Liposomale Formulierung von Mistelextrakten durch Zentrifugationsverfahren und Analyse der Zellproliferation von Gesamtextrakten und isoliertem Mistellektin I

Liposomal formulations of mistletoe produced by centrifugal technologies and cell proliferation analysis of both mistletoe extracts and isolated mistletoe lectin I

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Zusammenfassung

Um Liposomen aus Nanoemulsionen herzustellen, wird ein Zentrifugationsverfahren entwickelt, das eine hohe Einkapselungseffizienz und asymmetrische Membranen ermöglicht. Heparin-Komplexe werden für die Bildung einer stabilen Schutzschicht verwendet. Um die Erprobung der liposomalen Formulierungen *in vitro* und *in vivo* zu ermöglichen, wurden Mistelpräparate mit unterschiedlicher Viscotoxin-(VT) und Mistellektin-(ML) Zusammensetzung sowie isoliertes ML°I an Maus-Zelllinien erprobt. Ein Proliferationstest wurde durchgeführt, um die inhibierenden Konzentrationen (IC50) zu ermitteln und sensitive Zelllinien für *in vivo* Experimente auszuwählen. abnobaVISCUM (AV) Pini Präparate, die den geringsten Gesamtgehalt an ML und eine Dominanz von ML III aufweisen, zeigten bei B16-F10 Melanomzellen eine stärkere Inhibierung der Proliferation im Vergleich zu den ML I reichen Präparaten AV Fraxini und Quercus. Für AV Fraxnini und AV Quercus wurde gezeigt, dass die Zytotoxizität überwiegend auf ML I zurückzuführen ist und ML I daher als potentieller Wirkstoff zur Verkapselung in Liposomen geeignet ist. Auf isoliertes ML I reagiert die getestete Kolonkarzinomzelllinie C26 deutlich empfindlicher als die aggressive B16-F10 Melanomzelllinie. Diese Ergebnisse

erlauben den Vergleich eines sensitiven mit einem aggressiven Tumormodell *in vivo*. Im Vergleich zu C26 ist die Makrophagenzelllinie RAW264.7 relativ unempfindlich gegenüber isoliertem ML I. Die Ergebnisse deuten auf die Möglichkeit einer gezielten Therapie von z.B. Kolontumoren hin, bei der die Immunfunktionen intakt bleiben.

Schlüsselwörter: Liposomen, Heparin Immunschutz, Mistelextrakte, Mistellektin, Zytotoxizität, Wachstumsinhibition, Proliferationstest, Mauszellinien.

Summary

A centrifugal technology is under development to produce liposomes from a nanoemulsion and to enable high encapsulation efficiencies in asymmetric membranes. Heparin complexes are used for a robust protective coating of liposomes. To enable *in vitro* and *in vivo* testing of liposomal formulations, mistletoe preparations with differences in viscotoxin (VT) and mistletoe (ML) lectin composition were tested on murine cell lines. A proliferation assay was performed to determine the half maximal inhibitory concentration (IC50) and select sensitive cell lines for *in vivo* experiments. abnobaVISCUM (AV) Pini preparations, with the lowest total ML content and a ML°III dominated composition, showed a stronger inhibition of proliferation on B16-F10 (mouse melanoma) cells relative to the total ML content compared to AV Fraxini and Quercus. ML I is the major cytotoxic component in AV Fraxini and AV Quercus and therefore a potential API for encapsulation in liposomes. The tested colon carcinoma cell line C26 is significantly more sensitive to isolated ML I than the aggressive B16-F10 melanoma cell line. In regard to animal models, this allows to compare a sensitive to a relatively non-sensitive and more aggressive tumor cell line. In comparison to C26, the macrophage cell line RAW264.7 is relatively insensitive to isolated ML I, suggesting the possibility of selecting therapeutic concentrations that target e.g. colon tumours, but leave immune functions intact.

Keywords: Liposomes, heparin immune protection, mistletoe extracts, mistletoe lectin, cytotoxicity, growth inhibition, proliferation assay, murine cell lines

Introduction

Pharmaceutical flow processes were invented for the production of mistletoe preparations in the 1920s (Steiner 1923, Steiner 1924), many decades before liposomal preparations in cancer therapy were introduced in 1974 (Gregoriadis *et al.* 1974). The processes inaugurated for the preparation of mistletoe injectables bear a high potential for a new class of liposomal drug formulations of plant extracts, see Vrânceanu *et al.* 2012. Especially heat and solvent sensitive active pharmaceutical ingredients like mistletoe lectins cannot be encapsulated in liposomes by any conventional method, but could be loaded into liposomes by adapted flow processes initially developed for mistletoe.

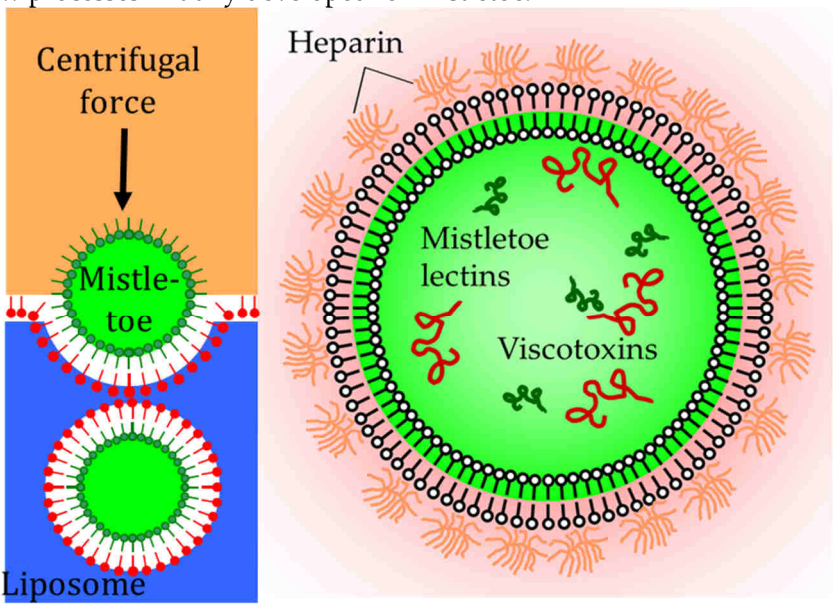


Fig. 1: Concept of the pharmaceutical flow process to embed mistletoe extracts in liposomes. Extracts are dispersed in an oil phase as an emulsion which is centrifuged, thereby forming liposomes. To protect against rapid clearance of liposomes by components of innate and adaptive immunity and to prolong the half-life of encapsulated API in the blood circulation, subsequent surface modifications of liposomes, e.g.

by coating with heparin conjugates, can be employed (Dührkop *et al.* 2016, Han et al. 2006).

Fig. 1 shows the concept of dispersing mistletoe extracts in nanometer sized droplets in an oil where phospholipids act as emulsifiers. Once the emulsion droplets are transferred through the interface by centrifugal forces due to their density difference, they are covered by an additional phospholipid monolayer forming bilayers. These biological membranes constitute the shell of the liposomes which enclose mistletoe compounds and can act as drug delivery systems to reach the tumour tissue by extravasation and accumulation.

In an European project collaboration, the pharmaceutical process engineering of liposomal formulations of mistletoe by flow processes is studied intensively and major aspects of emulsification are published (Hildebrandt *et al.* 2015, Hildebrandt *et al.* 2016). The immune reactions provoked by liposomes were described by Klapper *et al.* 2014 with new modes of complement activation by phospholipid liposomes being elucidated. Approved liposomal formulations in cancer therapy use polyethyleneglycol (PEG) to produce "stealth liposomes" with increased circulation properties. However, evidence increases about the antigenicity of PEG upon repeated injection leading to the so-called "accelerated blood clearance". Our European collaboration succeeded to provide evidence that liposomes coated with a novel heparin conjugate do not lead to complement activation (Dührkop et al. 2016), which is considered to be a central component of rapid liposome clearance from the blood circulation. Current research focuses on *in vitro* and *in vivo* research to investigate possible advantages of liposomal formulation of mistletoe in a murine cancer model. In the following, results for the selection of murine cancer cell lines are presented which suitably respond to mistletoe compounds.

European mistletoe (*Viscum album* L.) contains glycosylated proteins of type II ribosome-inactivating proteins (RIP II) (Barbieri *et al.* 1993, Stirpe 2004), which can be differentiated into the 3 isoforms of ML I, ML II and ML III (Luther *et al.* 1987). ML are distributed in the parenchymal cells of the plant and inside of vacuoles, mostly in close contact with protein

bodies (Blascheck et al., 2013). ML, viscotoxins (VT) and polysaccharides are major bioactive components of mistletoe extracts. The composition of mistletoe extracts depends on the species of the host tree, geographical location, and time of harvesting (Urech *et al.* 2006). ML I is a 63 kDa heterodimeric protein consisting of two chains, a toxic A-chain and a B-chain. The toxic A-subunit is able to inactivate eukaryotic ribosomes via targeting the N-glycosidase activity, resulting in apoptotic cell death (Dietrich *et al.* 1992). The B-subunit of ML I recognizes specific terminal sugar residues of glycolipids and glycoproteins on the cell membrane and allows the toxic A-chain to enter into the target cell (Franz 1986).

Extracts of mistletoe play an important role in adjuvant cancer therapy due to the improvement of the quality of life and the global health status of cancer patients (Kim *et al.* 2012, Eisenbraun *et al.* 2011, Kienle and Kiene 2010), which is attributed to their immune activating effect. The aim of this study was to determine the inhibitory effect of different AV preparations and isolated ML I in a dose-response experiment and to identify sensitive tumor cell lines for testing liposomal formulations of ML in animal models.

The highly metastatic murine melanoma cell line B16-F10 and the metastatic murine colon carcinoma cell line C26 were tested regarding growth inhibition and cytotoxic effects by mistletoe extracts with different ML and VT compositions and isolated ML I.

Materials and Methods

Purification of mistletoe lectins and abnobaVISCUM extracts

For protein isolation mistletoe was harvested in June from ash tree (*Fraxinus excelsior* L.) and extraction was performed as described previously (Eifler *et al*. 1993).

Three different mistletoe preparations, AV Fraxini, Quercus and Pini were selected. ML I is the most characterized mistletoe lectin and generally regarded a major API in mistletoe extracts. The three selected preparations represent high and very low ML and VT contents,

respectively, but also differ in their ML composition. For AV Fraxini and AV Quercus, ML I is the predominant lectin, whereas AV Pini almost exclusively contains ML III, as summarized in Table 1.

Table 1. Differences in AV mistletoe lectin (ML) and viscotoxin (VT) composition and concentrations:

	Host tree	ML [µg/ml]	VT [µg/ml]	Concentration ratio VT/ML	Predominant lectin
AV Fraxini 20 mg	ash	10.9	79.9	7.3	ML I
AV Quercus 20 mg	oak	8.4	66.4	7.9	ML I
AV Pini 20 mg	pine	0.27	1.2	4.4	ML III

Cell lines

The murine melanoma cell line B16-F10 was obtained from the American Type Culture Collection (ATCC No. 59123188, March 2013) and cultured in DMEM with high glucose supplemented with 10% heat-inactivated FBS and sodium pyruvate. The murine C26 colon carcinoma cell line was kindly provided from O. Kranenburg, Med. Oncology UMC Utrecht CLS 400156 and cultured in RPMI1640 medium with 2 mM L-glutamine and 10% heat-inactivated FBS. Cell cultures were handled under standard conditions (37°C, 5% CO2 and humid atmosphere) in cell culture media without added antibiotics.

Cell proliferation assay

The proliferation of cells was quantified by the colorimetric tetrazolium compound 3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent phenazine ethosulfate (PES) combined in one assay (CellTiter 96®Aqueous One Solution Cell Proliferation Assay, Promega, Madison, USA) according to supplier´s instructions.

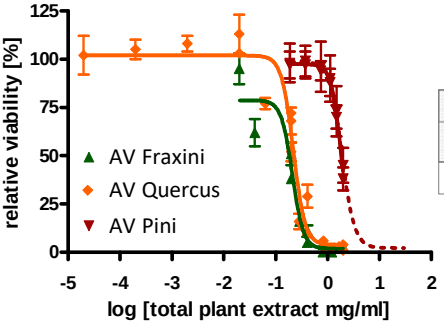
B16-F10 and C26 cells were detached with trypsin, washed with phosphate buffered saline (PBS) and counted after incubation with 0.5%

tryphan blue in a Bürker-Türk chamber. A defined number of cells was transferred into each well of a 96-well microtiter plate (Greiner, Frickenhausen, Germany). Cells were cultured for 24 h before incubation with AV Fraxini, Quercus, Pini (20 mg, respectively) or isolated ML I (concentration range from 10 pg/ml - 100 µg/ml) for 48 h. The AV dosage of 20 mg contains 15 mg homogenized extract from 20 mg mistletoe herb (fresh weight). Concentrations and IC50 for AV were calculated either based on mistletoe herb weight or based on ML concentration of AV (see Table 1). All ML concentrations were determined by ELISA. Control cells received DMEM or RPMI, depending on the type of cell line, as well as the same amount of phosphate ascorbic acid (PAA) or PBS buffer present in each dilution of tested AV preparations or isolated ML I, respectively. Each concentration was tested in quadruplicate wells.

The MTS tetrazolium compound is bio-reduced by NADPH or NADH produced dehydrogenase enzymes in metabolically active cells into a colored formazan product that is soluble in tissue culture medium. Assays were performed by adding 20 µl of the CellTiter 96®Aqueous One Solution Reagent directly to the culture wells containing 100 µl of cell culture medium and incubated for 1 h under standard conditions. The quantity of formazan product was measured by absorbance at 490 nm (reference wavelength 630 nm) with a 96-well plate reader. The absorbance is directly proportional to the number of living cells in culture. Absorbance of control cells was taken as 100% and the AV or ML I-treated cells as percentage of survival. The IC50 was determined by non-linear regression analysis of dose response curves with GraphPad Prism.

Results and Discussion

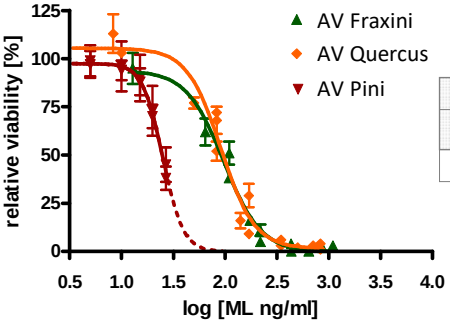
In Figure 2, the results of the proliferation assay comparing different AV extracts are shown (melanoma cell line B16-F10). The results indicate that IC50 values for AV Fraxini and AV Quercus are 6 times lower compared to AV Pini. AV Fraxini and AV Quercus have high ML and VT concentrations and are, therefore, more cytotoxic than AV Pini, which was an expected result.



AV Fraxini	AV Quercus	AV Pini
IC50 plant weight [mg/ml]		
0.3 ± 0.1	0.3 ± 0.1	1.8 ± 0.1

Fig. 2: Proliferation of B16-F10 melanoma cells after 48 h incubation with abnobaVISCUM (AV) of different host trees. Concentrations and IC50 values correspond to plant weight used for the extraction (= dose strength, see Materials and Methods).

Interestingly, if the different extracts are compared in regard to their lectin concentration (Figure 3), AV Pini appears approx. 4 times more effective than the other two extracts. Since AV Pini almost exclusively contains ML III and VT concentration is low (most likely in a subtoxic range), the increase in effectiveness might potentially be attributed to ML III. However, other compounds in AV Pini, or a higher background of plant material due to lower dilutions of plant extract, may contribute to this effect.



AV Fraxini	AV Quercus	AV Pini
IC50 ML [ng/ml]		
98.9 ± 2.4	95.2 ± 17.4	25.2 ± 0.9

Fig. 3: Comparison of AV extracts in regard to ML concentration (B16-F10).

Furthermore, B16-F10 and C26 were compared in regard to their sensitivity to isolated ML I (Figure 4 and Table 2). The colon carcinoma cell line C26 is the most sensitive of the cell lines tested with an IC50-value in the low ng/ml range. The very aggressive melanoma cell line B16-F10 is markedly less sensitive, with IC50 values 45 times higher than those of C26.

A comparison of ML I-rich mistletoe extracts (AV Fraxini, AV Quercus) and isolated ML I on B16-F10 show that the extract is approx. 30% more effective than ML I (Figure 3 and Table 2). As abnobaVISCUM is a complex mixture of bioactives and also rich in viscotoxins, this result is expected. Regarding the direct anti-proliferative effect on tumor cells, it may be inferred that ML I is the major bioactive in AV Fraxini and AV Quercus.

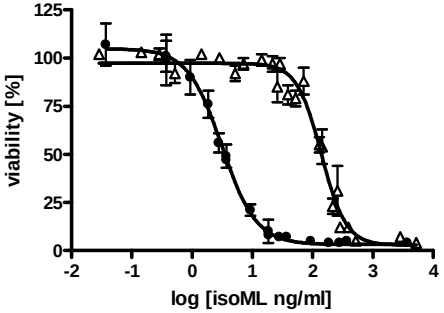


Fig. 4: The effect of various concentrations of ML I on two different murine cell lines: ● C26 colon cancer cells and Δ B16-F10 melanoma cells. Presented curves were established from n = 2 independent experiments out of 2 independent extract batches of ML I and show the mean value ± standard deviation of 4 measured wells per experimental run, respectively.

Table 2: IC50 of cell proliferation induced by extracts of ML I in murine cell lines. Numbers (± standard deviation) were established from n = 2 independent experiments (presented in Fig. 4):

Cell line	**C26**	**B16-F10**
Mouse strain	BALB/c	C57BL/6J
IC_{50} ± SD of ML I [ng/ml]	3.4 ± 0.3	151.8 ± 14.0

Conclusion

By investigating anti-proliferative effect of different AV preparations, we found that the colon carcinoma cell line C26 is much more sensitive to isolated ML I than the very aggressive B16-F10 melanoma cell line. This allows to compare a sensitive to a relatively non-sensitive and more aggressive tumor cell line in animal models. Also, the inflammatory cell model RAW264.7 (macrophage) is comparatively

insensitive to isolated ML I (as we are about to show in Hildebrandt 2016b), suggesting the possibility of selecting therapeutic concentrations that target e.g. colon tumours, but leave immune functions intact.

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