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„Design of liposomes containing Amphotericin B and 2-propylquinoline and *in vivo* evaluation against *Leishmania donovani*“

verfasst von / submitted by

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“Wherever the art of medicine is loved, there is also
a love of humanity.”
(Hippocrates)

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Abbreviations

2-PQ	2-propylquinoline/2-Propylchinolin
Abs	Absorption
AmB	Amphotericin B
Balb/c	Albino, laboratory-bred strain of the House Mouse
CC_{50}	Concentration that leads to cell death in 50% of the cells
$CHCl_3$	Chloroform
DDS	Drug delivery systems
DMEM	Dulbecco's Modified Eagle Medium
EC_{50}	Concentration that gives half-maximal response
EE	Encapsulation efficiency
EPC	Egg Phosphatidylcholine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Concentration that inhibits 50 % of the parasitic growth
IFN-Y	Interferon gamma
IL-4	Interleukin 4
IL-10	Interleukin 10
LUV	Large unilamellar vesicles
LDU	*Leishmania donovani* units
LV9	*Leishmania donovani* strain 9
MeOH	Methanol

mM	Millimolar
mmol	Millimole
nm	Nanometer
OLV	Oligolamellar vesicles
PDI	Poly dispersity index
VL	Visceral leishmaniasis
SUV	Small unilamellar vesicles
SI	Selectivity index
MUV	Multilamellar vesicles
PCR	Polymerase chain reaction
RAW 264.7	Macrophage-like cell line derived from BALB/c mice
RPM	Revolutions per minute
ULV	Unilamellar vesicles
UV/Vis	Ultraviolet/visible spectroscopy
WHO	World Health Organization

Abstract

Visceral leishmaniasis is a neglected tropical disease caused by the protozoan agent *Leishmania donovani* and mostly leads to death if left untreated. The increasing spread of resistance towards commonly used drugs leads to a constant need of better therapies. The alkaloid 2-propylquinoline and the antibiotic Amphotericin B are effective substances against visceral leishmaniasis. In order to optimize their pharmacokinetics and toxicological profile respectively, a liposomal formulation containing these two antileishmanials was prepared and tested *in vitro* on axenic and intra-macrophagic amastigotes and *in vivo* in female BALB/c mice. The *in vivo* results revealed a synergistic effect between the two substances while *in vitro* no synergy was found. With a reduction of parasitic burden by more than 80% liposomes can be seen as an optimal drug delivery system for these two agents in the treatment of leishmaniasis.

Die viszerale Leishmaniose ist eine durch den Protozoen Leishmania donovani verursachte Erkrankung, die unbehandelt meist einen tödlichen Verlauf nimmt. Durch steigende Resistenzbildung der Erreger ist ein ständiger Bedarf an neuen Behandlungen und an der Verbesserung bisheriger Therapien gegeben. Das Alkaloid 2-Propylchinolin und das Antibiotikum Amphotericin B sind effiziente Wirkstoffe gegen die Leishmaniose. Um deren pharmakologisches Profil und die Pharmakokinetik zu optimieren wurden Liposomen hergestellt und in vitro und in vivo in weiblichen BALB/c Mäusen auf einen synergistischen Effekt getestet. In vivo konnte ein synergistischer Effekt zwischen den beiden Wirkstoffen gezeigt werden, allerdings wurde dieser in den in vitro Tests nicht bestätigt. Da alle Formulierungen eine Reduktion der Parasitenlast von über 80% erzielten, stellen sie einen interessanten Ansatzpunkt für weitere Forschung auf diesem Gebiet dar.

Introduction

Neglected tropical diseases

Affecting the poorest populations with the lowest living standards, 17 illnesses are known under the term neglected tropical diseases. Besides being almost completely ignored by research institutions and major companies, these illnesses have various issues in common: they do not spread out into the world, as it has been the case for HIV, but stay endemic in poor countries and thus only get minor attention in global public health programs. This invisibility is problematic even within the affected countries, since it mainly concerns the poorest populations who are often isolated and not taken into account by the authorities or not able to take part in political and administrative decisions. Many of these diseases lead to unaesthetic lesions, causing stigmatization and discrimination in return. Mainly young women suffer badly from this consequence, as it makes finding a husband difficult and leads to isolation. The economic impact of these diseases is considerable, due to limitations of work power and immense costs of treatment. [10]

Leishmaniasis

One of the illnesses mentioned above is the parasitic disease leishmaniasis. It is caused by more than 20 protozoan agents from the *Leishmania* species and exists in three different forms: cutaneous leishmaniasis is the most benign form and leads to lesions that can be disabling when occurring multiply or lead to lasting scars. The mucocutaneous form causes mutilating lesions through partial or total destruction of mucous membranes of the nose, mouth and throat. The most severe form is the visceral leishmaniasis (VL), also known as Kala Azar and caused by *Leishmania donovani*. It is fatal when left untreated. [25]

Distribution

The WHO estimates the amount of people at risk of *Leishmania* to be 350 million in 88 countries. For visceral leishmaniasis alone, the number of new infections per year is estimated to be 200 000-400 000 of which more than 90% occur in Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. [25] Still, since numerous infections stay undiagnosed,

misdiagnosed or unreported and leishmaniasis isn't always notifiable, the actual number of cases is expected to be considerably higher. Also in Southern Europe cases of visceral leishmaniasis were reported: especially the transmission of *L. infantum* via dogs to humans causes problems. [33]

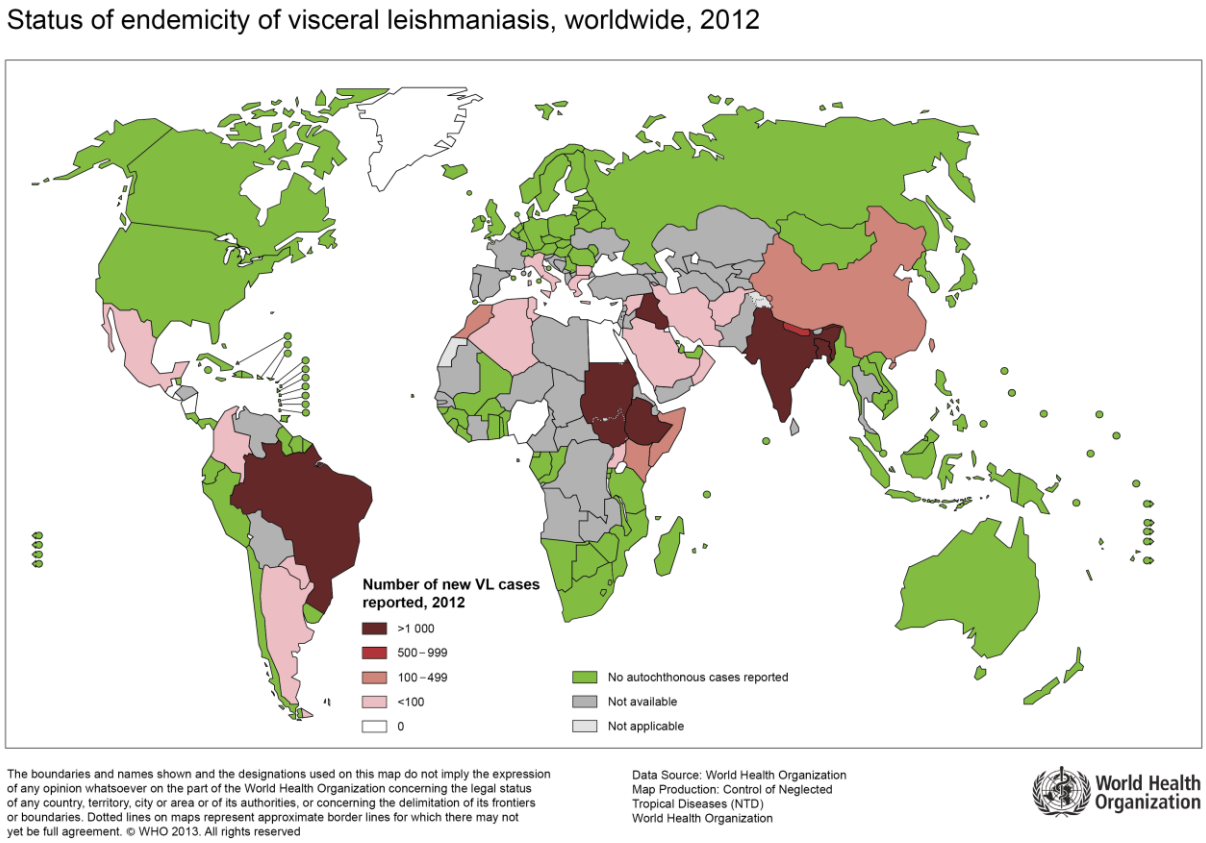

Figure 1: Distribution of visceral leishmaniasis worldwide
Source: http://www.who.int/leishmaniasis/en/

Development cycle of *Leishmania donovani*

The transmission of the protozoan agents takes place in their promastigote form (15-20 µm) via infected female sand flies. Transmission can be both anthroponotic and zoonotic. Having entered the human blood stream, the promastigotes adhere to macrophages and are phagocytised. Around 12-14 hours later they transform into their amastigote form and reproduce via cell division. Afterwards, they are set free from the cells and adhere to new macrophages. During another blood meal they get newly ingested by sand flies and return in their promastigote forms. [5] The stages of the development cycle are shown on the next page in Figure 2.

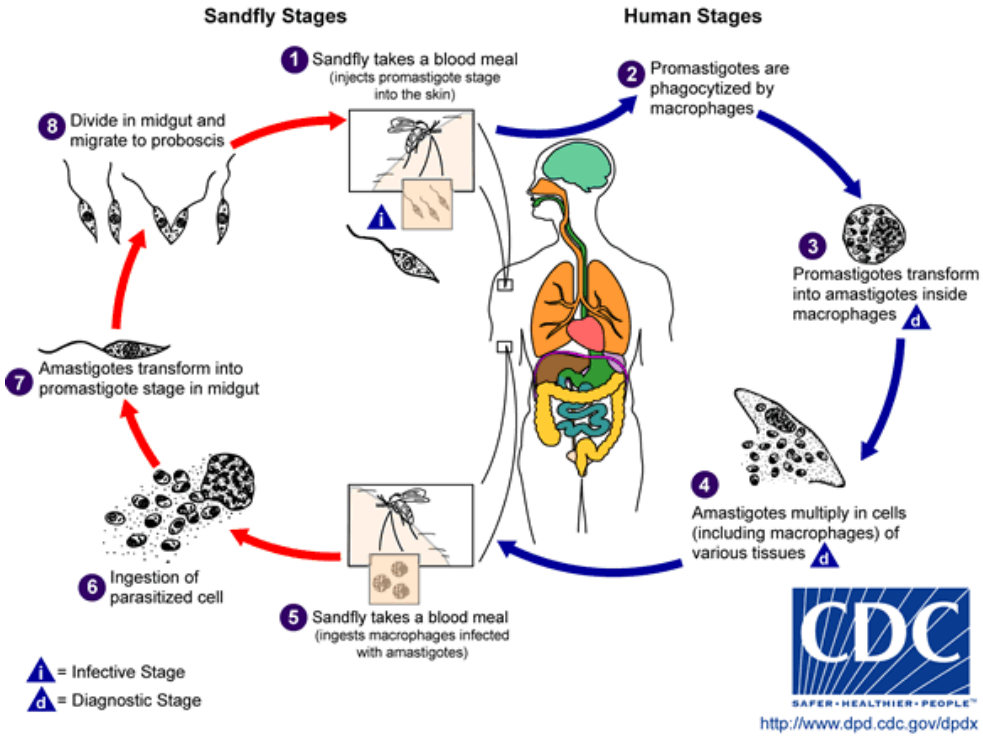

Figure 2: Development cycle of *Leishmania*
Source: http://www.cdc.gov/parasites/leishmaniasis/biology.html

Pathophysiology

During the infection, the T lymphocytes T_H1 and T_H2 are activated. T_H1 cells lead to the production of IFN-γ, which activates macrophages and thus has a protective function. At the same time, the formation of T_H2 cells leads to the production of huge quantities of IL-4 and IL-10. These mediators on the other hand inhibit the activation of macrophages and thus limit or even inhibit the elimination of *Leishmania* from the blood stream.

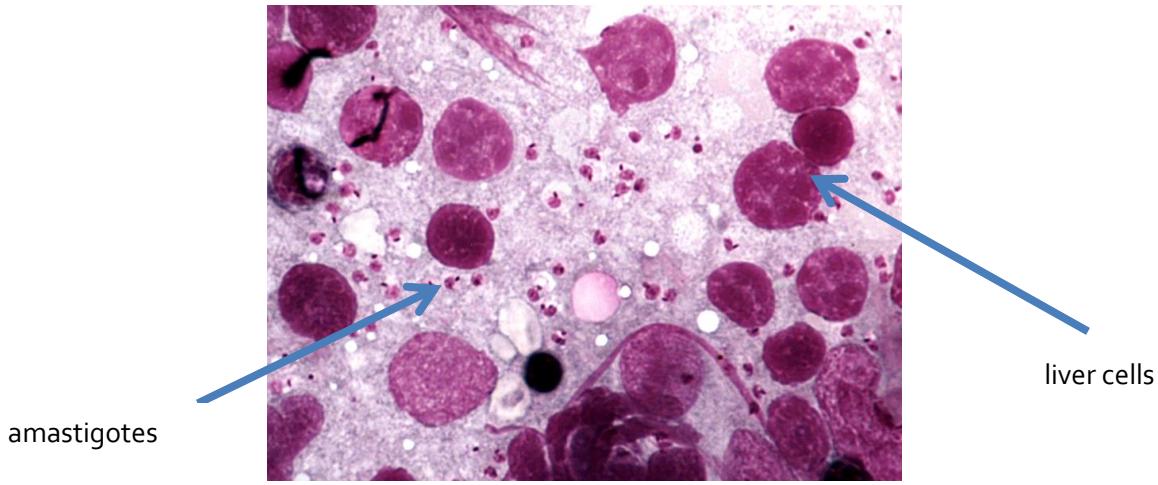

Figure 3: Amastigotes of *L. donovani* from liver tissue
Source: http://micros-vita.blogspot.co.at/2006/11/leishmania-donovani.html

The symptoms of Kala Azar involve irregular bouts of fever, enlargement of spleen and liver, anaemia, weight loss, thrombocytopenia, leukopenia with neutropenia and extreme paleness. [5]

Treatment today – problems

The drugs used so far show several disadvantages: toxicity, high costs and lack of efficiency limit their use, particularly in countries where the risk for leishmaniasis is the highest. Antimony derivatives had been used over centuries until, due to decreasing sensitivity of *Leishmania* species, second line drugs such as Miltefosine, Amphotericin B, Pentamidine and Paromomycin were introduced. Miltefosine has the advantage that it can be administrated orally, but it possesses side effects such as gastrointestinal toxicity and teratogenicity. Because of the reproductive toxicity, its use in women in the child bearing age group also raises costs for contraception. Liposomal AmB has shown excellent efficacy and considerable reduction of the side effects, however due to its high costs it remains hardly available in developing countries. To make matter worse, indication that resistance could develop was reported for all of the treatments mentioned above. [15]

Development of resistance

Similar to bacterial, viral and fungal infections, resistance is a widespread problem in the treatment of parasitic diseases. The parasites develop mutants that are resistant to the agent. These mutants cannot be eradicated by the agent and will maintain the infection in spite of therapy. Also, through a variety of mechanisms such as decreased uptake, increased export and inactivation by metabolism or sequestration, the parasite can manage to lower the drug level at the target site of action. Another way for a parasite to become resistant to drugs is decreased target affinity or complete loss of the target. [4] The development of resistance can be due to administration of sub therapeutic doses, incomplete duration of treatment and use of substandard drugs. [12]

The advantages of combination therapy

From experience with handling resistance in other diseases it is known that combination therapy gives good results in order to prevent the emergence of this problem. The likelihood of developing resistance to two compounds with different targets is very low. Furthermore, the activity can be increased by using compounds with a synergistic effect. Using more than one active substance allows to lower the required doses and therefore leads to

a reduction of costs and side effects. These advantages have been well studied in the treatment of Malaria, from which for instance it is known that spread and development of drug resistance have been decreased through combination of Artemisin with other drugs. [4,8] A common approach is the combination of strong antileishmanials with a weaker substance. [4]

Amphotericin B (AmB)

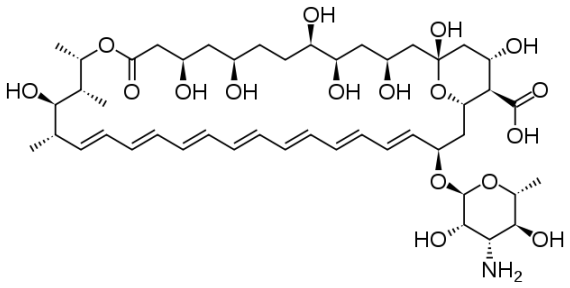

Figure 4: Chemical structure of Amphotericin B
Source: http://pipeline.corante.com

Amphotericin B belongs to the group of polyene antibiotics. It is a large amphiphilic molecule originated from *Streptomyces nodosus* with a broad antibiotic spectrum. Its activity is due to complexation with ergosterol in the parasitic membrane which leads to perforation. [1] Because of its amphiphilic nature, AmB tends to aggregate in aqueous media, where it is not soluble at all. This is thought to be the reason for the toxicity of AmB. [22] The most common adverse effect occurring during AmB treatment is nephrotoxicity. Other main side effects are hypokalemia, hypomagnesemia and bone marrow suppression. [23]

2–propylquinoline (2-PQ)

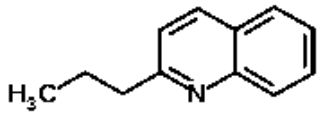

Figure 5: Chemical structure of 2-propylquinoline
Source: www.chemnet.com

Propylquinoline derivatives are new substances of hope in treating leishmaniasis. They are found in *Galipea longiflora*, a Bolivian plant from the Rutaceae family. In Bolivia, *Galipea* has been used traditionally against cutaneous leishmaniasis by the native Indians. Former studies on its *in vivo* activity have confirmed the antileishmanial potential. The active compounds are isolated from root bark, stem bark and leaves, and were found to be twelve 2-substituted quinoline alkaloids. Fournet et al [21] showed in *in vivo* experiments that 2-PQ and Chinamine D gave particularly hopeful results in the reduction of ulcerations caused by *Leishmania amazonensis*. Experiments also revealed remarkable bioavailabilities of the alkaloids while no apparent signs of drug toxicity, weight loss or hair loss were induced. [14] The drawbacks of 2-PQ are its very short *in vivo* half-life and its lipophilic nature that makes it difficult to prepare an intravenous formulation.

Liposomes

Liposomes are drug delivery systems that consist of one or more bilayers of amphiphilic molecules and are formed by self-assembly of lipids in aqueous media. Due to surface tension they result in round vesicles whose inside forms an aqueous space. They allow for encapsulation of amphiphilic, hydrophilic, and lipophilic molecules, of which the lipophilic and amphiphilic ones are located in the lipid bilayer whereas the aqueous space contains hydrophilic substances. [30]

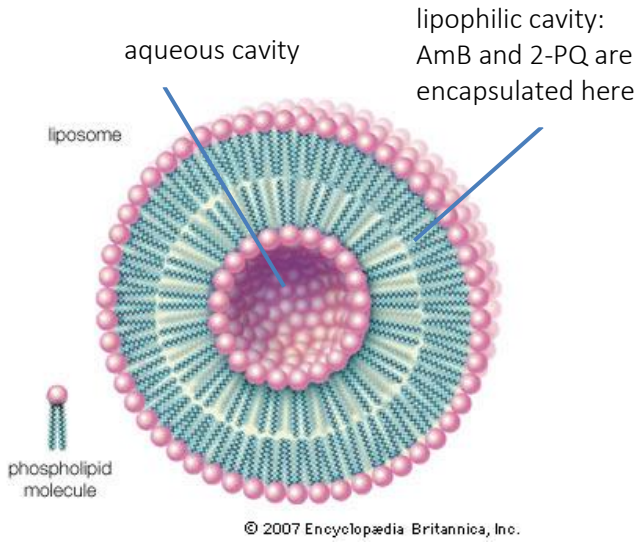

Figure 6: Structure of liposomes
Source: http://www.pharmainfo.net/nandini/blog/introduction-liposomes

Through their interactions with the cell membrane and uptake by endocytosis, liposomes play an important role as a carrier for all kinds of agents into cells. [18] They are designed to decrease side effects and to improve the drug's pharmacokinetics and biodistribution. [7] In the treatment of leishmaniasis with AmB they have various benefits: they carry AmB in its solubilized form and thus inhibit the formation of toxic aggregates. Furthermore, they naturally target cells of the mononuclear phagocytic system (MPS), particularly macrophages. [27] Liposomes are engulfed via the same mechanism as the parasites. Through targeting the reticuloendothelial system they reach high concentration levels in liver and spleen, whereas in the kidneys only low concentrations are achieved.

There are three lipid based formulations containing AmB on the market: AmBisome®, Abelcet®, and Amphotec®. Thereof, AmBisome® is the only true liposomal formulation. It almost completely erases the toxic side effects of Amphotericin B and is indicated as first line therapy in some developed countries. [31]

Figure 7: Lipid based formulations containing AmB: Ablecet®, Ambisome® and Amphotec®

Sources:
https://ssl.adam.com/ctent.aspx?productId=50&pid=50&gid=1125&site=welldynerx.adam.com&login=well1815
http://www.kranticancer.com/antifungal-medicines.html
http://www.drugs.com/pro/amphotec.html

Depending on the method of construction used, the liposomes can result in small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV) or oligolamellar vesicles (OLV).

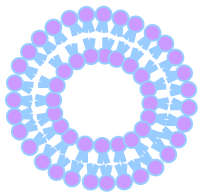	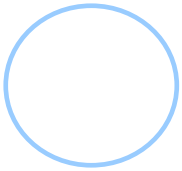		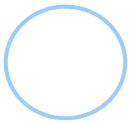
Name	LUV	MLV/OLV	SUV
Size (nm)	80-1 000	100-4 000	20-80

Figure 8: Classification of liposomes

Various types of lipids and other amphiphiles are used for the preparation of liposomes. The main component of the bilayer is usually a neutral phospholipid. Thereof, egg phosphatidylcholine (EPC), which is a neutral lipid derived from hen egg yolk, is the most commonly used lipid due to its availability in large quantities, high purity and bioadaptability. [18] However, charged lipids are sometimes also included; AmBisome®, for example, contains distearoylphosphatidylglycerol. Through addition of cholesterol, the *in vitro* and *in vivo* stability of the liposomes increases, whereas the permeability of the phospholipid bilayer can be decreased. [7, 17]

Objective

The research carried out in this project is part of a collaboration between the Institut Galien Paris-Sud, Faculty of Pharmacy, Université Paris-Sud 92290 Chatenay Malabry and the Department of Biotechnology of the Indian Institute of Technology Madras, Chennai 600 036, India.

The project titled “Novel nanotechnological approaches for treatment of leishmaniasis using 2-propylquinoline” aims to design nanoformulations that allow an intravenous administration of 2-propylquinoline. The objective of the following study was to develop a formulation of liposomes containing a combination of Amphotericin B and 2-propylquinoline and to examine *in vivo* and *in vitro* whether they have a synergistic effect.

Experimental Part

Methods

Material

L-α-Phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids. AmB, Cholesterol and HEPES were purchased from Sigma. 2-PQ was synthesized by Dr. K. Balaraman.

Preparation of Liposomes

Figure 9 shows the principal steps for the preparation of liposomes via high pressure homogenization method.

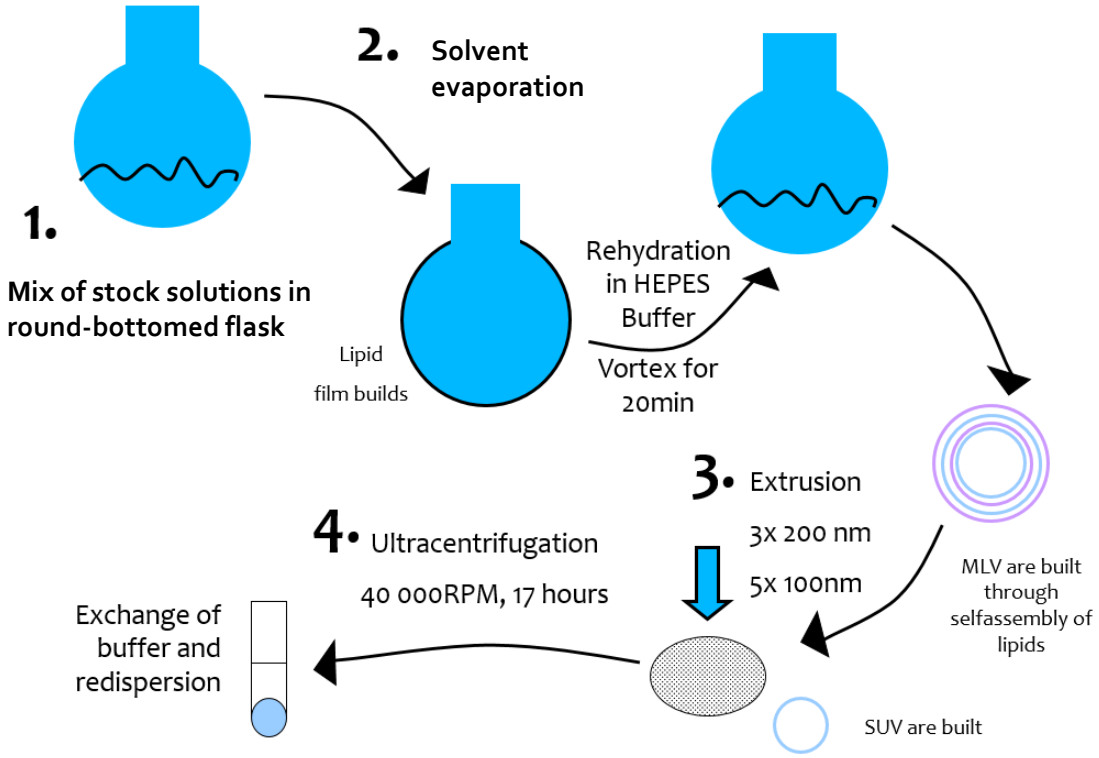

Figure 9: Preparation of liposomes

1. Preparation of stock solutions

Stock solutions of cholesterol and 2-PQ in chloroform were prepared. AmB was solubilized in MeOH. In order to dissolve it completely it was sonicated.

AmB	0.5 mg/ml
2-PQ	1 mg/ml
Cholesterol	10 mg/ml
EPC	25 mg/ml

Table 1: Concentrations of stock solutions

From previous liposomal formulations that contained 2-PQ alone, the optimal ratio of egg phosphathidyl choline, cholesterol and 2-PQ was known to be 80/10/10. This ratio has been used for all formulations. The amount of AmB added was compensated by reduction of EPC.

2. Solvent evaporation

The stock solutions were mixed in a round-bottomed flask and afterwards sonicated in order to achieve the best solubility of AmB. For a thorough homogenization of the compounds, the flask was rotated for 10 minutes at the rotary evaporator at room temperature. Afterwards, the solvents were removed by pressure reduction at 35°C. After the evaporation, the lipids were found as a thin film on the flask. This film is rehydrated in HEPES Buffer while vortexing for 20 minutes.

3. Homogenization and size reduction

The reduction of size and dispersion was facilitated via extrusion. In this method, the liposomes are extruded under pressure through a membrane filter. This was done three times through a polycarbonate membrane with a pore size of 200 nm and afterwards five times through 100 nm.

4. Purification

Afterwards, the liposomes were centrifuged at 9 000 g for 10 minutes at 25°C to remove large-sized aggregates and in the next step ultracentrifuged over night at 4°C and 150 000 g for 17 hours. After ultracentrifugation over night the liposomes were found as a pellet at

the bottom of the tube. As the buffer also contained active substance, it was removed and replaced by fresh buffer in which the pellet was dispersed again. The liposomes were kept in a glass flask wrapped in aluminium foil and parafilm at 4°C.

Characterization

Determination of particle size and poly dispersity index (PDI)

The particle size and PDI were determined by quasi-elastic light scattering method in a Malvern Zetasizer.

Encapsulation Efficiency

The quantification of encapsulated 2-PQ and AmB was achieved by UV/VIS spectroscopy. For 2-PQ, a calibration curve with concentrations between 0.003125 and 0.05 mg/ml in ethanol was made. The absorbance was recorded at λ_{max}=315 nm against ethanol as blank. For AmB, dilutions between 0.05 and 0.003 µg/ml in methanol were prepared and measured at λ_{max}=405 nm against methanol.

Quantification of phospholipids

Size and PDI are of importance to conclude whether the liposomes were prepared correctly. Another important determinant is the amount of phospholipids that could be retained in the liposomal preparation. The amount of phospholipids was evaluated by a phospholipid assay purchased from Biolabo (France). The assay is based on an enzymatic reaction that leads to a color change from colorless to pink in presence of phosphatidylcholine. During this enzymatic reaction, phosphatidylcholine is metabolized and oxidized in three steps to quinone imine and then quantified by UV spectroscopy at λ_{max}=500 nm. The measured absorption of the sample is compared with the absorption of a standard reagent.

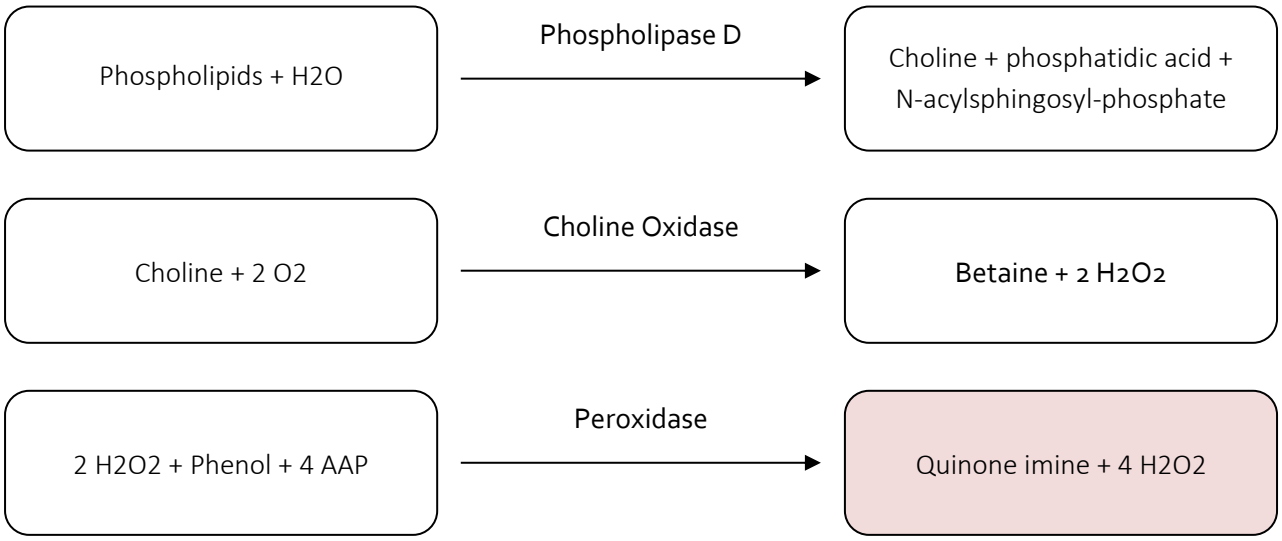

Figure 10: Enzymatic reaction of Biolabo phospholipid assay

The formula for the calculation of the concentration of lipids is

$$\text{Result} = \frac{\text{Abs (Assay)}}{\text{Abs (Standard)}} \times \text{Standard concentration (3g/l)}$$

In vivo experiment

In order to conclude whether there is a synergistic effect in a combination of drugs, doses of about a fourth of their EC 50 were administered.

Compound/formulation	Regimen	Number of mice
Liposomal 2PQ	3 mg/kgx5 days	8
	1,5 mg/kgx5 days	8
	0,75 mg/kgx5 days	8
Liposomal 2-PQ+AmB	0,75 mg 2PQ + 0,006mg AmB/kg x 5	8
	0,37 mg 2PQ + 0,003mg AmB/kg x 5	8
AmBisome ®	1 mg AB/kg x 5 days	8
	0,25 mg AB/kg x 5 days	8
Blank liposomes	Same suspension	8
Control (vehicle)	0,2ml x 5 days	8

Table 2*: In vivo* study design

Female BALB/c mice weighing about 20 g were selected for this study. The mice were infected with the *L. donovani* strain LV9. The liposomes were sterilized via sterile filtration with a pore diameter of 100 nm under laminar air flow before application. One week after infection, the treatment with the liposomal formulations was started. The doses administered are shown in Table 2.

The mice were sacrificed after the treatment. Liver and spleen were weighed. Swabs of liver were taken and fixed in MeOH on microscope slides for three minutes. Afterwards, the amastigotes were colored with Giesma stain for 20 minutes and then rinsed with water and dried. The liver cells and amastigotes were counted under a microscope: ten different fields of each slide were counted (at least 100 liver cells for each field) to determine the number of parasites per liver cell. The number of amastigotes in the liver was calculated by Stauber's Formula (Stauber, Franchino and Grun, 1958) [34]:

$$\textit{L. donovani}\ \text{units (LDU)} = \frac{\text{number of amastigotes per organ per nucleus}}{\text{1000 liver cells x weight of liver (mg) x } (2\text{x}10^{50\,000})}$$

Parasite suppression was calculated according to the following formula:

$$\% \text{ parasite suppression} = \frac{\text{mean liver amastigote counts of treated mice}}{\text{mean liver amastigote counts of untreated mice}} \times 100$$

The results were analyzed by the statistical test of Kruskal Wallis ($p<0.000001$) followed by an analysis to determine the significant differences ($P<0.05$). The statistical tool used was MedCalc 12.7.7.0.

In vitro experiment

The liposomal preparations were tested *in vitro* in terms of their inhibiting concentration towards intra-macrophagic and axenic amastigotes and their toxicity towards macrophages. Each evaluation was done in triplicates. The *in vitro* evaluations were conducted according to the protocol in Table 3.

	Anexic Amastigotes	Intra-macrophagic Amastigotes	Cytotoxicity
Cell line	*L. donovani* promastigotes LV9	Uninfected murine macrophages RAW 264.7	Uninfected murine macrophages RAW 264.7
Incubation	24 hours, 37°C, 5% CO_2, dark		
Centrifugation	2000 g for 10 minutes at 20°C	1200 g for 10 minutes at 20°C	1200 g for 10 minutes at 20°C
Counting of cells		via hemacytometer	via hemacytometer
Medium	M199 with 200µM $CaCl_2$ and 200µM MgCl2	Dulbecco's Modified Eagle's Medium (DMEM) with 10% SVF and 1% Penicillin/Streptomycin	Dulbecco's Modified Eagle's Medium (DMEM) with 10% SVF and 1% Penicillin/Streptomycin
Incubation	24 hours at 37°C, 5% CO_2 atmosphere, in darkness		
Distribution on 96-well microtiter plates	Distribution of dilution series of the liposomal preparations and miltefosine as a reference substance	Distribution of macrophages using DMEM as medium	Distribution of macrophages using DMEM as medium
Incubation		24 hours at 37°C, 5% CO_2 atmosphere, in darkness	24 hours at 37°C, 5% CO_2 atmosphere, in darkness
Infection/Addition of test substance	Parasitic suspension was added to the vials in order to achieve a concentration of 2x105 parasites in 200µl	Infection with $3{,}2x10^5$ amastigotes in order to obtain a ratio parasites/macrophages of 16:1.	Dilution series of the liposomal preparations were added to the macrophages
Incubation	3 days at 37°C, 5% CO_2 atmosphere, in darkness	24h at 37°C, 5% CO_2 atmosphere, in darkness	72h at 37°C, 5% CO_2 atmosphere, in darkness
Addition of test substance		Addition of dilution series of liposomal preparations	
Incubation		60h at 37°C, 5% CO_2 atmosphere in darkness	
Chemical reaction	Colorimetric test: Resazurine test 20µL Resazurin (c= 450µM) added to each vial and afterwards incubation at 37 °C in the dark with 5% CO_2 for 4 hours	Essay was stopped by addition of 100µL of direct PCR Lysis Reagents Cat # 302-C VIAGEN®, followed by a series of freezing at 80°C/ defreezing in order to dissolve the cells. To further dissolve the cells 5µL Proteinase K (c=1mg/ml) was added and incubated for 5 hours at 55°C	Colorimetric test: Resazurine test 20µL Resazurine (c=450µM) added to each vial and afterwards incubated at 37 °C in the dark with 5% CO_2 for 4 hours
Incubation	24 hours at 37°C, 5% CO_2 in darkness		
Evaluation	Analysis via UV/Vis (λ_{max}=595 and λ_{max}=570 nm).Determination of IC_{50} by measuring the difference at λ_{max}=570 and λ_{max}=590 nm	Evaluation by SYBR® I Green method (Invitrogen, France; Audisio et *al.*, 2012). The plates are afterwards transferred to a transparent film and fluorescence is measured via Mastercycler® and IC_{50} is calculated.	Analysis of difference in wavelengths (570nm-595nm) for calculation of CC_{50}

Table 3: Methods for *in vitro* tests

Results

The liposomes were prepared as explained in the method chapter. The preparations were analyzed via UV/VIS spectroscopy after a dilution of 1+100 in MeOH. Calibration curves for both active substances were made.

Concentration	Absorption at λ=315 nm
0	0
0,05	1,593
0,0375	1,156
0,025	0,804
0,01875	0,6
0,0125	0,41
0,00937	0,306
0,00625	0,209
0,00468	0,153
0,003125	0,106
mg/ml	nm

Table 4: Concentrations of 2-PQ for calibration curve

Amount of 2-PQ =

Absorption/31,687

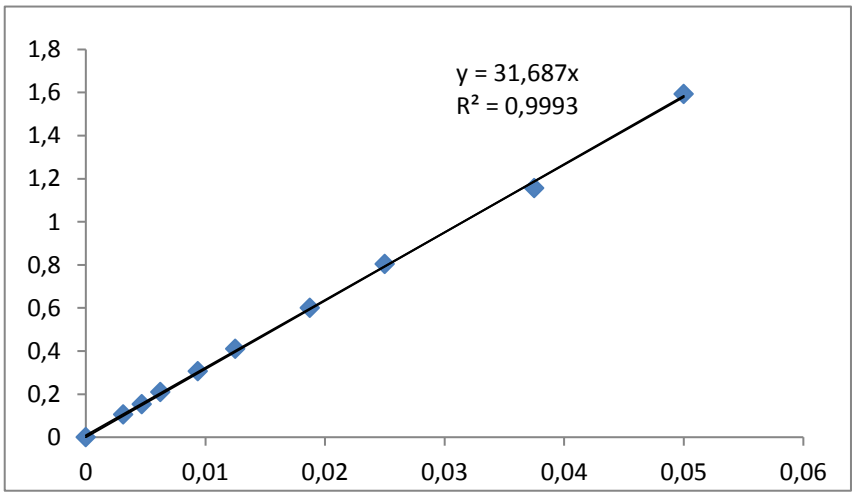

Figure 11: Linear regression curve of 2-PQ in ethanol at λ_{max}=315nm

Concentration	Absorption at λ=405nm
0	0
10	1.1841
5	0.5666
2.5	0.30248
1.25	0.1388
0.625	0.068066
0.313	0.033033
0.156	0.018796
0.078	0.0076546
µg/ml	nm

Table 5: Concentrations of AmB for calibration curve

Amount of AmB =

Abs/0118+0.0034

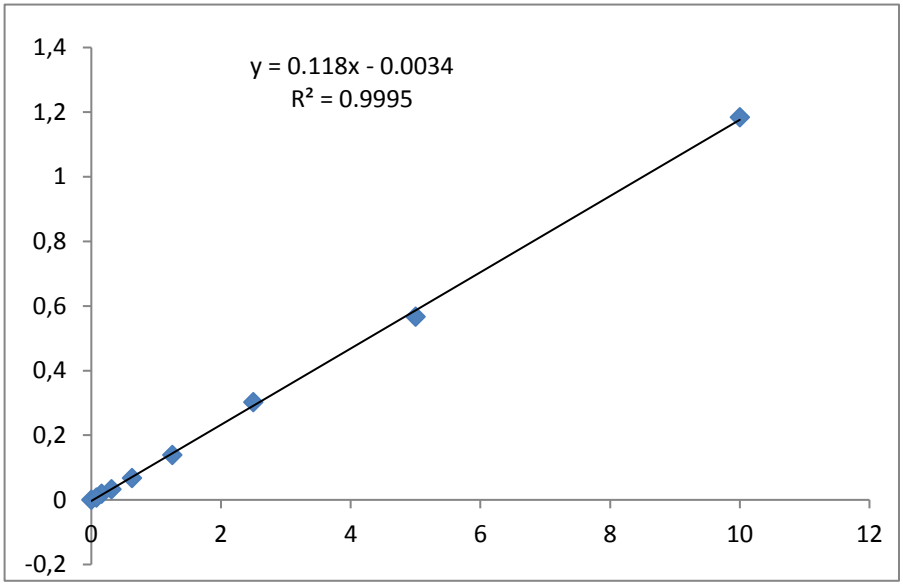

Figure 12: Linear regression curve of AmB in methanol at λ_{max}= 405nm

The encapsulation efficiency was calculated according to the following formula:

$$\text{Encapsulation efficiency} = \frac{\text{Mass of drug in liposomes}}{\text{Mass of drug used in formulation}} \times 100$$

Preparation 1-4

Based on their weights, AmB is about 10 times more effective than 2-PQ and its molecular weight is about 4-5 times higher. In the first experiment it was our aim to combine them in an equivalent effect which would be at a molar proportion of 1:50 AmB/2-PQ. As the extent

of the encapsulation was yet unknown, preparations containing 0.1, 0.2, 0.5 mol% AmB and 10mol% 2-PQ were made as well as a control batch without AmB. The encapsulation efficiencies for the drugs as well as the recovery of lipids are shown in Tables 6-8.

Recovery of phospholipids			
	Mass of drug used in formulation	Mass of lipids in Liposomes	Encapsulation Efficiency
AmB 0.1 mol%	6.15 mg/ml	2.28 mg/ml	37 %
AmB 0.2 mol%	6.14 mg/ml	3.32 mg/ml	54 %
AmB 0.5 mol%	6.12 mg/ml	3.29 mg/ml	54 %
Control	6.16 mg/ml	4.18 mg/ml	68 %

Table 6 : Recovery of phospholipids

Recovery of 2-PQ			
	Mass of drug used in formulation	Mass of drug in lipo-somes	Encapsulation efficiency
AmB 0.1 mol%	0.17 mg/ml	0.005 mg/ml	3 %
AmB 0.2 mol%	0.17 mg/ml	0.012 mg/ml	7 %
AmB 0.5 mol%	0.17 mg/ml	0.032 mg/ml	19 %
Control	0.17 mg/ml	0.073 mg/ml	43 %

Table 7 : Recovery of 2-PQ in preparations 1-4

Recovery of AmB			
	Mass of drug used in formulation	Mass of drug in lipo-somes	Encapsulation efficiency
AmB 0.1 mol%	0.01 mg/ml	0.003 mg/ml	29 %
AmB 0.2 mol%	0.02 mg/ml	0.006 mg/ml	32 %
AmB 0.5 mol%	0.05 mg/ml	0.015 mg/ml	32 %

Table 8: Recovery of AmB in preparations 1-4

In all formulations a certain amount of active substance and lipids was lost. The loss of active substances can partly be explained by the loss of phospholipids. Also during the process there always remains some dead volume in the tubing. The size was between 130 and 150 nm whereas the polydispersity index was about 0.1.

	Size	PDI
AmB 0.1	136 nm	0.07
AmB 0.2	149 nm	0.10
AmB 0.5	138 nm	0.09
Control	132 nm	0.07

Table 9: Size and Polydispersity Index

From previous liposomal formulations containing 2-PQ that have been prepared for this project, the average encapsulation is known to be around 50 %. The control without AmB gave the best result with 43% of encapsulated 2-PQ. In contrast, in the formulations that contained both drugs, the 2-PQ concentration was very low. (Table 7) This could be due to an interaction between AmB and 2-PQ.

Preparation 5-6: 0.5mol% AmB at a lipid concentration of 50 mM

In order to investigate where the loss of active substances and phospholipids respectively has taken place, a batch of liposomes with 0.5 mol% AmB at a higher lipid concentration of 50 mM was prepared. Small aliquots were taken after each preparation step:

- After mixing the stock solutions in the round-bottomed flask
- After evaporation and resuspension in buffer
- After extrusion
- After centrifugation and exchange of buffer.

These aliquots were quantified in terms of their concentration of active substance and lipids.

Recovery of AmB			
	Mass of drug used in formulation	Mass of drug in liposomes	Encapsulation efficiency
Mixture of stock solutions	0.07 mg/ml	0.07 mg/ml	100 %
After evaporation and dilution in buffer	0.23 mg/ml	0.20 mg/ml	85 %
After extrusion	0.23 mg/ml	0.12 mg/ml	51 %
After centrifugation	0.23 mg/ml	0.10 mg/ml	43 %

Table 10: Recovery of AmB

During the extrusion, a yellow layer was found on the membrane filter, which already indicated a certain loss of AmB through absorption during this step. This was confirmed in this experiment, where a loss of about 34 % occurred during extrusion. Overall the AmB encapsulation has significantly increased in comparison to the first formulations. While the maximum drug encapsulation in the former experiment amounted to 16%, this time it reached 43%. The increased encapsulation of AmB could be explained by the increased amount of lipids used: the losses were proportionally less when the total amount of material was higher. (50 mM and 10 mM respectively)

Recovery of 2-PQ in combination with AmB			
	Mass of drug used in formulation	Mass of drug in liposomes	Encapsulation efficiency
Mixture of stock solutions	0.25 mg/ml	0.25 mg/ml	100 %
After evaporation and dilution in buffer	0.86 mg/ml	0.51 mg/ml	60 %
After extrusion	0.86 mg/ml	0.3 mg/ml	36 %
After centrifugation	0.86 mg/ml	0.24 mg/ml	28 %

Table 11: Recovery of 2-PQ in liposomes that contain a combination of 2-PQ and AmB

Recoveryof 2-PQ in mg/ml in control			
	Mass of drug used in formulation	Mass of drug in liposomes	Encapsulation Efficiency
Mixture of stock solutions	0.25 mg/ml	0.25 mg/ml	100 %
After evaporation and dilution in buffer	0.86 mg/ml	0.66 mg/ml	77 %
After extrusion	0.86 mg/ml	0.48 mg/ml	56 %
After centrifugation	0.86 mg/ml	0.35 mg/ml	41%

Table 12: Recovery of 2-PQ in control

For 2-PQ, a loss of 40% has already occurred during evaporation and resuspension in buffer. Since 2-PQ is a volatile molecule, it seems that it evaporated during this step. Again, the amount of encapsulated 2-PQ was very low in the combined batch, as only 28 % could be retained in the final preparation. The encapsulation in the control batch was significantly

higher (41%), while the loss of active substance showed similar patterns as the loss of 2-PQ in the combination.

Recovery of Phospholipids			
	Mass of lipids used in formulation	Mass of lipids in liposomes	Encapsulation efficiency
After evaporation and dilution in buffer	30.61 mg/ml	34.27 mg/ml	100 %
After extrusion	30.61 mg/ml	26.34 mg/ml	86 %
After centrifugation	30.61 mg/ml	16.53 mg/ml	54%

Table 13: Recovery of phospholipids

The greatest loss of lipids took place after centrifugation. This result suggested that two different sized liposomes were formed during centrifugation: in addition to a pellet, a colored phase was observed that was not completely recovered during the exchange of buffer. The graph in Figure 13 describes how the substances were lost at each preparation step.

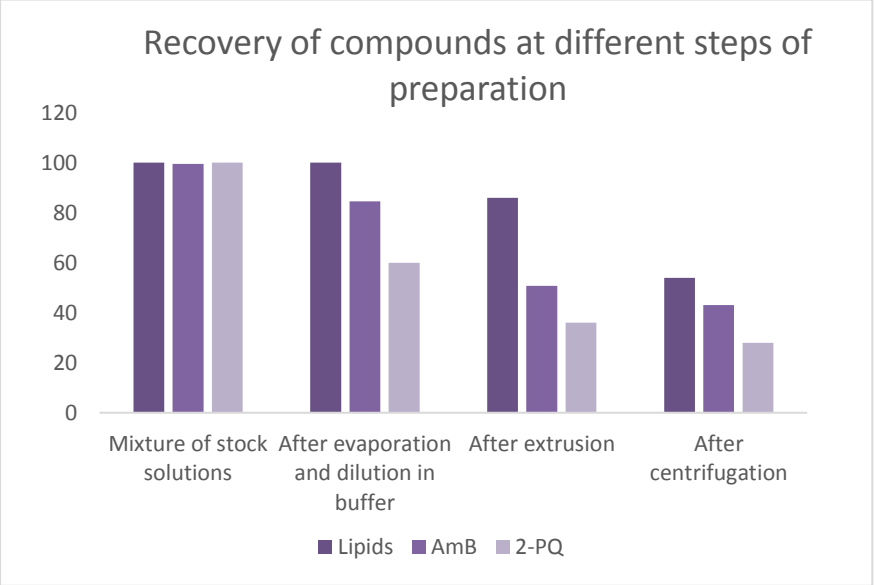

Figure 13: Recovery of compounds at different steps of preparation

Influence of the AmB-absorption on the analysis of 2-PQ

It was conspicuous in all results obtained so far that the 2-PQ encapsulation was always lower when AmB was involved. At the same time, however, it rose with the increased concentration of AmB.

Concentration of AmB	Encapsulation efficiency of 2-PQ
0 mol%	43 %
0.1 mol%	3 %
0.2 mol%	7 %
0.5 mol%	19 %

Table 14: Summary of encapsulation efficiencies of 2-PQ at differing concentrations of AmB

This led to concerns as to whether it was due to an analytical problem: AmB shows four peaks in the UV-spectrum, of which the smallest one is reached at λ_{max}= approximately 300 nm (Figure 14). Since we measured 2-PQ at λ_{max}=315 nm, the absorption at this point might be influenced by Amphotericin B.

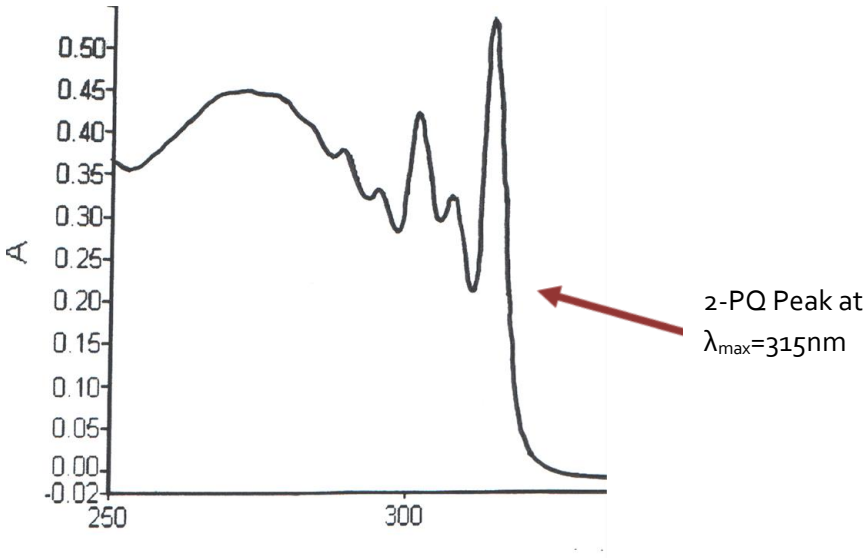

Figure 14: UV-spectrum of 2-PQ

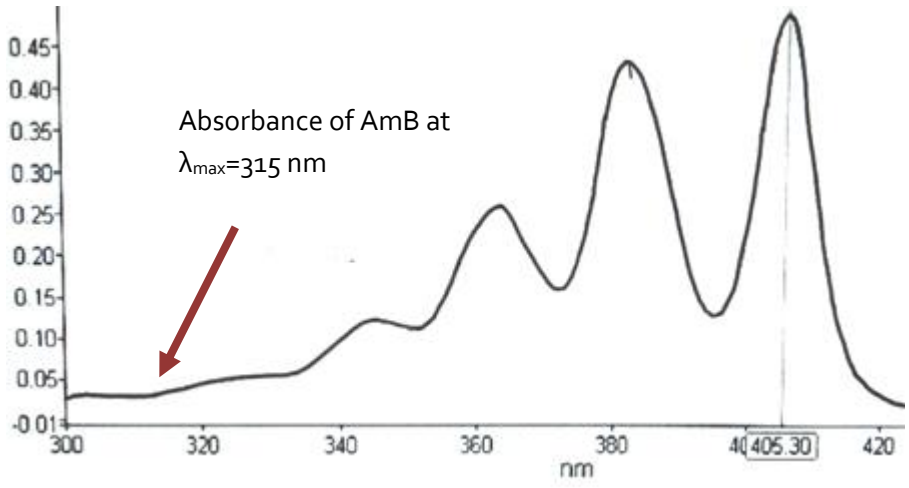

Figure 15: UV-spectrum of AmB

To evaluate the effect of AmB on the 2-PQ peak at λ_{max}=315 nm, a liposomal preparation of 2-PQ and AmB at a lipid concentration of 200 mM was prepared. The AmB concentration of this preparation was measured and a solution of AmB in MeOH at the same concentration was prepared. The absorption of AmB at λ_{max}=315nm was measured and the 2-PQ peak of the liposomal preparation corrected by this amount. Therefore, the correct encapsulation efficiency for 2-PQ is 48% in this preparation.

Preparation 7: 0.2 mol% AmB at a lipid concentration of 200 mM

	Absorbance	Mass of drug used in formulation	Mass of drug in liposomes	Encapsulation efficiency
2-PQ (λ_{max}=315nm)	0.542	3.42 mg/ml	1.71 mg/ml	50 %
AmB (λ_{max}=405nm)	0.234	0.37 mg/ml	0.2 mg/ml	54 %
2-PQ corrected by Abs of 0.2 mg/ml AmB solution	0.542-0.019= 0.523	3.42 mg/ml	1.65 mg/ml	48 %

Table 15: Encapsulation efficiency of Preparation 7 when 2-PQ is corrected by the absorbance of AmB at λ_{max}= 315 nm

This preparation of liposomes, prepared at a lipid concentration of200 mM, provided very promising encapsulation efficiencies around the 50% mark for both substances. To conclude whether a synergistic effect in a combination of drugs takes place, doses of about a fourth of their EC_{50} needed to be administered.

According to Yardley and Croft, the EC_{50} of AmBisome® in a single dose against visceral leishmaniasis is 0.73 mg/kg. [29] The EC_{50} for 2-PQ in this project's previous experiments was between 50 and 80 mg/ml. When administered to mice weighing around 20 g over a period of 10 days, the daily dose needed to be 0,0004 mg for AmB and 0,03 mg for 2-PQ respectively per mouse. According to this, to administer a single liposomal formulation to mice with both compounds at their EC_{50}, the compounds needed to be entrapped in a ratio of around 1:100 AmB: 2-PQ. In the previous liposomal formulation the AmB needed to be decreased by a factor 10, assuming that the EE would be about the same as in the previous experiment. In order to do so, another preparation with 0,02mol% at a lipid concentration of 200mM was prepared.

Preparation 8: 0,02mol% AmB at a lipid concentration of 200 mM

	Mass of drug used in formulation	Mass of drug in Liposomes	Encapsulation efficiency
2-PQ	3.42 mg/ml	1.78 mg/ml	52 %
AmB	0.04 mg/ml	0.01 mg/ml	26 %

Table 16: Drug recovery in preparation 8

This was the first attempt to prepare liposomes with such a low concentration of AmB. Unfortunately, the encapsulation efficiency of AmB was only 26 % at this concentration. At 0.01 mg/ml this was merely half of the desired amount for the *in vivo* experiment. It seems that through its low concentration a substantial part of it was lost through absorption during the extrusion. In a new attempt to get the right ratio, the AmB concentration was doubled from 0.02 to 0.04 mol%.

Preparation 9: 0.04 mol% AmB at a lipid concentration of 50 mM

	Mass of drug used in formulation	Mass of drug in Liposomes	Encapsulation efficiency
AmB 0,04mol%	0.019 mg/ml	0.009 mg/ml	49 %
2-PQ 10 mol%	0.86 mg/ml	0.52 mg/ml	61 %

Table 17: Drug recovery in preparation 9

At this concentration the encapsulation efficiency of AmB was almost 50%. Up to this point it has been assumed that the low encapsulation efficiency of the former preparation was due to the huge change in the AmB concentration by a factor of 10. Considering the new result this seemed to be less credible. To determine whether the low encapsulation efficiency of preparation 8 was due to a mistake during the preparation, another batch containing 0.02mol% lipids was prepared. As there might be a critical point between 0.02 and 0.04 mol% at which the encapsulation efficiency suddenly increases, a batch with 0.03mol % at 50mM lipids was also prepared.

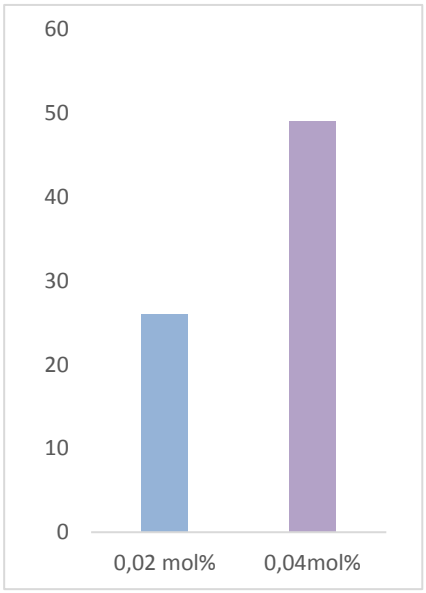

Figure 16: Comparison of AmB encapsulation efficiency at differing initial concentrations

At these low concentrations a lower dilution of 5+1 MeOH/liposomes needed to be used for the UV/Vis analysis. Since there was a considerably high amount of water in this low dilution, a new calibration curve under consideration of these new conditions was made. The absorption spectrum of AmB in 5:1 MeOH/water was checked to ensure that AmB was still in its monomeric form under these conditions and that the absorption maximum remained at λ_{max}=405nm.

Concentration	Absorption at λ=405nm
0	0
10	0,6111
5	0,3416
2,5	0,1826
1,25	0,0938
0,625	0,0445
0,313	0,0206
µg/ml	Nm

Amount of AmB =Abs-0.0121/0.0614

Table 18: Concentrations of AmB for calibration curve

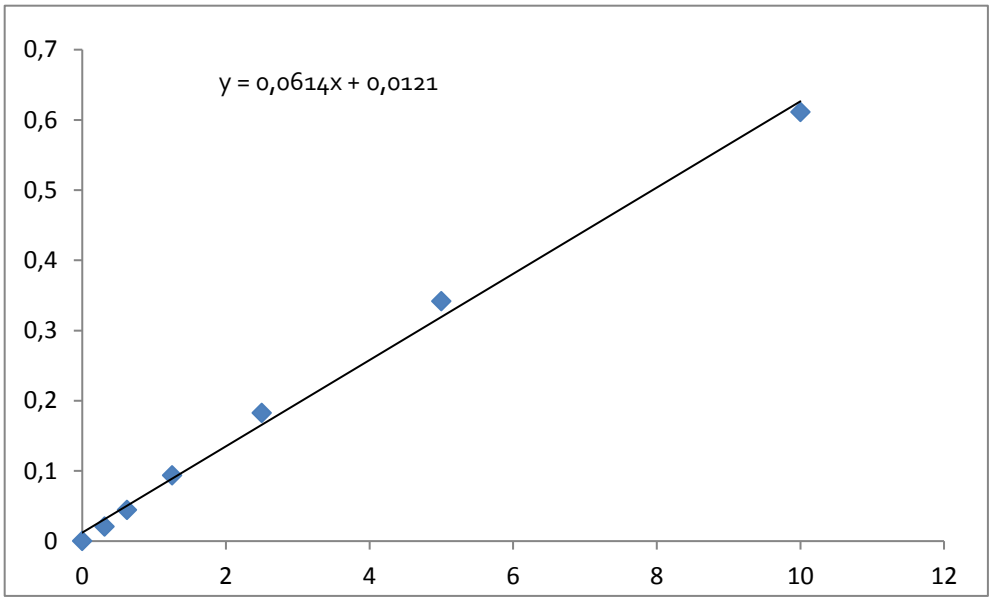

Figure 17: Calibration curve of AmB in MeOH/water 1+5

Preparation 10-11: 0.03 & 0.02 mol% AmB at 50 mM lipids

	Mass of drug used in formulation	Mass of drug in liposomes	Encapsulation Efficiency
AmB 0.03 mol%	0.014 mg/ml	0.008 mg/ml	60 %
2-PQ 10 mol%	0.86 mg/ml	0.3 mg/ml	35 %
AmB 0.02 mol%	0.009 mg/ml	0.003 mg/ml	34 %
2-PQ 10 mol%	0.86 mg/ml	0.31 mg/ml	36%

Table 19: Drug recovery preparation 10-11

The encapsulation efficiency for 0,02mol% was similar to the last experiment that was done at this concentration (Preparation 8). It seems that AmB is highly absorbed during extrusion when used at such a low concentration. This confirms that there must be a point between 0.02 and 0.04 mol% at which the encapsulation of AmB increases considerably. The desired concentration is found somewhere in the middle. In the last attempt to achieve the ratio that was aimed for, 0.025mol% AmB at 50 mM lipids were used.

Preparation 12: 0.025mol% AmB at a lipid concentration of 50 mM

	Mass of drug used in formulation	Mass of drug Found in liposomes	Encapsulation efficiency
2-PQ	0.86 mg/ml	0.18 mg/ml	22 %
AmB 0.025mol%	0.01 mg/ml	0.006 mg/ml	46 %

Table 20: Encapsulation efficiency of preparation 12

Although the required concentration of AmB was achieved, the 2-PQ encapsulation was too low. The encapsulation of 2-PQ is hard to predict, in the control batches without AmB it usually oscillates between 40 and 60%. However, when combined with AmB the concentration of lipids used seemed to play a crucial role in the encapsulation efficiency of 2-PQ: at a lipid concentration of 50 mM or less, the encapsulation efficiency of 2-PQ has always been very low. (Table 21), whereas at 200 mM it was around 50%. Therefore, preparations at 200 mM lipids, as used for the *in vivo* experiment, were deemed sufficiently concentrated in order to obtain the desired ratio in the *in vivo* experiment.

	Lipid concentration	2-PQ Encapsulation efficiency
Preparation 1	10 mM	19 %
Preparation 8	200 mM	52 %
Preparation 7	200 mM	54 %
Preparation 11	50 mM	34 %
Preparation 12	50 mM	36 %

Table 21: Comparison of encapsulation efficiency of 2-PQ at varying lipid concentrations in liposomes containing AmB and 2-PQ

For the *in vivo* experiment, three liposomal preparations of 4 ml at a lipid concentration of 200 mM each were prepared:

- Combination of AmB and 2-PQ
- Control with 2-PQ alone
- Control with AmB alone

Preparations for *in vivo* experiment			
	Mass of drug used in formulation	Mass of drug in Liposomes	Encapsulation efficiency
Combination			
2-PQ	3.42 mg/ml	2.68 mg/ml	78 %
AmB 0.025mol%	0.05 mg/ml	0.02346 mg/ml	51 %
2-PQ	3.42 mg/ml	2.59 mg/ml	76 %
AmB 0,025mol%	0.05 mg/ml	0.02856 mg/ml	62 %

Table 22: Drug recovery in the preparations for the *in vivo* evaluation

As expected for a lipid concentration of 200mM, the 2-PQ encapsulation was high. Table 23 shows size and polydispersity index of the liposomes.

	Size	PDI
Combination	143 nm	0.05
AmB Control	137 nm	0.096
2-PQ Control	148 nm	0.243

Table 23: Size and polydispersity index of preparations for *in vivo* experiment

Size is a critical parameter for intravenous application, since large particles (diameter >5µm) can lead to life-threatening embolisms. Also a homogenous size distribution is necessary to avoid such complications. The sizes and PDI of the preparations for the *in vivo* experiment were suitable for intravenous use.

Results of *in vivo* and *in vitro* experiment

Table 24 shows the *in vivo* as well *in vitro* results and also the doses given to the mice.

Compound / Formulation	*In vitro* activity on *L. donovani* IC_{50} (µM ± SD)		Regimen	*In vivo* activity		Reduction of parasite burden (%)
	Axenic amastigotes	Intramacrophage amastigotes		Number of mice	Route	
Liposomal 2PQ	3.10±0.25	5.84±0.31	3 mg/kg x 5 days	8	iv	83.8[a]
			1.5 mg/kg x 5 days	8	iv	32.5[a]
			0.75 mg/kg x 5 days	8	iv	5.2
Liposomal 2PQ+AmB	6.08±0.85 Eq 2PQ	13.5±1.93 Eq 2PQ	0.75 mg 2PQ + 0.006 mg AmB/Kg x 5	8	iv	86.5[a]
			0.37 mg 2PQ + 0.003 mg AmB/kg x 5	8	iv	10.3
AmBisome®	2.54±0.70	1.51±0.22	1 mg AmB/kg x 5 days	8	iv	88.7[a]
			0.25 mg AmB/kg x 5 days	8	iv	27.1
Blank liposomes	Inactive	Inactive	Same suspension	8	iv	5.7
Control (vehicle)	Inactive	Inactive	0.2 mL x 5 days	8	iv	0

[a] Versus control mice: $P<0.05$

Table 24: Results of *in vitro* and *in vivo* activity

The results showed that all three liposomal formulations have a strong antiparasitic effect. They all gave a high inhibition of parasitic growth (88.77% for AmB, 86.47% for the combination and 83.80% for 2-PQ). There has been synergy between AmB and 2-PQ *in vivo*, since the dose adjusted for the combined batch was much smaller than for 2-PQ on its own. A closer look at the *in vivo* results is taken in Table 25.

Treatment	Medium *L. donovani* units (LDU)	Standard deviation	Reduction of parasite burden %
AmB	**7,02E+0,8**	**1,82E+08**	88,70%
2-PQ + AmB	**8,62E+08**	**8,92E+07**	86,74%
2-PQ	**1,03E+09**	**3,44E+08**	83,80%
No treatment	**6,36E+09**	**1,64E+09**	**0%**

Table 25: Results of *in vivo* evaluation of liposomal preparations

	(1)IC_{50} ± Standard error *L. donovani* (LV9) (µM)		(2)CC_{50} ±Standard error (µM) RAW 264.7	(3)Selectivity index (SI)
	Anexic amastigotes	Intra-macrophagic amastigotes		
2-PQ	3.10±0.25	5.84±3.1	11.4±1,28	1.95
AmB+2-PQ	7.06±1.64	11.28±3.64	9.81±1,39	0.86
AmB	2.5±0.7	1.5±0.2	/	/

IC_{50}: inhibiting concentration, concentration that inhibits 50 % of the parasitic growth
CC_{50}: cytotoxic concentration, concentration at which 50% of the macrophages lose their viability
SI: Selectivity index, CC_{50} divided by IC_{50}

Table 26: Results of *in vitro* evaluation

The *in vitro* experiment was carried out by Ms. Nalia Mekarnia. In the *in vitro* results the combination showed a weaker effect than the formulations containing 2-PQ and AmB respectively: the IC_{50} for both anexic and intra-macrophagic amastigotes are both considerably higher than the respective concentrations for the batches containing only one substance. The IC_{50} of the combined batch was higher than for the liposomal batches containing 2-PQ and AmB alone. The cytotoxicity towards the RAW 264.7 cell line for 2-PQ was remarkably high. This could be due to the high concentration of phospholipids that were used to encapsulate enough 2-PQ.

Discussion

Visceral leishmaniasis (VL) is a parasitic disease caused by *Leishmania donovani* that leads to death if untreated. The number of affected people worldwide is estimated to be between 200 000 and 400 000 per year. [25] Various drugs against *L. donovani* are on the market, but all of them show drawbacks concerning their toxicity and high prices that often make them unavailable for the countries with the highest incidence of VL. A huge problem coming with all available drugs is the spread of resistant strains. These drawbacks lead to a constant need for new drugs and research on how to improve the medicines available.

The polyene antibiotic Amphotericin B (AmB) and the alkaloid 2-propylquinoline (2-PQ) are two effective drugs in the treatment of VL. However, their administration is problematic. AmB is almost completely insoluble in water. This leads to its side effects, in particular nephrotoxicity. [22] In order to achieve sufficient bioavailability, it needs to be administered parenterally. 2-PQ is an alkaloid derived from the roots and stems of the Bolivian tree *Galipea longifolia*. It has been used traditionally to treat cutaneous leishmaniasis by native Indians and its remarkable antileishmanial potency has been confirmed and in scientific research. [14] However, it also shows difficulties in its pharmacokinetics due to its short *in vivo* half-life. Because of these drawbacks, we used a liposomal formulation for both drugs: lipid based formulations could increase the *in vivo* half-life of 2-PQ and could also lead to an almost complete loss of AmB's nephrotoxicity through targeting of the reticuloendothelial system instead of the kidneys. As for all drugs used against VL, resistance poses a problem here as well. In order to overcome this difficulty, combination therapy is a commonly used approach, since it is unlikely for a strain to build mutants resistant against two different active substances at the same time. [4,8] In some cases, the combination of two drugs can lead to a synergistic effect and thus allow to decrease the doses used of the active substances. We combined AmB and 2-PQ in liposomes and investigated *in vitro* and *in vivo* whether a synergistic effect would occur.

AmB is a large, amphiphilic molecule, whereas 2-PQ is small and lipophilic. Due to their chemical nature they are both expected to be incorporated in the lipid bilayer of the liposomes. In our first preparation with both drugs, our goal was to evaluate how these two

drugs interact in the liposomes and whether the interaction would inhibit the incorporation of one of the substances. The basic findings in all studies were that their combination leads to different characteristics in encapsulation efficiency. This needed to be considered in order to choose the right ratio of components and active substances for the desired preparation. When incorporated together with AmB, the 2-PQ encapsulation efficiency depended on the amount of lipids used: at a low lipid concentration the 2-PQ recovery was lower than with a higher lipid concentration. In the preparation without AmB, this was not found to be the case. This could be due to the fact that both substances are incorporated in the lipid bilayer and there might thus be competition of some kind. During analysis via UV/Vis the AmB-peak at λ_{max}=405nm overlapped with the 2-PQ-Peak at λ_{max}=315nm. We concluded that the 2-PQ peak should be corrected when high amounts of AmB are involved. We also found that the drug recovery of AmB in liposomes strongly depended on the initial quantity of AmB. This is because a lower initial mass leads to proportionally higher losses through absorption, especially during the extrusion, the step where the most AmB was lost.

The size of the liposomes was measured using the quasi-dynamic light scattering method and found to be between 100 and 150 nm, which is a satisfying result for intravenous preparations. Three liposomal formulations (2-PQ, AmB and a combination of both drugs) were tested *in vitro* and *in vivo* in female BALB/c mice. All batches showed a high reduction of the parasite burden in the liver where amastigotes have been reduced by more than 80 % (Table 23). Of particular interest, however, was the finding that the liposomes with 2-PQ alone and with 2-PQ+AmB showed a similar result in parasite reduction despite the lower doses of the latter. This confirms that there is a synergistic effect *in vivo* between AmB and 2-PQ. The physiological target of *L. donovani* are macrophages. In the *in vitro* tests the IC_{50} for anexic and intra-macrophagic amastigotes as well as the cytotoxicity towards macrophages were evaluated. The IC_{50} in the *in vitro* tests revealed a smaller inhibition of amastigotes performed by liposomes with 2-PQ and AmB and thus no synergy. This was true for the anexic as well as the intra-macrophagic amastigotes. The test of cytotoxicity revealed a large CC_{50} in the batches where 2-PQ was involved. For a satisfying concentration of 2-PQ, high quantities of phospholipids needed to be used, which might be the reason for the increased cytotoxicity we found. Also, the combined batch showed a weaker *in vitro* reduction of parasite burden than the formulations containing only a single substance.

In the 2-PQ+AmB batch the CC_{50} was almost as high as the IC_{50} (Table 24). The *in vitro* evaluation therefore leads to the conclusion that such a formulation needs further improvement and attention should be paid to the cause of the cytotoxicity.

Within the framework of this research project, polymers were prepared in addition to the liposomes. Quinoline derivatives, covalently conjugated on polymers with lectin and polyglucose, have been prepared and tested *in vitro*. In comparison to these formulations, liposomes showed by far the best results in parasite reduction. The IC_{50} of the polymers was greater than 100 µM whereas the liposomal preparation containing 2-PQ had an IC_{50} of 3.10 µg. Thus, liposomes proved to be a reliable formulation for antileishmanial preparations for the intravenous route. Both AmB and 2-PQ require special formulations to make them suitable remedies. For intravenous application of 2-PQ and AmB liposomes seem to be an excellent drug delivery system.

If I would work again on the development of formulations, I would further investigate the stability of the preparations through size evaluation and evaluation of stability of the active substance in regular time intervals. Since VL has the largest incidence in less developed countries, it would be practical to create formulations that allow oral application, since they are more convenient to use and do not necessarily demand health staff and elaborate facilities to such an extent. Nanocochleates could be such a formulation.

Whenever resistance is lowering the effect of a single agent, the use of combination therapy is strongly indicated. Our *in vivo* results were very promising and indicate that there is synergy between AmB and 2-PQ, however this has not been confirmed *in vitro*. Both substances are difficult pharmaceuticals due to their pharmacokinetics and toxicity in the case of AmB. Liposomes cannot just overcome these problems but also give a high reduction of parasite burden.

Summary

Visceral leishmaniasis is a protozoan disease caused by *Leishmania donovani*. *L. donovani* is transmitted by female sand flies and is usually fatal when left untreated. The highest incidence is found in Bangladesh, Brazil, Ethiopia, India, Sudan and South Sudan. But cases in Southern Europe also occur, especially by zoonotic transmission of *L. infantum* from dogs to humans. [33] The increasing spread of resistant strains leads to a constant need of new substances and improvement of the therapies used so far. The alkaloid 2-propylquinoline (2-PQ) and the polyene antibiotic Amphotericin B (AmB) are effective substances against leishmaniasis. However, 2-PQ cannot be administered over intravenous route due to its high lipophilicity and has a short *in vivo* half-live. AmB aggregates in aqueous media, leading to its side effects, of which nephrotoxicity is the most severe. In order to overcome these drawbacks, we used liposomes as a drug delivery system. Liposomes could transport AmB in its soluble form directly to the macrophages that contain the amastigotes whereas the kidneys would be avoided. 2-PQ on the other hand could become available intravenously and its half-life duration could increase. Combination therapy is a valid approach in order to decrease the development of resistance. [4,8]

Furthermore, we investigated a synergistic effect between AmB and 2-PQ. We found, that for manufacturing and analysing liposomes with AmB and 2-PQ some characteristics need to be considered concerning the choice of the initial concentrations of 2-PQ and AmB for the desired drug recovery:

(i) The drug recovery of AmB depends strongly on its initial quantity, since a lesser initial mass leads to proportionally higher losses through absorption

(ii) 2-PQ is recovered to a lesser amount at a low lipid concentration and when AmB is involved; its recovery is high when the lipid concentration is high

(iii) During analysis via UV/Vis the AmB-Peak at λ_{max}=405nm overlaps with the 2-PQ-Peak at 315nm.

Three liposomal formulations (2-PQ, AmB and a combination of both drugs) were manufactured and tested *in vitro* and *in vivo* in female BALB/c mice. The *in vivo* results revealed a high reduction of parasite burden for all 3 batches (88, 70% AmB, 86, 74% AmB+2-PQ,

83, 80% 2-PQ). Furthermore, the *in vivo* tests revealed a synergy between AmB and 2-PQ, however, this has not been confirmed *in vitro*. Through the high reduction of parasite burden these liposomes are an interesting topic for future research.

Zusammenfassung

Die viszerale Leishmaniose ist eine tropische, durch *Leishmania donovani* verursachte Erkrankung. Der protozoische Erreger wird über Sandmücken übertragen. Unbehandelt nimmt die viszerale Leishmaniose meist einen tödlichen Verlauf. Die höchste Inzidenz findet sich in Bangladesch, Brasilien, Äthiopien, Indien, Südsudan und Sudan, aber auch in Südeuropa treten immer wieder Fälle von viszeraler Leishmaniose auf, vor allem durch Übertragung von *L. Infantum* von Hunden auf Menschen. (33) Durch steigende Resistenzbildung der Erreger ist ein ständiger Bedarf an neuen Substanzen und einer Verbesserung der vorhandenen Therapien gegeben. Das Alkaloid 2-Propylchinolin (2-PQ) und das Antibiotikum Amphotericin B sind bewährte Wirkstoffe gegen viszerale Leishmaniose, jedoch kann 2-PQ aufgrund seiner Lipophilie nicht parenteral angewendet werden. Amphotericin B (AmB) aggregiert im wässrigen Medium. Diese Aggregate führen zu der Hauptnebenwirkung Nephrotoxizität. Liposomen sind geeignete Drug Carrier, die einerseits AmB zu deren Zielzellen, den Makrophagen, bringen könnten und die Niere dabei umgehen. Andererseits könnte 2-PQ in dieser Form intravenös appliziert werden und auch die Halbwertszeit würde steigen. Durch die steigende Resistenzbildung ist der Einsatz von Kombinationstherapien nötig. Daher wurde in dieser Diplomarbeit in umfassenden Versuchen eine liposomale Formulierung mit einer Kombination von AmB und 2-PQ hergestellt und in vitro sowie in vivo auf einen synergistischen Effekt getestet. Bei der Herstellung von Liposomen mit AmB und 2-PQ sind einige Besonderheiten bezüglich der Wahl der Wirkstoffmenge für die gewünschte Ausbeute und der Analyse zu beachten:

(i) Die Einbaurate von AmB ist stark von der ursprünglich eingesetzten Menge abhängig, da bei weniger Ausgangssubstanz relativ höhere Verluste während der Herstellungsprozesse entstehen.

(ii) 2-Propylchinolin wird bei geringer Lipidkonzentration und gleichzeitiger Anwesenheit von Amphotericin B in geringerer Menge in die Liposomen aufgenommen als bei höherer Lipidkonzentration.

(iii) Weiters muss bei der UV-photospektrometrischen Analyse berücksichtigt werden, dass der AmB-Peak bei λ_{max}=405nm mit dem 2-PQ-Peak bei λ_{max}=315nm überlappt.

Drei liposomale Zubereitungen (2-PQ, AmB und eine Kombination aus beiden Substanzen) wurden erstellt und sowohl in vitro als auch in vivo in weiblichen BALB/c Mäusen getestet. Die in vivo Ergebnisse ergaben eine hohe Reduktion der Parasitenlast (84 % 2-PQ, 89 % AmB, 87 % AmB+2-PQ). Außerdem konnte in den in vivo Untersuchungen eine synergistische Wirkung dieser beiden Wirkstoffe bewiesen werden, allerdings nicht in den in vitro Tests. Mit einer Reduktion der Parasitenlast um über 80 % in vivo können diese Liposomen als vielversprechender Ansatzpunkt für weitere Forschungen auf diesem Gebiet gesehen werden.

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