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Solid lipid nanoparticle (SLN) formulations as a potential tool for the reduction of cytotoxicity of saponins

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The present pilot study explored the potential of solid lipid nanoparticles (SLN) to entrap saponins and reduce the membrane toxicity of these compounds. SLN composed of different types of solid lipid were prepared by the cold homogenisation technique. Combinations of anionic, cationic and non-ionic stabilisers were selected in order to obtain negatively, positively and neutrally charged SLN. Mean particle size and zeta potential of blank and saponin-loaded formulations were measured by Dynamic Light Scattering (DLS), Electrophoretic Light Scattering (ELS) and *in vitro* cytotoxicity on MRC-5 SV2 and J774 cells was assessed using a resazurin-based assay. The type of solid lipid used for the formulation influenced the mean particle size, while the zeta potential mainly depended on the kind of surfactant utilised. Blank SLN composed of hard fat and anionic or non-ionic surfactants did not result in cytotoxicity. After loading with saponin, the anionic hard fat SLN was found to be the optimal formulation.

1. Introduction

Among the wide range of pharmacological activities that have been attributed to saponins in general (Hostettmann and Marston 1995), the characteristics of recently described maesabalides (Germonprez et al. 2004) deserve particular attention since they were shown to possess promising *in vitro* and *in vivo* antileishmanial action (Maes et al. 2004). Since membrane toxicity is characteristic for many saponins due to their amphiphilic nature, stable incorporation in nanoparticle formulations could be a practical solution to reduce toxicity and at the same time to enhance cell-targeting potential. This principle has been extensively exploited for several anticancer drugs (Torchillin 2007; Yuan et al. 2008) and for the antifungal and antileishmania drug amphotericin B (Mullen et al. 1997). The enhanced antileishmania action of liposomal amphotericin B has clearly been linked to a higher accumulation in the macrophage (Yardley and Croft 2000), however, this phenomenon still largely depends on the particular composition of the formulation (Gupta and Vyas 2007). Adding specific macrophage ligands to the nanoparticle formulation may further improve this cell-targeting effect (Vyas and Khatri 2007).

As no formal toxicity studies were yet performed with the maesabalides, a feasibility study of nanoparticle pharmaceutical forms with macrophage-targeting potential became a logical first step in the advanced study of their pharmacodynamics. Among the range of injectable colloidal carrier systems, such as liposomes, biodegradable polymeric nanoparticles and solid lipid nanoparticles (SLN), the latter were withheld for further evaluation in view of their excellent tolerability, good physical stability, protection of labile drugs, high drug load capacity, controlled drug release, low cost and ease of production (Müller et al. 2000; Mehnert and Mäder 2001; Wissing et al. 2004; Sapino et al. 2005).

Since too limited amounts of pure maesabalides were available to run the different pilot studies, the model amphiphilic molecules quillaja and aescin with chemical similarity to the maesabalides, were loaded into SLN of various composition. The physical characteristics and the *in vitro* cytotoxicity of the preparations on different cell lines were evaluated.

2. Investigations, results and discussion

The main preparation methods of SLN are high shear homogenisation and/or ultrasound techniques; hot and cold high-pressure homogenisation; solvent emulsification/evaporation and micro-emulsification (Üner et al. 2006; Date et al. 2007). In view of the amphiphilic nature of the model molecules quillaja and aescin, emulsification methods were not considered. Instead, the saponins were dispersed in the lipid melt to achieve full incorporation into the matrix upon subsequent cooling. Particle size reduction was obtained by ultrasound. Different mixtures of triglycerides, diglycerides and monoglycerides, i.e. hard fats (Ph. Eur.), were included to determine the influence of

matrix core material on physical and cytotoxic properties of the nanoparticles.

In order to evaluate the influence of SLN surface charge, combinations of stabilisers were selected to confer negative, positive and neutral net charges after dispersion in water. Anionic nanoparticles (A) were obtained with Lipoid® E80 + deoxycholic acid; cationic particles (C) with Lipoid® E80 + stearylamine and non-ionic particles (N) with sorbate 85 alone.

2.1. Physical characterisation

The mean particle size (Z_{ave}) of the various SLN varied between 205 and 911 nm for the blank formulations, between 170 and 777 nm for quillaja-loaded SLN and between 248 and 312 nm for aescin-loaded SLN. Stearic acid SLN were larger in comparison to hard fat SLN (Fig. 1), which can be explained by the fact that the various Witepsol® types contain considerable amounts of mono- and diglycerides with surface active properties (Mehnert and Mäder 2001). In view of similar properties of saponins, these molecules could reduce the particle size as well. However, such an effect was not observed.

Merely based on size, SLN have good potential as carriers for passive targeting of incorporated drugs to cells of the reticuloendothelial system. Indeed, internalisation of particles larger than 300nm is primarily performed by phagocytes as phagocytosis is essential for the removal of foreign particles within an organism (Watson et al. 2005). The uptake by phagocytosis of SLN composed of stearic acid plus several cosurfactants has been studied earlier in

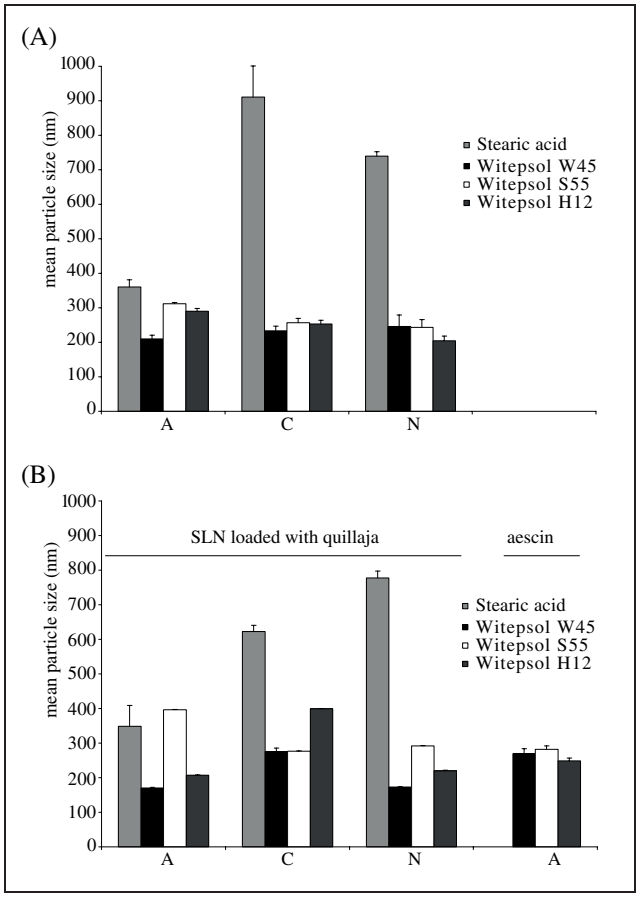

Fig. 1: Mean particle size ± standard deviation (nm) (n = 3) as determined by PCS of (A) blank SLN formulations and (B) saponin-loaded SLN formulations
A anionic; C cationic; N non-ionic

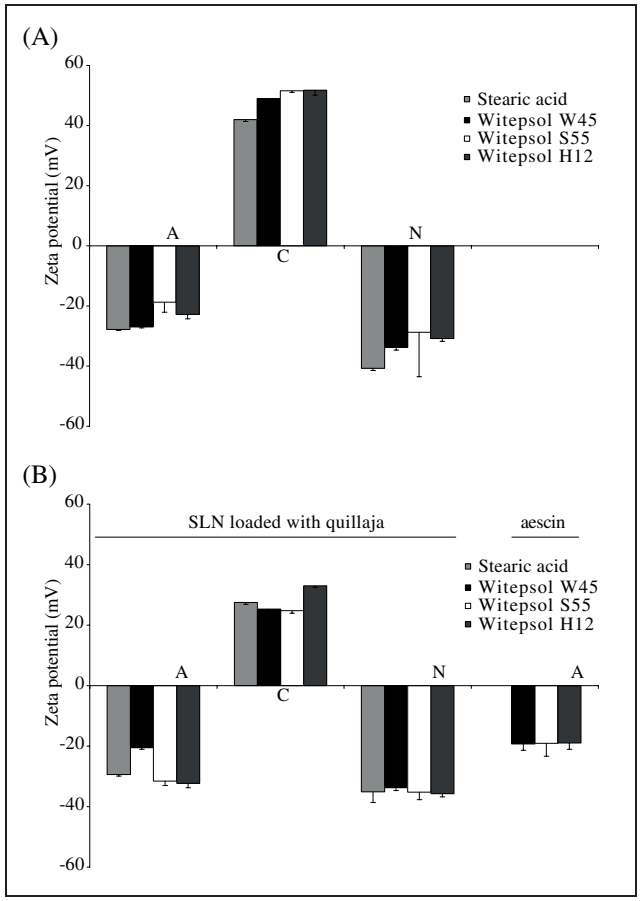

Fig. 2: Mean zeta potential ± standard deviation (mV) (n = 10) as determined by ELS of (A) blank SLN formulations and (B) saponin-loaded SLN formulations
A anionic; C cationic; N non-ionic

J774 cells, where 30 to 40% of the initial dose of lipid particles was taken up within 90 min (Bocca et al. 1998).

The zeta potential of stearylamine SLN was positive (Fig. 2). Quillaja-loaded cationic particles were less positive than blanks, suggesting the presence of at least part of the saponin onto the particle surface. Interestingly, SLN prepared with non-ionic sorbate 85 were negatively charged to the same or even greater extent than SLN prepared with deoxycholic acid. It has been described that SLN composed of stearic acid or Compritol® 888 ATO and the non-ionic emulsifier Tween® 80 had an average zeta potential between −30 and −35 mV in deionised water (Estella-Hermoso de Mendoza et al. 2008), thereby confirming our results. No sedimentation occurred with all SLN dispersions after storage at 5 °C for at least one week in deionised water, suggesting that the measured zeta potentials provide adequate electric repulsion between the particles, rendering the colloidal dispersion stable.

2.2. Cytotoxicity

The *in vitro* cytotoxicity of the different SLN formulations was assessed on two cell lines with different characteristics. MRC-5 SV2 cells were used as a model for non-phagocytising cells, also because the cytotoxic action of the maesabalides had been investigated on these cells (Maes et al. 2004). Since the primary aim of this study was to formulate SLN with saponin for the passive targeting of macrophages, cytotoxicity on phagocytising cells was an important criterion as well. J774 cells were used as they

Table: In vitro cytotoxicity of various SLN formulations on MRC-5 and J774 cells a CC50 expressed as concentration of SLN; b CC50 expressed as equivalent concentration of saponin present in SLN

SLN formulation or reference saponin		In vitro cytotoxicity (μg/ml)					
		Blank[a]		Quillaja[b]		Aescin[b]	
		MRC-5	J774	MRC-5	J774	MRC-5	J774
Stearic acid	A	136	79	6.1	2.9		
	C	13	5.0	3.9	0.6		
	N	>250	>250	>35	11.7		
Witepsol W45	A	>250	>250	>35	13.1	>17	>17
	C	29	9	5.4	<0.3		
	N	>250	>250	0.7	<0.3		
Witepsol S55	A	>250	>250	24	2.3	>17	>17
	C	32	10	3.7	0.7		
	N	250	>250	0.9	<0.3		
Witepsol H12	A	194	170	35	2.6	>17	>17
	C	73	34	2.7	0.3		
	N	>250	>250	1.9	0.3		
Quillaja				3.0	<0.3		
Aescin						11.5	5.8

are routinely applied for *in vitro* cytotoxicity and phagocytosis studies (Lemarchand et al. 2006; Nakano et al. 2007).

To evaluate the *in vitro* cytotoxic potential of the nanoparticulates, a resazurin-based cytotoxicity assay (O'Brien et al. 2000; Crouch and Slater 2001) was preferred over the conventional methyl-thiazol-tetrazolium (MTT) colorimetric test (Olbrich et al. 2004; Lemarchand et al. 2006). The former provides a higher degree of sensitivity and is more simple and rapid. Indeed, resazurin (blue and non-fluorescent) is enzymatically reduced in viable cells to resorufin (pink and highly fluorescent), which can be measured both colorimetrically and fluorometrically. In contrast, MTT is reduced to an intensely coloured, water insoluble formazan dye, only permitting absorbance measurements. Furthermore, resazurin can be considered as an 'add-and-read' substrate, permitting direct reading after addition. MTT-derived formazan crystals on the other hand require prior extraction in an organic solvent, such as isopropanol or dimethyl sulfoxide, thereby rendering the test protocol more complex and prone to variation (Mosmann 1983; McMillian et al. 2002).

In a first experiment, the cytotoxic potential of blank formulations was investigated. As shown in the Table, the nature of the lipid matrix influences cytotoxicity on both MRC-5 and J774 cells. Anionic and non-ionic SLN comprised of hard fat did not reveal any cytotoxic effect, whereas the anionic stearic acid preparations did. Schöler et al. (2002) demonstrated that slightly reduced viabilities of peritoneal mouse macrophages treated with SLN might be due to the release of free fatty acids upon intracellular enzymatic degradation. Likewise, free stearic acid may most likely be the main cytotoxic component of the stearic acid SLN, since it has been shown that stearic acid induces loss of membrane integrity in J774 cells, resulting in cell death (Martins de Lima et al. 2006). Interestingly, non-ionic stearic acid SLN were not cytotoxic in the concentration range tested.

Irrespective the type of solid lipid, SLN containing stearylamine as stabilising agent were highly cytotoxic. This has also been observed by Schöler et al. (2001) for SLN stabilised by the surfactant cetylpyridinium chloride (CPC), which caused significant viability reduction of mouse peritoneal macrophages. Moreover, the cytotoxicity of CPC incorporated in SLN was higher than that of equally concentrated solutions of surfactant only. This finding could be explained by enhanced adherence of the positively charged particles to the negatively charged cell membrane. A closer interaction of the SLN with the cell may result in higher (intra)cellular concentrations of the cytotoxic surfactant upon degradation of the lipid matrix. In a more recent publication, the same authors report that the cytotoxic effects of CPC-stabilised SLN could be antagonised with cytochalasin B, which blocks phagocytosis (Olbrich et al. 2004), indicating that cytotoxicity is primarily a consequence of internalisation of SLN.

The main goal of this study was to reduce the membrane toxic effects of saponins by incorporating them in stable particles. The potential toxicity of saponins is attributed to their amphiphilic nature, since quillaja and aescin both consist of a hydrophobic triterpene aglycone and a hydrophilic sugar moiety (Fig. 3) (Hartke and Mutschler 1986; van Setten and van de Werken 1996).

The *in vitro* cytotoxicity of SLN formulations loaded with saponins was assessed in a similar fashion as for blanks. The amount of saponin, i.e. quillaja or aescin, present in the formulations was calculated referring to the initial amount of drug dispersed in the lipid melt assuming no loss during cold homogenisation technique. In this way, the toxic effect of saponin-loaded SLN was compared to that of an equally concentrated solution of quillaja or aescin. When comparing CC_{50}-values of drug-loaded SLN with those of free drug, information can be obtained about the efficiency at which the saponin is incorporated into the lipid matrix. Indeed, the observed cytotoxicity of saponin-loaded particles becomes a measure of the percentage of free saponin in the culture medium (i.e. initially not-entrapped in SLN and/or released as a consequence of high burst release).

Results show that anionic hard fat SLN efficiently entrap quillaja as the CC_{50} on both cell lines is decreased in comparison to the CC_{50} of the free saponin (Table). For anionic stearic acid SLN, this trend is much less obvious. The fact that stearic acid is less suitable than Witepsol® as core material for the manufacturing of SLN endorses the observations of Estella-Hermoso de Mendoza et al. (2008), who found that the type of solid lipid was a critical parameter

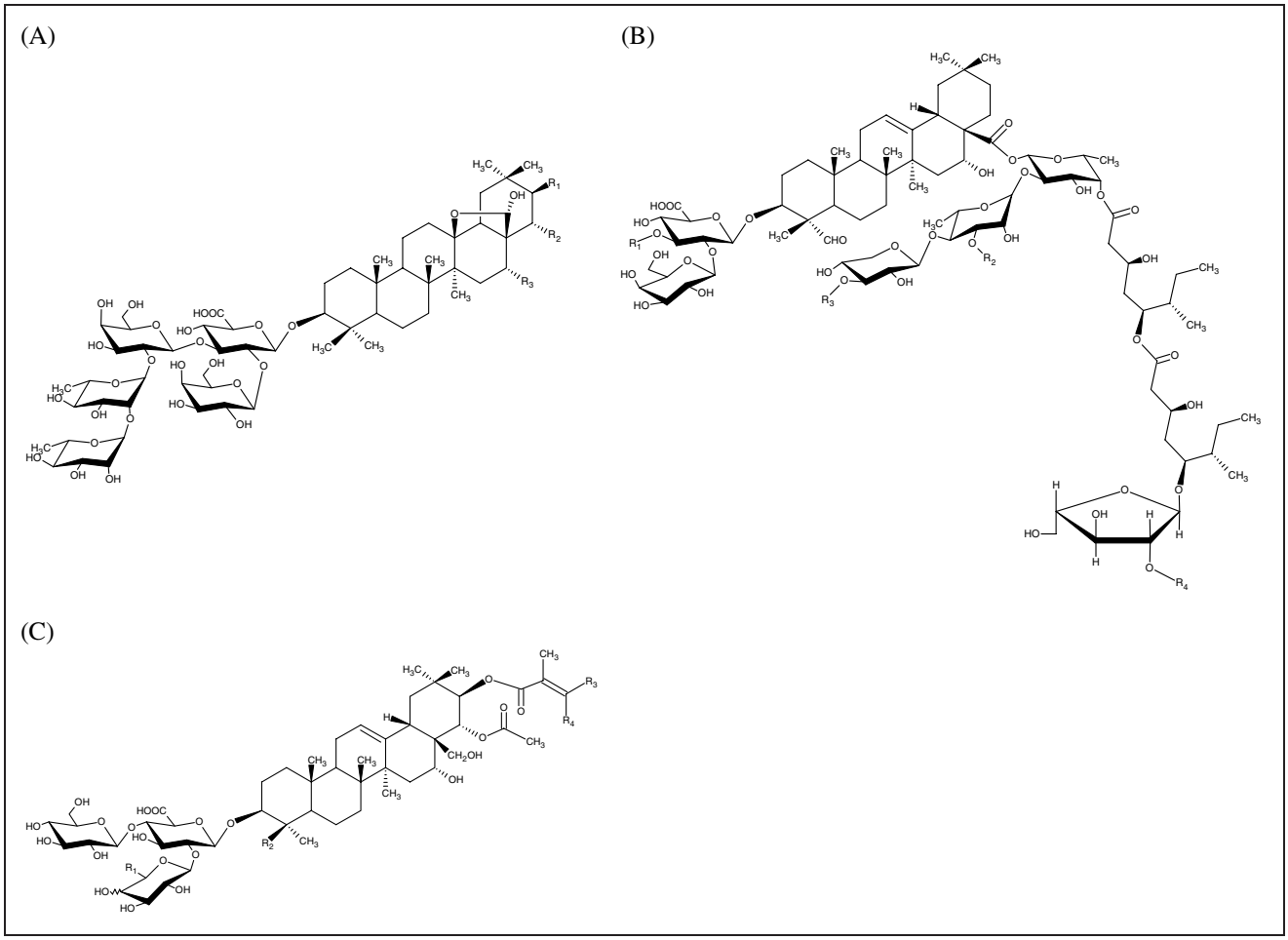

Fig. 3: Chemical structure of (A) maesabalides, (B) quillaja saponins and (C) aescin. R1, R2, R3 and R4 represent different substitution patterns of individual molecules

influencing the entrapment efficiency of edelfosine, an amphiphilic alkyl-lysophospholipid analogue with antitumor and antileishmania activity. By using Compritol® 888 ATO instead of stearic acid, entrapment efficiency increased and burst release decreased remarkably. The drug loading capacity of selected lipids obviously depends on several factors of which the solubility of the drug in the lipid melt and the chemical and physical structure of the solid lipid matrix have critical importance (Müller et al. 2000). Most likely, stearic acid forms highly crystalline particles with a perfect lattice, leading to drug expulsion, whereas the more complex mixtures of mono-, di- and triglycerides (i.e. various Witepsol® types) form crystals of lesser quality, facilitating drug incorporation.

Quillaja would be expected to be incorporated less efficiently in SLN than aescin, since quillaja saponins are known as bidesmosides, with sugar moieties attached to the aglycone at two positions (Fig. 3). Quillaja can consist of six to ten monosaccharide residues (van Setten and van de Werken 1996) rendering these molecules more hydrophilic than aescin. Consequently, aescin would have a higher affinity for the lipid melt than quillaja. However, by comparing the CC_{50}-values of both saponins incorporated in the anionic hard fat SLN, this effect could not be observed.

This pilot study indicated that SLN prepared by the cold homogenisation technique can efficiently entrap saponins and consequently reduce the *in vitro* cytotoxicity of this type of compounds. SLN composed of hard fat and a combination of the surfactants Lipoid® E80 and deoxycholic acid were found to be the optimal formulation for the reference saponins aescin and quillaja. Further investigations will focus on the evaluation of the *in vitro* toxicity and antileishmanial efficacy of maesabalide-loaded SLN and on the mechanism of uptake by phagocytising cells and the intracellular fate of the internalised SLN.

3. Experimental

3.1. Materials

The semi-synthetic glycerides (Ph. Eur.) Witepsol® H12, S55 and W45 were obtained from Sasol (Witten, Germany). Egg lecithin (Lipoid® E80) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Stearic acid, deoxycholic acid, stearylamine, resazurin, saponin from quillaja bark (purified by ultrafiltration to reduce low molecular weight contaminants, sapogenin content 24%) and aescin (mixture of saponins, purity $\geq$96.0% calculated on the dried material) were supplied by Sigma. Sorbate 85 was obtained from ICI Chemicals (Brussels, Belgium). Milli-Q water (Millipore Co., Bedford, USA) was used throughout the experiments. All reagents were of pharmaceutical or analytical grade.

3.2. Cell cultures

J774A.1 (murine macrophage-like cell line) and MRC-5 SV2 (human fetal lung fibroblast cell line transfected with SV 40) were grown in tissue culture flasks (Sarstedt) or multiwell microtiter plates (Greiner Bio-One) in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS) (InVitrogen) at 37 °C and in a 5% CO_2 atmosphere. The choice of both cell types is justified as J774 cells do phagocytise and MRC-5 do not, thereby mimicking the differential susceptibility of macrophages and other host cells. Several researchers have reported that J774 cells are able to take up large particles up to 1 μm (Catelas et al. 1999; Schrijvers et al. 2004).

3.3. Preparation of SLN

SLN were prepared by the cold homogenisation technique. The solid lipid (0.8 g) (i.e. Witepsol® H12, S55, W45 or stearic acid) and stabilisers (Lipoid® E80 0.2 g + deoxycholic acid 0.2 g, Lipoid® E80 0.2 g + stearylamine 0.2 g or sorbate 85 0.4 g for anionic, cationic and non-ionic particles respectively) were melted under stirring at 69–72 °C. After dispersion of the saponins (0.2 g quillaja or aescin) into the lipid melt, mixtures were cooled down and the resulting solid was grounded in a mortar to particles of about 100 µm. These were subsequently dispersed in 100 ml Milli-Q water under sonication at 22–26 W (amplitude 60%) (Branson Sonifier 450-D 102-C, Danbury, USA) for 5 min at 5 °C. The resulting nanosuspensions were freeze-dried (Leybold-Heraeus D8B, Germany) and sterilised by means of gamma-irradiation at a dose of 25 kGy from a Co^{60} irradiation source (Gammir I-Sulzer irradiation unicell, IBA-Mediris, Sterigenics, Fleurus, Belgium).

3.4. Physical characterisation

The mean particle size (Z_{ave}) of the nanoparticles was determined by Photon Correlation Spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, UK). Freshly prepared SLN were diluted with deionised water. Measurements were performed in triplicate.

The zeta potential of the nanoparticles was determined using Electrophoretic Light Scattering (ELS) utilising the same instrument as for PCS. Averages and standard deviations result from ten consecutive measurements on the same sample.

3.5. Cytotoxicity

The different types of SLN were suspended in RPMI medium and added to confluent monolayers of MRC-5 SV2 and J774 cells in 96-well plates. The tested concentrations ranged from 375 to 2.92 µg/ml using two-fold dilution steps. After 72 h of incubation, the viability of cells was assessed quantitatively by resazurin conversion (10 µg/ml). Resazurin reduction to fluorescent resorufin was measured (excitation 550 nm; emission at 590 nm) after 4 h of incubation (McMillian et al. 2002). All plates contained untreated controls and blanks. The cytotoxic concentrations that reduced cell viability by 50% (CC_{50}-values) were calculated by linear regression analysis between the two concentrations of SLN that resulted in growth inhibition higher and lower than 50%.

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