SMCC-modification step, because the maleimide groups were already added to the lipid mixture during liposome preparation, using DPPE-PEG-Mal.

Table 7: Relative amount of cytotoxic drug contained in liposomes analysed by UV-spectroscopy.

<table>
<thead>
<tr>
<th>Cytotoxic drugs</th>
<th>Encapsulated after extrusion</th>
<th>Encapsulated after Antp-coupling and dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FdU-NOAC</td>
<td>100%</td>
<td>58%</td>
</tr>
<tr>
<td>NOAC-ETC</td>
<td>100%</td>
<td>53%</td>
</tr>
<tr>
<td>EPO-B</td>
<td>100%</td>
<td>85%</td>
</tr>
</tbody>
</table>

4.6.2 EPO-B: comparison of cytotoxic effect between EPO-B in solution and EPO-B in liposomes

EPO-B was encapsulated into liposomes and its cytotoxic effect was compared to an EPO-B solution in PB at the same concentration. EPO-B showed no difference in its toxicity on B16 cells either encapsulated into liposomes or in solution. After 30 h of incubation time, an IC\textsubscript{50} of 0.5 nM was obtained in both cases (see Figure 14).

Figure 14: Cytotoxic effect of EPO-B in solution and in liposomes on B16F1 cells after 30 h of incubation. Each value is the mean ± S.D., n=4.
EPO-B was very stably incorporated into liposomes. After 3 months, the mean diameter of liposomes did not vary as illustrated in Figure 15.

![Figure 15: Stability of the EPO-B liposomes. The diameter of liposomes was analysed by dynamic laser scattering.](image)

4.6.3 Comparison of IC\textsubscript{50} of Antp modified liposomes and Antp free-liposomes

The cytotoxic effect of liposomes and Antp modified liposomes carrying different cytotoxic drugs on B16F1 and F9 cells was tested with the WST-1 test. Table 8 shows IC\textsubscript{50} values of the different liposomes loaded with three cytotoxic drugs. The most effective compound was EPO-B followed by NOAC-ETC and 5-FdU-NOAC. Indeed, after 4 h of incubation, the IC\textsubscript{50} of EPO-B was 100 fold lower than that of NOAC-ETC and 300 fold lower than that of 5-FdU-NOAC on B16F1 cells. After 18 h of incubation, the IC\textsubscript{50} of EPO-B was about 20 nM, 50 fold lower than that of NOAC-ETC and still 150 fold lower than that of 5-FdU-NOAC.

Table 8: Overview of IC\textsubscript{50} values in µM of liposomes and Antp peptide modified liposomes at different incubation times on B16F1 and F9 cells. Cytotoxicity was measured by incubation with WST-1 dye followed by fluorometric determination.

<table>
<thead>
<tr>
<th>Incubation times</th>
<th>B16F1 cells</th>
<th>F9 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>1 h</td>
</tr>
<tr>
<td>Lipo-O</td>
<td>5-FdU-NOAC lipo</td>
<td>no IC\textsubscript{50} reached</td>
</tr>
<tr>
<td>Lipo-P</td>
<td>5-FdU-NOAC lipo + Antp</td>
<td>-</td>
</tr>
<tr>
<td>Lipo-Q</td>
<td>NOAC-ETC lipo</td>
<td>-</td>
</tr>
<tr>
<td>Lipo-R</td>
<td>NOAC-ETC lipo + Antp</td>
<td>-</td>
</tr>
<tr>
<td>Lipo-S</td>
<td>EPO-B Lipo</td>
<td>-</td>
</tr>
<tr>
<td>Lipo-T</td>
<td>EPO-B lipo + Antp</td>
<td>-</td>
</tr>
</tbody>
</table>

The effect of Antp peptide modification on cytotoxicity was also assessed. Antp increased the cytotoxicity of the liposomes containing NOAC-ETC only on B16F1 cells by about a factor of 2 after 2 h of incubation and a factor of 3 after 4 h. After 18 h incubation, the cytotoxicity of NOAC-ETC in Antp liposomes was similar to that of control liposomes (see Figure 16).
For the other anticancer drugs, the presence of Antp peptide on the surface of liposomes did not improve their cytotoxicity on B16F1 and on F9 cells.

![Figure 16: Cytotoxic effect of NOAC-ETC contained in Antp modified (liposomes Q) and control liposomes (liposomes R) after different incubation times with B16F1 cells.](image)

### 4.7 DNA-loaded liposomes

The lipid composition of DNA-liposomes was different, using cationic lipids interacting with the negatively-charged DNA. First, DNA incorporation into liposomes was analysed, followed by transfection of cells.

#### 4.7.1 Determination of DNA concentration in liposomes

The Pico Green test is based on fluorescence staining nucleic acids for quantitating double-stranded DNA in solution. The plasmid concentration of the pEGFP-NH2 in liposomes was quantified at different stages of the preparation and modification of liposomes calibrated against a standard curve of Lambda phage DNA. Plasmid-loaded-liposomes were solubilised with 10% Triton X-100. As shown in Table 9 after extrusion only about 10% of the initial amount of plasmid was detected. After the digestion with DNAase and separation of the non-encapsulated plasmid using a Biogel A 15m column, the collected fractions were concentrated and analysed. 30% of the plasmid were still in the liposomes W after digestion and separation of non-encapsulated plasmid, whereas only 3% were detected after Antp-coupling.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Initial concentration of plasmid mg/ml</th>
<th>After extrusion mg/ml</th>
<th>After digestion and separation of non-encapsulated plasmid mg/ml</th>
<th>After modification with Antp peptide mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes V</td>
<td>1.3</td>
<td>0.107</td>
<td>0.018</td>
<td>-</td>
</tr>
<tr>
<td>Liposomes W</td>
<td>1.3</td>
<td>0.151</td>
<td>0.049</td>
<td>0.005 (liposomes X)</td>
</tr>
</tbody>
</table>

The encapsulation efficiency of the plasmid in liposomes was analysed by gel electrophoresis. The results obtained were difficult to interpret.
4.7.2 Expression of plasmid delivered by liposomes into PAE cells

Despite the low efficiency of plasmid encapsulation in liposomes measuring with the Pico Green test, liposomes (liposomes V and W) and Antp modified liposomes (liposomes X) were added to PAE cells. The amount of liposomes required to deliver 5 µg of DNA per $2 \times 10^5$ cells was determined according to the theoretical quantity of DNA contained in liposomes and according to the determination using the Pico Green test. The identical quantity of free plasmid alone was used as positive control. As negative control, the cationic liposomes U without plasmid were incubated according to the procedure described in chapter 3.7.5. GFP expression was then determined by flow cytometry and fluorescence microscopy. All PAE cells incubated with control cationic liposomes were dead. The results obtained were difficult to analyse, because of the high proportion of dead cells. However, the fluorescence of the positive control was the highest. Improved delivery of DNA by Antp modified liposomes could not be showed and the encapsulation of DNA into liposomes has to be optimised in future experiments (see chapter 5.4, Discussion).
5 DISCUSSION AND PERSPECTIVES

Antp and TAT peptides, two representatives of "cell penetrating peptides" were extensively studied for their ability to translocate across the plasma membrane and to promote cellular uptake of various cargo molecules. The mechanism of cellular entry is still not understood in detail. In this project, the capacity of TAT and Antp peptides to enhance cellular uptake of liposomes was studied.

5.1 Labelling of peptide modified liposomes

Liposomes were labelled by adding a fluorescent dye, DiO or Texas-red, to the lipid mixture to be able to measure the concentration of lipids during the preparation and the different modification steps. Due to the high fluorescence quantum yield of these dyes, very low concentrations are sufficient to obtain highly fluorescent liposomes. However, Texas-red was inadequate to measure the concentration of liposomes, because it got adsorbed to the filters of the extruder. Other analytical methods were used in earlier work, such as radioactive labelling of liposomes with e.g. $[^3H]$-cholesterol [32] or labelling with other fluorescent dyes, which stably incorporated into the lipid bilayer. A third method to determine the lipid content used is an anorganic phosphate determination method, in which a complex of molybdenel is formed.

DiO labelling allowed the detection of cellular association of liposomes using standard fluorescent optical filters in flow cytometry or with the fluorescence microscope. Other fluorescent dyes or markers were used in the literature in the same way, such as rhodamine or fluorescein-dextran [16].

Instead of labelling liposomes, other authors labelled the peptides attached to the surface of liposomes with FITC [35].

5.2 Cellular association of Antp- or TAT- liposomes

The results obtained by flow cytometry and fluorescence microscopy, reflecting the measurements of cell fluorescence after a 1 h incubation with peptide modified liposomes do not allow to allude to "cellular uptake", because of the inherent methodological limitations of these two methods that do not permit to distinguish between cell surface binding and liposome translocation into cells. Therefore, as no detailed analysis of the intracellular localisation of liposomes by confocal microscopy after short (i.e. 1 h) incubation was performed in this study, the term "cellular association" is used to describe the interaction between cells and peptide modified liposomes.

5.2.1 Effect of peptide number per liposome on the association efficiency of liposomes

The results obtained demonstrate that after a 1 h incubation Antp and TAT peptide liposomes showed significantly improved cellular association with various cells provided that a sufficiently high number of peptides is attached to the surface of liposomes (about 110 Antp peptide molecules per liposome) (Figure 9 B). When liposomes modified with only a low number of peptide (about 36 Antp peptide molecules per liposome) were used, uptake into cells was reduced dramatically and could not be improved by increasing the amount of liposomes added to cells (Figure 10).

From a certain number of TAT peptide attached to the surface of liposomes, cellular association was not improved (Figure 9 A). This results from the tendency of TAT peptide liposomes to form aggregates.
The average number of Antp molecules attached per liposome was calculated from fluorescence data using trace labelling with FITC-Anpt and assuming that FITC labelled Antp is attached to the surface of liposomes similar to unlabelled Antp. The determination of the number of attached peptide molecules is based on the following assumptions: The calculation is made with the measured mean diameter and the lipid concentration of a liposome assuming an even distribution of maleimide groups between the inner and outer lipid monolayer. A further detailed analysis of the distribution of reactive groups on the surface of liposomes would be required to explain the low peptide binding efficiency obtained (24 to 39%) (Figure 11 A).

Another strategy to attach peptides to the surface of liposomes would be to first form micelles with maleimide containing phospholipids (e.g. DPPE-PEG-maleimide) and peptides and then to bind these micelles to liposomes.

In the literature, only TAT peptide binding has been quantified using radioactivity [16]. TAT peptide was labelled with $^{111}$In. The coupling strategy was also different using an amphiphilic PEG derivative, which links TAT to liposomes through stable carbamate bonds. With this method, approximately 500 TAT peptide molecules were coupled to a single liposome of 200 nm, corresponding to the reactive groups available on the liposome surface. Tseng and collaborators estimated the number of peptide molecules per liposome to be in the range of 5 to 200 peptide molecules, based only on calculations from the average number of phospholipid molecules per liposome and ignoring thus the coupling efficiency [35].

5.2.2 Mechanism of cellular association of Antp- or TAT- liposomes

As already shown, cell penetrating peptides significantly enhance cellular association of liposomes compared to control liposomes. However, cellular association of TAT modified liposomes and Antp derivatised liposomes vary and are cell-type dependent (Figure 12). In order to extend these data and to investigate the cellular entry mechanism of these peptides, cell association of peptide modified liposomes was studied using CHO cells deficient in surface expressed polysaccharides such as glycosaminoglycans. Recent studies showed that the uptake of Antp peptide conjugates or peptide modified liposomes is blocked by heparan sulfate glycosaminoglycans and that TAT-mediated uptake is inhibited by heparin and dextran sulfate [6].

The results obtained here (Figure 13) show very clearly that cellular association of peptide modified liposomes requires expression of glycosaminoglycans, because a significant reduction of cellular association was observed in CHO mutant cells deficient in glycosaminoglycan synthesis. These data confirm the possible role of cell-surface glycosaminoglycans in peptide-dependent cellular uptake, which was already proposed in other publications [36]-[39]. Direct ionic interactions between the strong positively charged peptides with negative charges of sulphated polysaccharides expressed on the cell surface may be the molecular basis of this interaction. However, these results do not exclude direct interaction with other negatively charged components of the cell membrane, such as phospholipids. It is also possible that several mechanisms are involved in peptide-mediated cellular uptake.

5.3 Cytotoxicity of antitumor drugs

The antitumor activity of cytotoxic drugs depends greatly on their metabolic profile in tumor cells. In this project, the cytotoxicity of three different lipophilic antitumor drugs incorporated into liposomes was compared in B16F1 and F9 tumor cells (Table 8). The cytotoxic effect of these drugs increases with the incubation time. 5-FdU-NOAC was the drug with the lowest efficiency (IC$_{50} = 3$ µM after 18 h exposure), whereas NOAC-ETC
and EPO B were 3- and 150-fold more active, respectively. EPO B, a new natural cytotoxic drug is by far the most active compound with an IC\textsubscript{50} in the nM range measured after a few hours of exposure. These results can be explained by the different metabolic profiles of these cytotoxic drugs. The advantage of the mechanism of NOAC-ETC over 5-FdU-NOAC is the very rapid phosphorylation of ETC by cytidine kinase to a cellular metabolite, which strongly inhibits RNA-synthesis. The metabolic profile of ETC and its analogs has a cytotoxic effect by inhibiting cell growth on slowly growing solid tumors, not only in G1-phase, but also during the entire cell cycle with the exception of M-phase [27].

Unlike 5-FdU-NOAC and NOAC-ETC, the mechanism of action of EPO B does not interfere with DNA or RNA synthesis, but on binding to tubulin, followed by stabilisation of microtubules, thus causing a mitotic block, similar to taxols. Another potential advantage of EPO B as an anticancer drug is its activity in multidrug resistant cells. No resistance has been observed yet. Recent publications have also shown the very potent cytotoxicity of EPO B in a variety of human cancer cell lines 24 h and 144 h after exposure, obtaining similar results as shown here [40]. However, \textit{in vivo} tests have shown that EPO B possesses a narrow therapeutic window and might be too toxic to become a clinically useful anticancer agent [41]. Therefore, the use of liposomes as an EPO-B delivery carrier, as done for the first time in this project, may help to protect host cells from the toxicity of EPO B. As illustrated in the following figures, \textit{Figure 14} and \textit{Figure 15}, EPO-B liposomes are very stable and as potent as EPO-B in solution. In summary, the use of Antp peptide to improve the cytotoxicity of liposomes loaded with these three cytotoxic drugs was studied. Although Antp peptide significantly improves cellular association of liposomes, the results of the cytotoxicity assays demonstrate the superiority of Antp modified liposomes tested on B16F1 cells over plain liposomes only when loaded with NOAC-ETC 2 and 4 h after exposure (\textit{Figure 16}). The beneficial effect of Antp peptide on cells occurs very rapidly during the first hours of incubation while the cytotoxic drug needs a certain time to exert a toxic effect on the tumor cells depending on their mechanism of action. After long incubation times the unmodified liposomes, which enter cells slowly, also inhibit cell growth at IC\textsubscript{50} values comparable to those with Antp-liposomes at short incubation times (see \textit{Table 8}).

Therefore, cytotoxicity tests are not adequate to demonstrate the beneficial effect of PTDs on liposome encapsulating anticancer drugs. In the literature, the improvement of uptake of liposomal doxorubicin by PTD modification has also not been reflected by increased cytotoxicity \textit{in vitro} and \textit{in vivo} [35].

5.4 DNA-loaded liposomes

The use of PTD-modified cationic liposomes for plasmid DNA delivery across cellular membranes would be an interesting strategy for the treatment of various diseases including cancer, AIDS and cystic fibrosis. To investigate this possibility, a plasmid encoding the Green Fluorescent Protein was incorporated into cationic liposomes and, after modification with the Antp peptide, liposomes were transfected into PAE cells. No expression of the plasmid could be detected. This reflects the complexity of this transfection system which was already observed during the preparation of peptide modified liposomes. The loading efficiency of liposomes with plasmid was very low when analysed with the Pico Green test, presumably because the plasmid was not pure enough. It is possible that the presence of the positively charged fluorescent dye Texas-red disturbed the separation of the non-encapsulated plasmid from the liposomes on a Biogel A 15m column. Control liposomes without plasmid were toxic on cells,
presumably because of their positive charge. A possible strategy to neutralise them would be to form a complex with negatively charged molecules. In future experiments, various steps of the transfection protocol should be optimised: the targeted delivery to cells, the release of the plasmid DNA, entry of the plasmid into cells and into the nucleus and finally the expression of the protein encoded by the plasmid DNA. The detection method of GFP is not the most sensitive. The detection of expression of others proteins such as β-galactosidase or chloramphenicol acetyltransferase would be also appropriate [42].

Even though this investigation would have required more time to obtain more reliable results, it clearly appears that the use of PTD liposomes opens a promising way for gene therapy. In the same perspective, Torchilin has presented important results which demonstrate the efficiency of TAT peptide-liposome-DNA complexes in both in vitro and in vivo transfection experiments [43], [44]. Nevertheless, slight differences appear between Torchilin’s experiments and those presented here, especially concerning the PTD peptide used, the transfected cells, the omission of treatment with DNAase to digest non-encapsulated plasmid, and the concentration of the cationic lipid (10 mol % DOTAP versus 20 mol % DDAB in this study).

Further investigation should take these differences into account in order to optimise the DNA delivery system.

5.5 Perspectives

In this project, we demonstrated a significant improvement in cellular uptake of liposomes by modification with PTDs peptides provided a sufficient number of peptide molecules is attached to the liposome surface. These experiments in vitro showed, that cellular association is peptide- and cell-type dependent. A prerequisite for translocation was the interaction with cell surface-expressed glycosaminoglycans for both Antp and TAT peptide modified liposomes. The results obtained in this work encourage the use of cell penetrating peptides attached to liposomes for improved drug delivery in therapeutic applications.

In vivo, these peptide modified liposomes will encounter various cells during their circulation through the bloodstream of an organism and this will complicate their efficient delivery of pharmaceuticals into a diseased tissue. Therefore, it might be interesting to modify liposomes with both target-specific antibodies and PTDs peptides with the goal to make them cell-type specific and capable of improving cellular uptake. The number of antibodies and PTDs peptides attached per liposome must be optimised to achieve the best antibody to peptide ratio necessary to target liposomes to specific tissues followed by improved efficiency of cellular uptake.

A second perspective concerns the application of peptide modified liposomes for drug delivery to dendritic cells. Due to the presence of PTDs peptides, liposomes are expected to be taken up rapidly by these cells after subcutaneous injection, avoiding a distribution into the whole body. This represents a promising approach for vaccine development.

Another perspective regarding EPO-B encapsulated into liposomes would be to test this system in vivo in mouse tumor models, in order to analyse the toxicity and the pharmacological profile of such liposomes. The liposomal formulation of this drug might represent a promising anticancer agent, which could be successfully used to cure tumors which are resistant to other antitumor drugs.
6 ABBREVIATIONS

5-FdU-NOAC 2'-deoxy-5-fluorouridylyl-N\textsuperscript{4}-octadecyl-1-β-
D-arabinofuranosycytosine

Antp Basic peptide of Antennapedia

B16F1 cells Mouse melanoma cells

CHO cells Chinese hamster ovary cells

CPP Cell Penetrating Peptide

DC cells Dendritic cells

DDAB Didodecyldimethylammonium bromide

DiO 3,3'-dioctadecyloxacarbocyanine perchlorate

DPPE 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine

EPO-B Epothilone B

ETC 1-(3-C-ethinyl-β-D-ribopentafuranosyl)cytosine or
3'-C-ethinylcytidin

F9 cells Murine teratocarcinoma cells

FITC Fluorescein isothiocyanate isomer I

GFP Green fluorescent protein

IC\textsubscript{50} fifty percent growth-inhibitory concentration

Mal Maleimide groups

MW Molecular weight

MLV Multi Lamellar Vesicles

N Avogadro's number

NOAC-ETC N\textsuperscript{4}-octadecyl-1-β-D-arabinofuranoslyctosine-
(5' -5')-3'-C-ethinylcytidine

OPPC 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine

PAE cells Porcine aortic endothelium cells

PB Physiologic phosphate buffer, 67mM, pH 7.4

PBS Phosphate buffered saline

PE-PEG-Mal 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-
N-[maleimide(polyethylene glycol)2000]

PTD Protein Transduction Domain

RT Room temperature

SPC Soy phosphatidylcholine

Sulfo-SMCC Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-
carboxylate

SUV Small Unilamellar Vesicles

TAT Basic peptide of transactivator of transcription (TAT) protein

µM micromolar [µmol/l]

nM nanomolar [nmol/l]

W38 cells Fibroblasts

WST-1 4-(3-(4-iodophenyl)-2-(4-nitropheryl)-2H-5 tetrazol)
1,2-benzol disulfonate
7 REFERENCES

[34] American Type Culture Collection (ATCC), Home Page.


8 APPENDIX

Appendix I

Figure 17: Standard curve of 5-FdU-NOAC, measured by UV-spectrophotometer at 270 nm wavelength.

Figure 18: Standard curve of NOAC-ETC, measured by UV-spectrophotometer at 273 nm wavelength.

Appendix II

Table 10: Overview of the different liposome types prepared for cellular association experiments.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic components</th>
<th>Cysteine or peptide</th>
<th>Mean diameter [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lipo-A</td>
<td>SPC</td>
<td>-</td>
<td>84 ± 30²</td>
</tr>
<tr>
<td>Control lipo-B</td>
<td>Cysteine</td>
<td></td>
<td>90 ± 40²</td>
</tr>
<tr>
<td>Antp-lipo-C</td>
<td>Anp 1:1.2</td>
<td></td>
<td>66 ± 35²</td>
</tr>
<tr>
<td>Antp-lipo-D</td>
<td>Anp 1:0.6</td>
<td></td>
<td>62 ± 34²</td>
</tr>
<tr>
<td>Antp-lipo-E</td>
<td>Anp 1:0.24</td>
<td></td>
<td>55 ± 28²</td>
</tr>
<tr>
<td>Antp-lipo-F</td>
<td>Anp 1:0.12</td>
<td></td>
<td>53 ±28²</td>
</tr>
<tr>
<td>TAT-lipo-G</td>
<td>TAT 1:1.2</td>
<td></td>
<td>52 (86%), 184 (14%)³</td>
</tr>
<tr>
<td>TAT-lipo-H</td>
<td>TAT 1:0.6</td>
<td></td>
<td>67 (90%), 235 (10%)³</td>
</tr>
<tr>
<td>TAT-lipo-I</td>
<td>TAT 1:0.24</td>
<td></td>
<td>102 (100%)³</td>
</tr>
<tr>
<td>TAT-lipo-J</td>
<td>TAT 1:0.12</td>
<td></td>
<td>93 (100%)³</td>
</tr>
</tbody>
</table>

¹ = corresponding to a molar ratio of maleimide groups to peptide.
² = Gaussian distribution
³ = Nicomp distribution
Table 11: Overview of different liposome types prepared for the determination of the number of Antp molecules per liposome.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic components</th>
<th>Antp&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mean diameter [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lipo-K</td>
<td>SPC</td>
<td>-</td>
<td>76 ± 35&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antp-lipo-L</td>
<td>Cholesterol α-tocopherol DPPE, Texas-red</td>
<td>Antp 1:1.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Antp-lipo-M</td>
<td>Antp 1:0.6</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Antp-lipo-N</td>
<td>Antp 1:0.24</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

n.d., not determined  
<sup>1</sup> = corresponding to a molar ratio of maleimide groups to peptide.  
<sup>2</sup> = Gaussian distribution

Table 12: Overview of liposomes prepared for cytotoxicity tests.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic components</th>
<th>Cytotoxic drug</th>
<th>Antp&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mean diameter [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>SPC Cholesterol α-tocopherol</td>
<td>5-FdU-NOAC</td>
<td>-</td>
<td>50 ± 22&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>P</td>
<td>Antp 1:1.2</td>
<td>35 (82%), 105 (18%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>NOAC-ETC</td>
<td>-</td>
<td>78 ± 32&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>Antp 1:1.2</td>
<td>90 (88%), 178 (12%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>EPO-B</td>
<td>-</td>
<td>87 ± 31&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>Antp 1:1.2</td>
<td>100 (83%), 156 (17%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> = corresponding to a molar ratio of maleimide groups to peptide.  
<sup>2</sup> = Gaussian distribution  
<sup>3</sup> = Nicomp distribution

Table 13: Overview of liposomes prepared for the transfection.

<table>
<thead>
<tr>
<th>Name of liposomes</th>
<th>Basic lipid composition</th>
<th>Plasmid</th>
<th>Antp</th>
<th>Mean diameter [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control U</td>
<td>OPPC DDAB Texas-red</td>
<td>-</td>
<td>+</td>
<td>61 ± 26&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>50 (85%), 165 (15%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>W</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>63 (82%), 190 (18%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>89 (90%), 308 (10%)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> = Gaussian distribution  
<sup>2</sup> = Nicomp distribution
## Appendix III

### Table 14: Yields of liposomes after preparation, modification and attachment.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>76 ± 35</td>
<td>1.3 * 10^15</td>
<td>1.1 * 10^15</td>
<td>1 * 10^15</td>
</tr>
</tbody>
</table>

---

1 = a * d^b * c  
2 = Yield calculation = [Initial] * 0.95 * 0.90, based on yield of liposome preparation (95%) and yield of liposome modification (90%).
3 = Yield calculation = [Initial] * 0.95 * 0.90 * 0.90, based on yield of liposome preparation (95%), yield of liposome modification (90%) and yield of liposome attachment (90%).

### Table 15: Calculations of reactive groups on liposomes.

<table>
<thead>
<tr>
<th>Mal:Antp</th>
<th>Amino groups/ml^4</th>
<th>Amino groups/lipo^5</th>
<th>Yield after sulfo-SMCC maleimide/ml^6</th>
<th>Yield after sulfo-SMCC maleimide/lipo^7</th>
<th>Initial Antp peptide (100%) [mg/ml]^8</th>
<th>Antp peptide attached [mg/ml]^9</th>
<th>Yield after Antp attachment [Antp/ml]^10</th>
<th>Yield after Antp attachment [Antp/lipo]^11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1.20</td>
<td>10^18</td>
<td>770</td>
<td>5.1 * 10^17</td>
<td>466</td>
<td>2.7</td>
<td>0.64 ± 0.01</td>
<td>1 * 10^17</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>1:0.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.35</td>
<td>0.40 ± 0.01</td>
<td>6 * 10^16</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>1:0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.54</td>
<td>0.21 ± 0.006</td>
<td>3.3 * 10^16</td>
<td>36 ± 1</td>
</tr>
</tbody>
</table>

---

4 = [mol(DPPE)/ml * N] / 2  
5 = (4) / (1)  
6 = [NH_2] * 0.95 * 0.90 * 0.6  
7 = (6) / (2), corresponding to 60% efficiency [32]  
8 = ((6) / N) * MW (Antp) * 1000  
9 = After attachment, measured by FITC fluorescence  
10 = ((9) / MW (Antp) / 1000 ) * N  
11 = (10) / (3)