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Cytotoxicity of Solid Lipid Nanoparticles as a Function of the Lipid Matrix and the Surfactant

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Purpose. Assessment of the in vitro cytotoxicity of solid lipid nanoparticles (SLNs) as a function of lipid matrix (Dynasan 114, Compritol ATO 888), and stabilizing surfactant (poloxamers, Tween 80, soya lecithin, and sodium dodecyl sulphate). Comparison with other colloidal carriers should determine their potential use in the clinic.
Methods. SLNs were produced by high pressure homogenisation. Cytotoxicity was assessed by measuring the viability of HL60 cells and human granulocytes after incubation with SLNs. Particle internalisation was quantified by chemiluminescence measurements.
Results. The nature of the lipid had no effect on viability; distinct differences were found for the surfactants. Binding to the SLN surface reduced markedly the cytotoxic effect of the surfactants, e.g., up to a factor of 65 for poloxamer 184. The permanent HL60 cell line—differentiated from cells with granulocyte characteristics by retinoic acid treatment—yielded results identical to freshly isolated human granulocytes. In general, the SLNs showed a lower cytotoxicity compared to polyalkylcyanoacrylate and polylactic/glycolic acid (PLA/GA) nanoparticles.
Conclusions. Because the results are identical when using human granulocytes, differentiated HL60 cells can be used as an easily accessible *in vitro* test system for i.v. injectable SLN formulations. The SLNs appear suitable as a drug carrier system for potential intravenous use due to their very low cytotoxicity *in vitro*.

KEY WORDS: solid lipid nanoparticles; cytotoxicity; HL60 cells; poloxamer; Tween 80; sodium dodecyl sulphate.

INTRODUCTION

Increasing attention is being focused on solid lipid nanoparticles (SLNs) as a colloidal drug carrier system combining the advantages of polymeric nanoparticles, fat emulsions, and liposomes but simultaneously avoiding their disadvantages (1–3). With regard to the physical characteristics, they fulfill the requirements for human use (4). A further prerequisite for human use is an acceptable low toxicity. Sufficient data are available for the use of lipids via the peroral route. In principle, all food lipids, GRAS substances, and the substances with accepted GRAS status can be used. However, there are little data on injected solid lipids (5). To assess the potential *in vivo* tolerability of lipids and excipients (e.g. stabilising surfactants), basic *in vitro* cell culture studies can be performed. As a test system, the human promyelotic cell line HL60 was employed. HL60 cells were differentiated in vitro with cells having characteristics of mature granulocytes by treatment with retinoic acid (RA) (6). The effects of lipids differing in degradation velocity and various surfactants used for stabilisation on the cytotoxicity are investigated in this study. Results obtained with differentiated HL60 cells and human granulocytes are compared to assess whether the more easily accessible HL60 cells are a suitable test system for replacement of human granulocytes. To judge the cytotoxicity of SLNs on a relative scale related to other colloidal carriers, they are compared with nanoparticles of known *in vitro* and *in vivo* cytotoxicity.

MATERIALS AND METHODS

Cell Line and Culture Conditions

Human promyelotic (HL60) cells were cultivated in RPMI 1640; differentiation was induced by the addition of 1×10^{-6} M retinoic acid (RA) for five days (6).

Human Granulocytes

Peripheral blood granulocytes from normal donors were isolated by the Ficoll-dextran method (7).

Cytotoxicity Assay

HL60 cells or granulocytes were adjusted with PBS to 2×10^6 cells/ml. 100 μl of this suspension were deposited onto microtiter plates (200,000 cells/well). The cells were incubated with the nanoparticle dispersion (stock dispersion diluted accordingly to give the desired final concentration in the well) or surfactant solution (100 μl) for 12 h, then 50 μl dimethylthiazolyl-diphenyltetrazolium (MTT) solution was added (2.5 mg/ml PBS) and further incubated for 4 hours (4). The particle/MTT mixture was removed and the blue salt formed by living cells dissolved in 50 μl basic SDS solution (25% w/w). The extinction at 550 nm was measured by an Easy Rider EAR 400 AT from SLT-Labinstruments, Grödig, Austria. The viability was expressed in percent compared to a control not treated.

For assessment of SLN cytotoxicity, the majority of free surfactant was removed by washing the SLN dispersion with water yielding SLN dispersion containing only 0.125% surfactant. For incubation with the cells, this SLN dispersion was diluted by at least 10-fold yielding a maximum surfactant content of 0.0125%. Reference solutions of surfactant without nanoparticles were obtained by equivalent dilution. For a better comparison, the actual surfactant concentration is not plotted on the x-axis in Fig. 3, but the SLN concentration from Fig. 2 which contains the identical amount of surfactant. The phagocytic uptake of particles was determined by luminol-enhanced chemiluminescence (CL) using an CL Amerlite Analyser (Amersham, UK) as described previously (8). The CL intensity was measured 70 times over a period of up to 3 hours. The uptake was quantified by calculating the area under the curve (AUC) which correlates linearly with the particle mass internalized (8). The experiments were performed in triplicate and repeated at least 3 times (relative standard deviation of 10% maximum).

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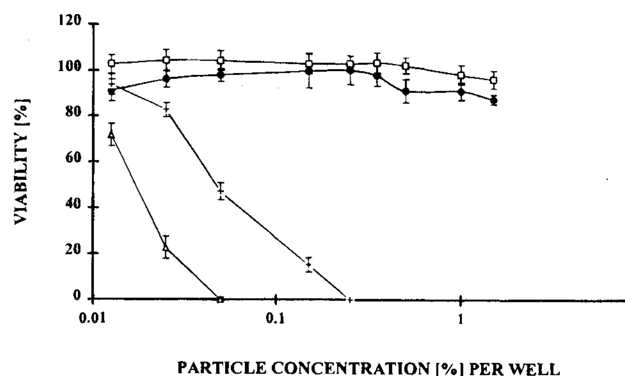

Fig. 1. Cytotoxicity of Dynasan-SLN (□), Compritol- SLN (•), and, as reference drug carriers, polyhexylcyanoacrylate- (+) and polymethylcyanoacrylate- (△) nanoparticles in HL60 cell suspension. The viability is expressed as % of the viability of untreated cells.

Solid Lipid Nanoparticles

SLNs were produced by high pressure homogenisation (LAB 40, APV Gaulin, Lübeck, Germany) of a pre-emulsion at 70°C (10% lipid, 0.5% surfactant) (3). Lipid phases used were Dynasan 114, a triglyceride of myristic acid (Hüls AG, Witten, Germany), or Compritol ATO 888, glycerolbehenate (Gattefossé, Weil am Rhein, Germany). Surfactants were Lipoid S75, soya lecithin with 68% phosphatidylcholine (Lipoid KG, Ludwigshafen, Germany), poloxamer 184, 188, 235, 335, and 407 (Synperonic® L64, F68, P85, P105 and F127, ICI Middlesborough, Cleveland, UK), and Tween 80 (Dr. Karl Thomae GmbH, Biberach, Germany). All surfactants were added directly in the production process, and no later adsorption was performed. Crystallinity of the SLN was verified after storage at 4°C for 7 days. Degree of crystallinity was at least 90% (3). The mean particle size was determined by photon correlation spectroscopy (PCS) (Zetasizer 4, Malvern Instruments, Malvern, UK). The homogenisation parameters were adjusted to yield mean particle diameters between 186 and 280 nm for all SLN batches (Table 1).

Polyalkylcyanoacrylate Nanoparticles

The particles were polymerized as described previously using methyl-monomer (surfactant: 0.36% Tween 20) and hexyl-monomer (stabilizer: 1% dextran 70, 5% glucose) (9,10). The polymethylcyanoacrylate (PMCA) and polyhexylcyanoacrylate (PHCA) nanoparticles were 237 nm and 179 nm, and the polydispersity indices 0.09 and 0.08, respectively.

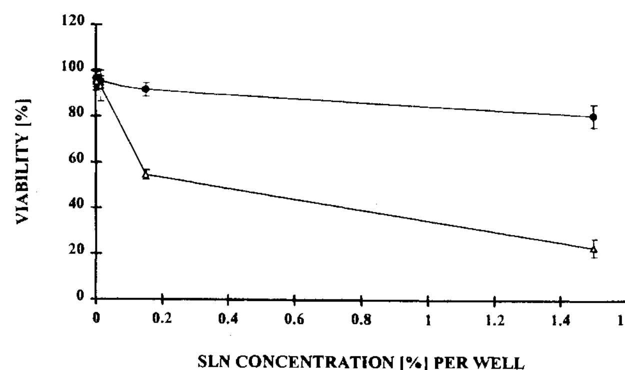

Fig. 2. Cytotoxicity of Compritol-SLN stabilized with poloxamer 407 (•) or Tween 80 (△) in HL60 cell suspension. The viability is expressed as % of untreated cells.

RESULTS AND DISCUSSION

Differentiated HL60 cells were incubated with increasing concentrations of SLN made from two lipids differing in their degradation velocity—Dynasan and Compritol. Previous work showed a distinctly faster *in vitro* enzymatic degradation in lipase/colipase solutions for Dynasan. The surfactant was found to affect the degradation velocity as well. To study solely the effect of the lipid, both SLNs were stabilized with Lipoid S75. In the concentration range 0.015 to 1.5%, no significant difference in viability compared to the control was found for both SLNs (Fig. 1). This proves that SLNs are well tolerated; the difference in degradation velocity of the lipids had no effect on the cytotoxicity in the cell culture. These *in vitro* data are in agreement with in vivo animal studies. Differences in systemic toxicity of more slowly degrading Compritol could only be created by high dosing of SLNs after multiple injections (5).

To estimate the possible in vivo tolerability of the SLN on the basis of in vitro cell culture data, they need to be compared with cytotoxicity data of nanoparticles with known *in vivo* toxicity. Polyalkylcyanoacrylate nanoparticles were chosen for comparison because intensive *in vitro* (9,10), but also *in vivo,* data are available (11). Clinical phase I studies were performed (12). Polymethyl- and polyhexylcyanoacrylate nanoparticles (PMCA, PHCA) were chosen representing the most and least toxic particles of this class. Full cell death occurred at 0.05% of PMCA, and at 0.35% of PHCA (Fig. 1). *In vivo,* an injected dose of 20 mg/kg PHCA nanoparticles was found to cause no damage to the liver, the organ with highest accumulation after i.v. injection (13). Comparing the *in vitro* data of both nanoparticle systems, a distinctly higher in vivo tolerance is expected for the SLN. In human granulocyte cultures, SLN proved even less cytotoxic than polyester nanoparticles (4). A 100% mortality was observed at 0.5% polylactic/glycolic acid (PLA/GA) nanoparticles in the cell suspension. In contrast, 5% cetylpalmitate SLN reduced the viability of the human granulocytes to only 85% (4). Therefore, the SLNs are expected to be better tolerated in vivo than polyester nanoparticles.

Cytotoxic effects can be caused by particles adhering to the cell membrane (9), particles internalized by the cell, and degradation products in the cell culture medium and inside the cell. Chemiluminescence (CL) measurements were performed to eliminate the possibility that the lower cytotoxicity of SLNs is due to a reduced uptake by the HL60 cells. The uptake was found to be similar for the investigated particles shown in Fig. 1, appr. 7000 AUC units (data not shown).

To assess the possible contribution of the stabiliser to the cytotoxicity, SLNs composed of the same lipid—Compritol—but with different stabilizers were investigated. Poloxamer 407 is well tolerated in cell cultures up to 10%. Tween 80 shows some cytotoxicity (14) although registered for i.v. use. Prior to incubation, free surfactant was removed. The poloxamer 407 SLN had little effect on the viability. In contrast, a distinct reduction was observed at $\geq 0.1\%$ SLN stabilized with Tween 80 (Fig. 2) indicating the importance of the surfactant.

Table I. Toxicity Reducing Factor (TRF) by Binding of SLN Stabilizers to the Particle Surface Calculated for Different SLN Formulations

Stabilizers in SLN-formulation	TRF for SLN concentrations [%]					
	0,00001	0,0001	0,001	0,01	0,1	1
poloxamer 407 (246 nm)	0,95	0,96	0,99	0,99	1,01	0,99
Tween 80 (214 nm)	0,95	0,99	1,87	4,63	10,09	24,90
poloxamer 184 (280 nm)	0,92	1,11	2,69	3,9	5,3	65,49
poloxamer 188 (268 nm)	0,99	0,99	1,00	1,22	1,41	2,5
poloxamer 235 (237 nm)	0,97	0,99	2,11	5,27	9,08	31,8
poloxamer 335 (254 nm)	0,91	1,17	2,22	2,98	4,74	17,32
SDS (186 nm)	1,06	1,72	7,59	37,17	2,88	1,89

Note: The TRF is the ratio of viability of cells (%) after incubation with surfactant-stabilized SLN to the viability (%) after incubation with a surfactant solution of identical concentration (Particle size is listed in brackets, left column)

The cell damaging effect of surfactants depends on the status of the molecules, e.g., free in solution or bound to surfaces, and also on their conformation on surfaces. As with polyhydroxybutyrate nanoparticles produced with sodium dodecyl sulphate, it is assumed that the surfactants are partially irreversibly bound to the surface (15). The cell damaging groups might be exposed or favourably shielded reducing the cytotoxicity compared to the free surfactant. To assess this, RA-treated HL60 cells were incubated with surfactant solution. The concentrations corresponded to the surfactant concentrations in the SLN dispersions from Fig. 2. In contrast to the Tween 80 bound to SLN (Fig. 2, lower curve), a higher cytotoxicity was observed for the free Tween 80. A viability reduction to 50% was obtained for free Tween 80 at amounts present in 0.001% SLN. Little cytotoxicity was observed for poloxamer 407 (Fig. 3, upper curve). Obviously, binding of a surfactant to the SLN surface reduces surfactant toxicity.

A reduced cytotoxicity might be due to a lack of internalisation of particles, e.g., reduced uptake caused by polyethoxylated surfactants (16). Uptake was assessed by CL measurements. The uptake of poloxamer stabilized SLN was appr. half of that of Lipoid S75 stabilized SLN (appr. 3000 compared to 6500 AUC units), i.e., appr. one third of the control of hydrophobic polystyrene particles. This proven uptake is considered to lead to detectable effects in the assay in the case of toxic compounds. Tween stabilized SLN showed an increasing reduction in the uptake at increasing particle concentration (2464 to 244 AUC units, 0.015% to 1.5% Tween). This corresponds to the observed cytotoxicity of Tween. The damaged or dead cells are not capable of particle phagocytosis.

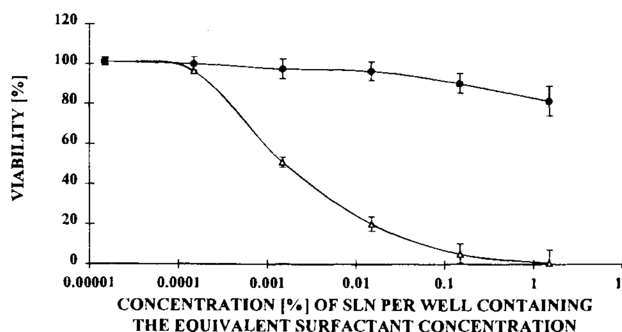

Fig. 3. Cytotoxicity of solutions of the SLN-stabilizers poloxamer 407 (•) and Tween 80 (△). The concentration is equivalent to the total stabilizer concentrations in the washed SLN dispersions investigated in Fig. 2. For reasons of comparison, the concentration plotted on the x-axis is the lipid concentration of the respective SLN dispersion from Fig. 2 (e.g. 1% SLN dispersion on the x-axis contains 0.0125% stabilizer, cf. Methods).

Blockcopolymers showed an increase in cytotoxicity with a decrease in molecular weight (17). To assess which poloxamers can be used for SLN production, a series with decreasing molecular weight was investigated. In addition, sodium dodecyl sulphate (SDS) was used. It is a surfactant with excellent dispersion properties in the homogenisation process—but its use is possibly limited to the toxicity. A decrease in viability was observed for all SLN stabilized with these surfactants above 0.01%, being most pronounced for the low molecular weight poloxamer 188 and the highly surface active SDS (Fig. 4A). It is remarkable that even the relatively toxic SDS leads to particles being well tolerated at a concentration of 0.01%. Surfactant concentrations identical to the SLNs, but free in solution instead being surface-bound, leads to distinct cytotoxic effects at very low concentrations of both the SDS and poloxamers (Fig. 5). For example, SDS-SLN show no cytotoxicity up to 0.01% (Fig. 4A). In contrast, the free surfactant shows no cytotoxic effects only up to concentrations which are present in a washed 0.00001% SLN dispersion (Fig. 5). A factor of appr. 1000 reflects the efficiency of toxicity reduction by binding to the surface of SLN.

A factor was calculated to quantify the cytotoxicity reducing effect of binding surfactants to the SLN surface. The toxicity reducing factor (TRF) is the ratio of viability of cells (%) after incubation with surfactant-stabilized SLN to the viability (%) after incubation with a surfactant solution of identical concentration. This calculation was performed for each of the increasing SLN concentrations from 10^{-5}% to 1% (Table I). For surfactants in which there is a lack of cytotoxicity at the investigated concentration, the TRF is necessarily 1, i.e., there is no difference between surface-bound and free surfactant (e.g. poloxamer 407). Medium toxic surfactants (e.g., Tween 80 and other poloxamers) show an increase in TRF. SDS, as a more toxic compound, shows an increase to 37 at 0.01%, and a decrease at higher concentrations due to cell death (Table I).

Different cells are susceptible to a different extent to cytotoxic effects caused by particulate carriers (19). To have a more relevant model, cells in contact with the blood (i.e., nanoparticles) are used; i.e., hepatocytes (17,19), Kupffer cells, and

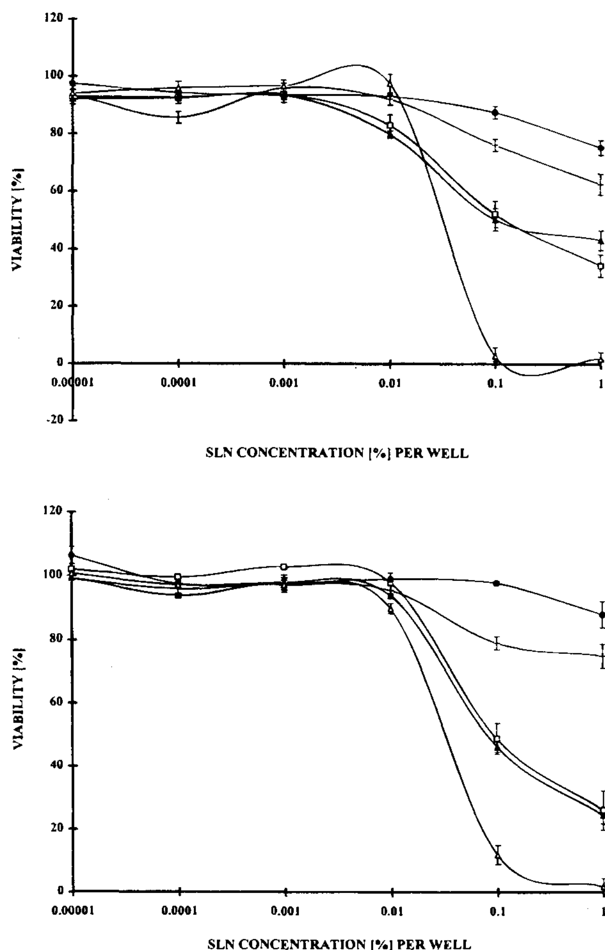

Fig. 4. Viability (expressed as % viability of untreated cells) of HL60 cells (upper) and freshly isolated human granulocytes (lower) as a function of increasing concentrations of SLN stabilized with poloxamer 188 (•), 235 (+), 335 (▲), 184 (□) and sodium dodecyl sulphate—SDS (△).

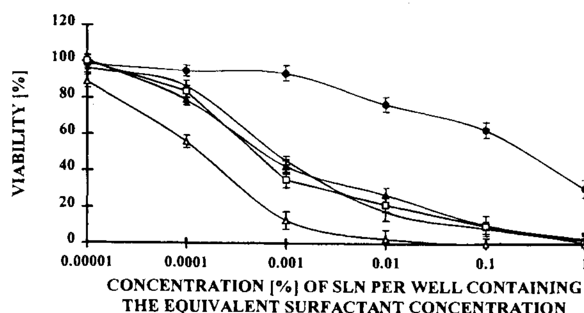

Fig. 5. Cytotoxicity of solutions of the SLN-stabilizers poloxamer 188 (•), 235 (+), 335 (▲), 184 (□), and SDS (△). The concentration is equivalent to the total stabilizer concentrations in the SLN dispersions investigated in Fig. 4. For reasons of comparison, the concentration plotted on the x-axis is the lipid concentration of the respective washed SLN dispersion from Fig. 4 (cf. Fig. 3).

human granulocytes are additional cells with phagocytic capacity (4,20). In fact, the hepatocytes tolerated 100-fold higher nanoparticle doses than, e.g., fibroblasts (19), demonstrating the importance of using a relevant cell model for testing. In contrast to Kupffer cells from a perfused rat liver, human granulocytes have the advantage of being available in larger quantities and originating from man. More convenient are donor-independent permanent human derived cell lines such as the HL60 cell line. To assess whether they are similarly susceptible to cytotoxic effects, the surfactant-stabilized SLNs were simultaneously incubated with human granulocytes (Fig. 4B). The viability/concentration profiles were practically identical to the ones obtained with differentiated HL60 cells (Fig. 4A). From the data obtained to date, RA-differentiated HL60 cells are an easily accessible and suitable in vitro test model with susceptibility similar to human granulocytes.

CONCLUSIONS

The cytotoxicity of SLN is relatively low in general, especially when compared with other nanoparticulate carriers such as polyalkylcyanoacrylates and PLA/GA. The investigated lipids differing in degradation velocity had no effect on the *in vitro* cytotoxicity, and distinct differences were observed regarding the surfactants used for stabilisation. However, binding of surfactants such as Tween 80 to the surface reduced their low toxicity further. This allows the use of higher concentrations of these surfactants when required (e.g. for physical stabilisation). Less well tolerated surfactants might also be used because of the reduction in toxicity after incorporation in the SLN surface. Differentiated HL60 cells are a suitable *in vitro* test model. Due to easy availability, it allows the conductance of extensive screening studies. From the *in vitro* data obtained to date, the SLNs appear to fulfill one of the essential prerequisites of a colloidal carrier for introduction into the clinic—a low cytotoxicity.

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