Colloidal Formulation of Mistletoe Extracts by a Pharmaceutical Flow Process for Targeted Cancer Therapy

Kolloidale Formulierung von Mistelextrakten durch einen pharmazeutischen Strömungsprozess für eine zielgerichtete Krebstherapie

Gero Leneweit, Maria B. C. de Matos, Nataliia Beztsinna, Christoph Heyder, Claudia Duehrkop, Bárbara S. Miranda, Kirsten Ullmann, Alberto Dessy, Kristina Nilsson Ekdahl, Bo Nilsson, Hermann Nirschl, Enrico Mastrobattista, Robbert Jan Kok

Summary

Complete remissions were reported upon intratumoural injections and high dosage intravesical administrations of mistletoe preparations. Liposomal formulations prepared for triggered release potentially allow drug accumulation and treatment of tumours unreachable by intratumoural injections. However, efficient encapsulation of macromolecular biologicals in liposomes has so far been challenging, especially if there is no interaction between the liposomal bilayer and the biological. Steps to overcome this problem and to provide liposomal formulations of mistletoe compounds are described here. The capacity of liposomal formulations for triggered release of mistletoe lectin 1 is demonstrated and details of endocytosis and endosomal escape mechanisms are provided. To enable long circulation of liposomes for their accumulation, a protective coating of heparin is under development to minimize innate immune reactions.

Keywords: liposomes, mistletoe, encapsulation of biopharmaceuticals, tumour targeting, triggered release, inhibition of complement activation

Zusammenfassung

Vollständige Remissionen wurden bei intratumoralen Injektionen und hochdosierten intravesikalen Verabreichungen von Mistelpräparaten berichtet.

Liposomale Formulierungen, die für eine induzierte Freisetzung vorbereitet werden, ermöglichen potenziell die Anreicherung von Medikamenten und die Behandlung von Tumoren, die mit intratumoralen Injektionen nicht erreichbar sind. Die effiziente Verkapselung makromolekularer Biologika in Liposomen war bisher jedoch eine Herausforderung, insbesondere wenn keine Wechselwirkung zwischen der liposomalen Doppelschicht und den Biologika besteht. Schritte zur Überwindung dieses Problems und zur Bereitstellung liposomaler Formulierungen von Mistelpräparaten werden hier beschrieben. Die Fähigkeit liposomaler Formulierungen zur induzierten Freisetzung von Mistellektin 1, Details zur Endozytose und zum Austritt aus den Endosomen werden dargestellt. Um eine lange Zirkulation der Liposomen für ihre Akkumulation zu ermöglichen, wird eine Schutzschicht aus Heparin entwickelt, um angeborene Immunreaktionen zu minimieren.
Schlüsselwörter: Liposomen, Mistel, Verkapselung von Biopharmazeutika, Tumor-Targeting, induzierte Freisetzung, Hemmung der Komplementaktivierung

Introduction

Cancer therapy by mistletoe was introduced clinically from 1917 onwards. It was based on three concepts which were vibrant at that time or in the decades to follow and continue to be under debate until now:

1. The induction of high fever, the positive prognostic relevance of which was assessed (Wiedmann, Starnes 1994);
2. The development of strongly anti-tumoural drugs with little adverse effects;
3. The use of nanoscopic formulations with colloidal states of matter of both drugs and excipients for parenteral administration, aiming for improved tumour targeting and reduced induction of innate and adapted immunity.

By putting these concepts into relation to concurrent developments in cancer therapy and parenteral drug formulations, the following milestones can be viewed:

Fever induction as a remedy in cancer therapy started systematically in the 1890s by William Coley. Until today, it offers many intriguing aspects to activate the immune system in order to fight cancer and still asks

for an elaboration of clinical settings to be applied (Forbes 2010; Mastrobattista 2013).

Chemotherapy only started from 1946 based on the experiences of chemical weapons in the two world wars (nitrogen mustard, see Gilman 1963). The use of biological drugs (targeted therapies) was introduced decades later with the aim to improve selectivity in their mode of action, to avoid cytotoxicity against healthy tissue and to overcome the formation of chemo-resistance by tumours.

First oil-in-water emulsions for the aim of parenteral nutrition injected subcutaneously (Friedrich 1904) caused severe pain and had to be abandoned. It took until 1962 for the first safe parenteral emulsion to be invented (Intralipid®, Wretlind 1981). It was in 1995 when Doxil® was approved, the first liposomal drug formulation for second line therapy of ovarian cancer. However, the method applied for the loading of liposomes with doxorubicin, a topoisomerase inhibitor, is based on the diffusion of the small drug molecule through the liposomal membrane and its precipitation inside due to pH change. This so-called "remote loading" is however impossible for macromolecular biological drugs.

The concept of forming asymmetric liposomes from W/O emulsions as shown in Fig. 1 (a) was first introduced by Träuble and Grell (1971). It took more than three decades (Pautot et al. 2003) for experimental proofs, but these were discussed controversially since then. It therefore remains an open challenge to encapsulate macromolecular drugs in liposomes for targeted therapies. Also, the protection of drug delivery systems like liposomes from an activation of innate and acquired immunity and their rapid elimination from the blood circulation are still faced with major difficulties. Although polyethylene glycol (PEG) attenuates immune reactions, accelerated blood clearance after repeated injection, anti-PEG antibody formation and anaphylactic reactions occur. These symptoms ask for a better immune protection to enable long circulation of liposomes and improved tumour targeting.

It is the second and third conceptual aspect of mistletoe therapy which will be mainly focused here.

Materials and Methods

Detailed descriptions of the materials and methods used are provided by the original publications of each of the results summarized here. For the characterization of the phospholipid emulsions, different tensiometric methods (profile analysis, du Noüy, spinning drop) were used. Quantification of average emulsion and liposome sizes was done by dynamic light scattering, visualization by transmission electron microscopy (TEM and cryo-TEM). Mistletoe lectins were isolated by use of affinity chromatography and purity was demonstrated by SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) and FPLC (Fast Protein Liquid Chromatography). Quantification was achieved by ELISA (Enzyme Linked ImmunoSorbent Assay). Immune activation was tested by a whole blood model from healthy donors without use of anticoagulants. Avoidance of innate immune activation was monitored by ELISA and QCM-D (Quartz Crystal Microbalance with Dissipation). Cell culture tests on murine CT26 colon carcinoma and 4T1 murine breast cancer cells were studied by fluorescence confocal microscopy using Yokogawa Cell Voyager CV7000s.

Results and Discussion

Fig. 1 (a) shows the concept to form liposomes from water-in-oil emulsions. Drug-loaded water droplets transfer a water/oil phase boundary with its monolayer covering the droplet monolayer to form asymmetric bilayers. Fig. 1 (d) and (e) show experimental proofs of this method with asymmetric liposomes. In contrast, Figs. 1 (b) and (c) demonstrate the development of macroscopically visible organogels. These are formed by self-organisation at the phase boundary by most oil phases (here: squalene) and oppose the formation of liposomes. Therefore, detailed studies of monolayer formation at oil/water interfaces were performed with squalene and squalene (Hildebrandt et al. 2016a; 2016b) and their adsorption kinetics was analysed (Hildebrandt et al. 2018). Water-in-oil emulsions were explored using DPPC and squalene (Sommerling et al. 2018)

and proved to be unstable because of initial coalescence, followed by Ostwald ripening (Ostwald 1897) of the droplets. Short-term stabilization could be achieved by repetitive mechanical treatment. To avoid the formation of organogels, the hydrophobic phase was replaced by perfluorinated oils which proved to be suitable to form monolayers (Ullmann et al. 2020) suitable for the formation of asymmetric liposomes.

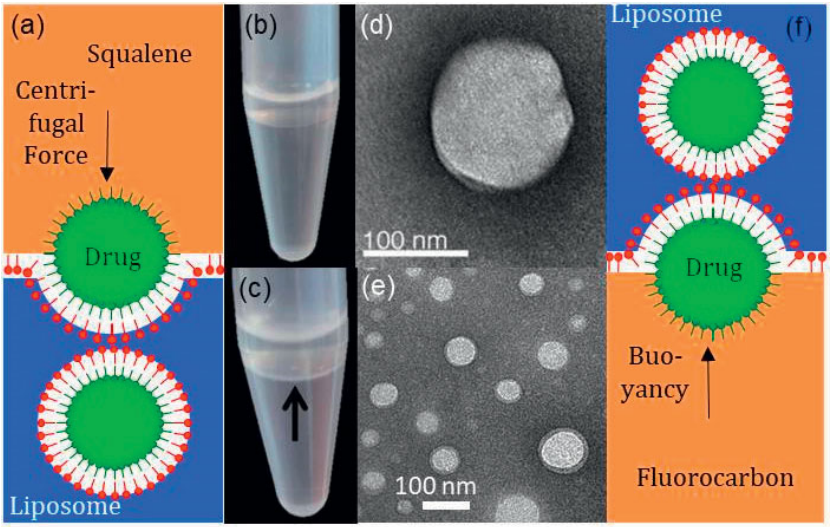

Fig. 1: Formation of asymmetric liposomes by water-in-oil (W/O) nanoemulsions. (a) Schematic sketch using squalene oil as hydrophobic phase. (b) W/O Emulsion placed on water phase to produce liposomes by centrifugation, age of interface: 30 min. (c) Age of interface: 150 min. (d) Transmission electron microscopy (TEM) of liposome produced with squalene as hydrophobic phase. (e) TEM of liposomes produced with a fluorocarbon as hydrophobic phase. (f) Due to the higher density of fluorocarbons, liposomes are produced by buoyancy in the centrifugal field. Figs. 1 (b), (c) and (d) are extracted from de Matos et al. 2019, for license see: http://creativecommons.org/licenses/by/4.0.

Lysolecithin was introduced as a thermosensitive membrane constituent (10 mol %) to enable controlled drug release when accumulated in the tumour (de Matos et al. 2018). Fig. 2 (a) shows a schematic sketch of the

membrane conformation when heated to 42 °C. Fig. 2 (b) shows a comparison of apoptosis induction when CT26 cells were incubated at 37 °C or 42 °C for 1 h with mistletoe lectin 1 (ML1) containing liposomes, yielding IC_{50} values of 25 μg/ml or 0.6 μg/ml, respectively. Here, cells were washed after 1 h and further incubated for 47 h at 37 °C.

Uptake of fluorescence-labelled ML1 in tumour cells was monitored by confocal microscopy, an example of which is shown in Fig. 2 (c) (Beztsinna et al. 2018). Imaging with fluorescently labelled antibodies of the endosomal pathway allowed to trace the mechanisms of endocytosis and endosomal escape. Uptake was proven to take place both by caveolin and by clathrin enabled endocytosis. Pearson's correlation coefficient was applied to detect the colocalization of mistletoe lectins with early endosomes, late endosomes, and lysosomes. It could be shown that a considerable fraction of ML1 escapes from early endosomes to reach the cytosol and the ribosomes to initiate apoptosis.

Liposomes were incubated in whole blood for 90 min. to test their haemocompatibility by measuring the cleavage of complement protein C3 (Duehrkop et al. 2016). Fig. 3 (a) shows the increase of C3a for unprotected liposomes. Liposomes protected by a heparin complex attached by electrostatic forces with 10 % of the cationic lipid DOTAP resulted in complete suppression of the production of C3a and strong reduction of liposomes' leakiness due to blood compounds, Fig. 3 (b).

Fig. 3 (c) shows the development of apoptosis over 72 h of parental 4T1 murine breast cancer cells, and their multi-drug resistant daughter cells treated with 200 μg/ml. Fig. 3 (d) shows a comparison of IC_{50} values for parental 4T1 and chemoresistant 4T1 cells with 1.4 and 9.4 ng/ml, respectively. This means that the increase in IC_{50} is about a factor 6.9 for ML1, while for doxorubicin a factor of 13.4 was reported (Chen et al. 2017).

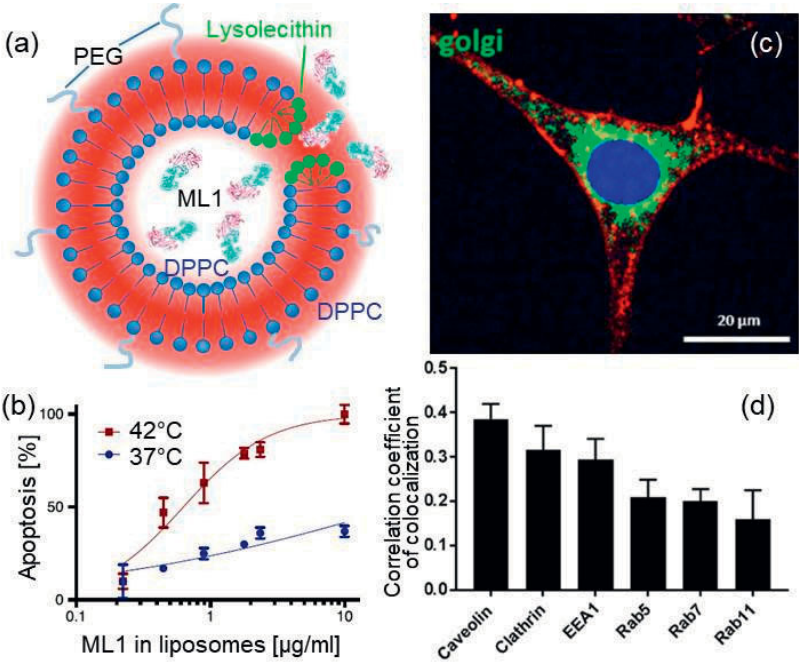

Fig. 2: Release of mistletoe lectins (ML1) from liposomes and endocytosis of mistletoe lectin in CT26 murine colon carcinoma cells. (a) Thermosensitive liposomes with DPPC/lysolecithin/PEG-DSPE = 86:10:4 mol %, causing membrane leakiness upon hyperthermia. (b) Incubation of CT26 cells at either 42 °C or 37 °C for 1h, followed by cell washing and culture for 47 h at 37 °C. (c) Confocal microscopy of CT26 cell after 1h incubation with ML1 with nucleus stained in blue, Golgi apparatus in green and ML1 in red. (d) Correlation coefficients for the colocalization of ML1 with antibodies of the endosomal pathway, proving ML1 uptake by both caveolin and clathrin enabled endocytosis. Colocalization decreases from early endosomes (EEA1 and Rab5) to late endosomes (Rab7) and lysosomes (Rab11), proving endosomal escape of a large fraction of ML1. Fig. 2 (b) is extracted from de Matos et al. 2018, Figs. 2 (c) and (d) from Beztsinna et al. (2018), http://creativecommons.org/licenses/by/4.0.

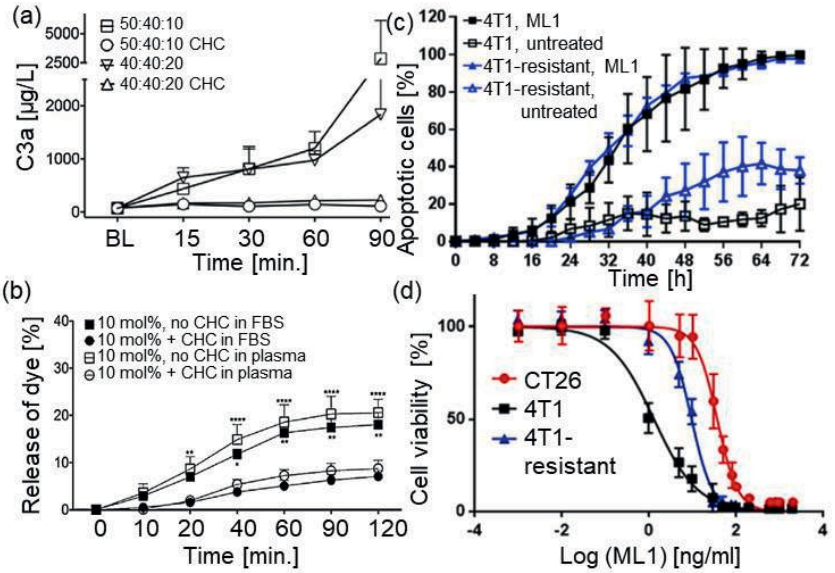

Fig. 3: Protection of liposomes against innate immune activation by coating with a heparin complex (CHC). Mistletoe lectins prove to be efficient both against the multi-drug resistant murine breast cancer cell line 4T1 and its parental cell line. (a) Activation of complement protein C3a by different liposomes within 90 min., shown against the baseline (BL). Numbers in the legend stand for molar fractions of DPPC, cholesterol and DOTAP with CHC or without. (b) Stability of coated or non-coated liposomes in serum and plasma, proven by the leakage of a fluorescent dye from the liposomes. (c) Percentage of apoptotic cells over 72 h of 4T1 parental and 4T1 multidrug resistant cells with ML1 (200 µg/mL), controlled by non-treated cells. (d) Cell viability measured at different ML1 concentations to determine IC_{50} = 1.4 ng/ml (4T1 parental) vs. 9.4 ng/ml (4T1-resistant). Figs. 3 (a) and (b) are extracted from Duehrkop et al. (2016), by courtesy of Elsevier B.V., Figs. 3 (c) and (d) from Beztsinna et al. (2018), http://creativecommons.org/licenses/by/4.0.

Conclusions and Outlook

The results indicate the feasibility of liposomal formulations of mistletoe extracts and their benefits. In the future, the research partners will concentrate on the treatment of Glioblastoma multiforme (GBM) and its treatment by liposomal drug formulations.

Acknowledgements

This work was supported by the People Programme (Marie Skłodowska Curie Actions) of the European Union's Seventh Framework Programme FP7/2007–2013 under REA grant agreement n° 324275. The authors gratefully acknowledge funding by the Phospholipid Research Center (Heidelberg).

Conflict of Interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

References

Beztsinna N, de Matos MBC, Walther J et al.: Quantitative analysis of receptor-mediated uptake and pro-apoptotic activity of mistletoe lectin-1 by high content imaging. Sci Reports. 2018; 8: 2768–2778.

Chen Y, Tezcan O, Li D, Beztsinna N et al.: Overcoming multidrug resistance using folate receptor-targeted and pH-responsive polymeric nanogels containing covalently entrapped doxorubicin. Nanoscale. 2017; 9: 10404–10419.

de Matos MBC, Miranda BS, Rizky Nuari Y et al.: Liposomes with asymmetric bilayers produced from inverse emulsions for nucleic acid delivery, J Drug Target. 2019; 27: 681–689.

de Matos MBC, Beztsinna N, Heyder C et al.: Thermosensitive liposomes for triggered release of cytotoxic proteins. Eur J Pharm Biopharm. 2018; 132: 211–221.

Duehrkop C, Leneweit G, Heyder C et al.: Development and characterization of an innovative heparin coating to stabilize and protect liposomes against adverse immune reactions. Colloids Surf B. 2016; 141: 576–583.

Forbes N: Engineering the perfect (bacterial) cancer therapy. Nature. 2010; 10: 785–794.

Friedrich PL: Die künstliche subkutane Ernährung in der praktischen Chirurgie. Arch klin Chirurg. 1904; 73: 507–516.

Gilman A: The initial clinical trial of nitrogen mustard. Am J Surg. 1963; 105: 574–578.

Hildebrandt E, Nirschl H, Kok RJ et al.: Adsorption of phospholipids at oil/water interfaces during emulsification is controlled by stress relaxation and diffusion. Soft Matter. 2018; 14: 3730–3737.

Hildebrandt E, Dessy A, Sommerling JH et al.: Interactions between Phospholipids and Organic Phases – Insights in Lipoproteins and Nanoemulsions. Langmuir. 2016a; 32: 5821–5829.

Hildebrandt E., Sommerling JH, Guthausen G et al.: Phospholipid adsorption at oil in water versus water in oil interfaces: Implications for interfacial densities and bulk solubilities. Colloids Surf A. 2016b: 56–63.

Klapper Y, Hamad OA, Teramura Y et al.: Mediation of a non-proteolytic activation of complement component C3 by phospholipid vesicles. Biomaterials. 2014; 35: 3688–3696.

Mastrobattista E: Artificial microbes to fight cancer. Nanomedicine. 2013; 8: 5–7.

Ostwald W: Studien über die Bildung und Umwandlung fester Körper. Z Phys Chem. 1897; 22: 289–330.

Pautot S, Frisken BJ, Weitz DA: Engineering asymmetric vesicles. Proc Natl Acad Sci. 2003; 100: 10718–10721.

Sommerling JH, de Matos MBC, Hildebrandt E et al.: Instability Mechanisms of Water-in-Oil Nanoemulsions with Phospholipids: Temporal and Morphological Structures. Langmuir. 2018; 34: 572–584.

Träuble H, Grell E: The formation of asymmetrical spherical lecithin vesicles. Neurosci Res Program Bull. 1971; 3: 373–380.
Ullmann K, Poggemann L Nirschl H et al.: Adsorption Process for Phospholipids of Different Chain Lengths at a Fluorocarbon/ Water Interface studied by Du Noüy Ring and Spinning Drop. J Colloid Polym Sci. 2020; DOI: 10.1007/s00396-020-04618-3.
Wiedmann B, Starnes CO: Coley's Toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmac Ther. 1994; 64: 529–564.
Wretlind A: Development of fat emulsions. J Parenter Enteral Nutr. 1981; 5: 230–235.

Dr. Gero Leneweit, ABNOBA GmbH, Pforzheim, Germany; Carl Gustav Carus Institute of the Association for the Promotion of Cancer Therapy, Niefern-Öschelbronn, Germany
Dr. Maria B. C. de Matos, Dr. Nataliia Beztsinna, Prof. Dr. Enrico Mastrobattista, Prof. Dr. Robbert Jan Kok, Utrecht University, Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht, The Netherlands
Dr. Christoph Heyder, Dr. Bárbara S. Miranda, Dr. Alberto Dessy, ABNOBA GmbH, Pforzheim, Germany
Dr. Claudia Duehrkop, Prof. Bo Nilsson MD, Prof. Kristina Nilsson Ekdahl PhD, Uppsala University, Department of Immunology, Genetics and Pathology, Uppsala, Sweden
Kirsten Ullmann, Prof. Dr. Hermann Nirschl, Karlsruhe Institute of Technology (KIT), Institute of Mechanical Process Engineering and Mechanics, Karlsruhe, Germany

Corresponding author:
gero.leneweit@carus-institut.de